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Use of PCR-based methods in microbiology and their specific applications

Habilitation thesis

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Acknowledgement

Firstly, I would like to thank my wife Marika who had to withstand all the late returns home from the laboratory and in the meantime raise two children. That's what you call a scientific success!

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Thanks to all of you who I have met in my life and who have shaped my attitude towards science and life.

MOTTO:

„Vyskytoval jsem se ve formách organických i anorganických, dělil jsem se, štěpil jsem se, ale přišel jsem než na jednu zkušenost, a totiž tu, že z ničeho se nemá dělat věda. Ani z vědy ne, natož ze života. Když už člověk jednou je, tak má koukat, aby byl. A když kouká, aby byl, a je, tak má bejt to, co je, a nemá bejt to, co není – jak tomu v mnoha případech je. To je celý.“

Jan Werich

Content

1. Abstract

This habilitation thesis is a curated selection of 17 publications that focus on the application of PCR-based methods in specific microbiological contexts. PCR-based methods have gained considerable popularity over time due to their efficacy in the detection, identification and typing of microorganisms. They have become an integral part of both research and routine diagnostics. The focus of my professional endeavors has been the advancement of PCR-based techniques, with an emphasis on their practical application within standard research laboratories. In this thesis, I will address three aspects of the application of PCR-based methods in microbiology that, while methodologically feasible, are predominantly used in research but rarely in practice. The first aspect concerns the ability of PCR (especially quantitative PCR) methods to determine the amount of DNA in samples. The second relates to the inability of PCR-based methods to assess the viability of microorganisms, since the presence of DNA/RNA alone does not provide information about the physiological state of the living cell. The third aspect concerns the technological limitations of PCR-based methods in terms of their multiplexity and the applications that can provide a solution to this problem. These three aspects will be presented and discussed in this habilitation thesis, and will be documented by the published data of myself and my colleagues in order to provide solutions.

2. Introduction

Molecular diagnostics has revolutionized microbiology by offering speed, sensitivity, and specificity often unattainable by traditional culture-based techniques. Among its versatile tools, PCR-based approaches utilizing *in vitro* amplification of DNA—particularly PCR and quantitative PCR (qPCR)—have become indispensable. Yet, as molecular diagnostics matures, its utility is increasingly shaped by how well it addresses three critical diagnostic needs: *how many* microbes are present (quantification), *which ones are viable* (viability assessment), and *how many targets* can be detected simultaneously (multiplexing). These three facets—quantification, viability, and multiplex detection—not only represent advanced applications of molecular methods but also offer synergistic potential for improving microbial diagnostics in both research and routine settings.

The first key application of PCR-based methods is quantification of target DNA, which is best exemplified by qPCR and partially digital PCR (dPCR). These methodologies empower researchers to make absolute determinations regarding the quantity of DNA copies present, thereby facilitating not only the qualitative assessment of microbial load but also its quantitative evaluation. However, the quantification of DNA by qPCR is a complex procedure that requires strict compliance with rigorous requirements for standards and their quantification. Failure to adhere to these principles can lead to erroneous conclusions.

Viability assessment, the second pillar, refers to the inability of PCR-based methods to assess the viability of microorganisms, as the presence of DNA/RNA alone provides no information about the physiological state of the living cell. This aspect is addressed through utilization viability PCR (vPCR), where chemical dyes such as propidium monoazide (PMA) or newer metal-based compounds like platinum or palladium salts selectively inhibit PCR amplification of DNA from dead cells. This strategy enables to selectively differentiate between live and dead cells of particular microorganism, circumventing the growth-dependency of cultures.

Finally, multiplex diagnostics with technologies such as xMAP enable the simultaneous detection of dozens of microbial targets. Conventional diagnostics procedures, such as those employed in research and routine qPCR, are capable of detecting up to four or five targets within a single analytical run. However, there exist certain applications for which a more

sophisticated targeted analysis is required. MOL-PCR, a particularly noteworthy example, capitalizes on the specificity of ligation and the versatility of bead-based xMAP platforms, enabling scalable, high-throughput assays for pathogen identification, single-nucleotide polymorphism (SNP) typing, and resistance profiling.

Each of these molecular applications addresses a limitation of traditional culture diagnostics. Quantification by qPCR or dPCR provides numerical insight into microbial load, in that requires a specific interpretation criteria. For example, in the case of *Mycobacterium avium* subsp. *paratuberculosis* (MAP), quantification allows veterinarians to distinguish infected animals from passive shedders using threshold models based on DNA copy numbers.

Viability assays offer a functional improvement over culture, which fails to detect difficultly culturable microorganisms or viable but non-culturable organisms. Using MAP as the model example, the ability to detect live MAP cells in milk, even after pasteurization, highlights the importance of distinguishing DNA from dead cells and infectious agents. The optimization of metal-based dyes has enhanced the efficiency and reliability of these methods, thereby reducing the complexity of light-activated protocols, such as PMA treatment.

Multiplexing, in contrast, addresses the need for efficiency and complex targeted analysis of the sample. Conventional PCR or qPCR is constrained in its capacity to target a limited number of genes, whereas MOL-PCR facilitates the concurrent detection of up to 50 targets, including specific DNA regions, resistance genes, and virulence factors. The assay's modular design facilitates rapid updates, scalability, and integration into diverse workflows, ranging from food safety to biothreat surveillance.

Together, these three diagnostic capabilities offer a complementary toolkit: quantification delivers clarity on microbial burden, viability testing ensures biological relevance, and multiplexing enhances diagnostic breadth without increasing resource use.

3. Structure of the habilition thesis

This thesis presents a collection of 17 peer-reviewed papers published between 2008 and 2022, which address three aspects of the utilization of PCR-based methods in microbiology. The publications are organized according to three primary categories: the utilization of qPCR in quantification of microorganisms (1-6), the determination of viability by culture-independent techniques (7-13), and multiplexing options to expand the possibilities of PCR (14-17).

To date, I have authored or co-authored 75 publications in peer-reviewed journals. A selection of 17 articles related to the three aspects of utilization of PCR based methods in microbiology I consider to be my the most significant contribution to the field. The following tables offer a synopsis of my contributions to these articles, with particular emphasis on my roles in experimental work, student supervision, manuscript preparation, and research guidance.

Slana, I., Kralik, P., Kralova, A., Pavlik, I., 2008. On-farm spread of Mycobacterium avium subsp paratuberculosis in raw milk studied by IS900 and F57 competitive real time quantitative PCR and culture examination. INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY 128, 250-257.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
50	-	40	20

Kralik, P., Slana, I., Kralova, A., Babak, V., Whitlock, R.H., Pavlik, I., 2011. Development of a predictive model for detection of Mycobacterium avium subsp paratuberculosis in faeces by quantitative real time PCR. VETERINARY MICROBIOLOGY 149, 133-138.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
80	100	80	50

Kralik, P., Pribylova-Dziedzinska, R., Kralova, A., Kovarcik, K., Slana, I., 2014. Evidence of passive faecal shedding of Mycobacterium avium subsp paratuberculosis in a Limousin cattle herd. VETERINARY JOURNAL 201, 91-94.

Experimental work (%)	**Supervision (%)**	**Manuscript (%)**	**Research direction (%)**
50	50	50	50

Kralik, P., Ricchi, M., 2017. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. FRONTIERS IN MICROBIOLOGY 8.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
80	100	70	50

Beinhauerova, M., Babak, V., Bertasi, B., Boniotti, M.B., Kralik, P., 2020. Utilization of Digital PCR in Quantity Verification of Plasmid Standards Used in Quantitative PCR. FRONTIERS IN MOLECULAR BIOSCIENCES 7.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	20	100

Beinhauerova, M., Beinhauerova, M., McCallum, S., Seller, E., Ricchi, M., O'Brien, R., Blanchard, B., Slane, I., Babak, V., Kralik, P., 2021. Development of a reference standard for the detection and quantification of Mycobacterium avium subsp. paratuberculosis by quantitative PCR. SCIENTIFIC REPORTS 11.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	30	100

Kralik, P., Nocker, A., Pavlik, I., 2010. Mycobacterium avium subsp paratuberculosis viability determination using F57 quantitative PCR in combination with propidium monoazide treatment. INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY 141, S80-S86.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
100	60	80	70

Pribylova, R., Kubickova, L., Babak, V., Pavlik, I., Kralik, P., 2012. Effect of short- and long-term antibiotic exposure on the viability of Mycobacterium avium subsp paratuberculosis as measured by propidium monoazide F57 real time quantitative PCR and culture. VETERINARY JOURNAL 194, 354-360.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
60	80	50	90

Kralik, P., Babak, V., Dziedzinska, R., 2014. Repeated cycles of chemical and physical disinfection and their influence on Mycobacterium avium subsp paratuberculosis viability measured by propidium monoazide F57 quantitative real time PCR. VETERINARY JOURNAL 201, 359-364.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
80	100	50	100

Ricchi, M., De Cicco, C., Kralik, P., Babak, V., Boniotti, M.B., Savi, R., Cerutti, G., Cammi, G., Garbarino, C., Arrigoni, N., 2014. Evaluation of viable Mycobacterium avium subsp paratuberculosis in milk using peptide-mediated separation and Propidium Monoazide qPCR. FEMS MICROBIOLOGY LETTERS 356, 127-133.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
10	50	40	40

Kralik, P., Babak, V., Dziedzinska, R., 2018. The Impact of the Antimicrobial Compounds Produced by Lactic Acid Bacteria on the Growth Performance of Mycobacterium avium subsp paratuberculosis. FRONTIERS IN MICROBIOLOGY 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
80	100	50	80

Cechova, M., Beinhauerova, M., Babak, V., Slana, I., Kralik, P., 2021. A Novel Approach to the Viability Determination of Mycobacterium avium subsp. paratuberculosis Using Platinum Compounds in Combination With Quantitative PCR. Frontiers in Microbiology 12.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	50	100

Cechova, M., Beinhauerova, M., Babak, V., Kralik, P., 2022. A viability assay combining palladium compound treatment with quantitative PCR to detect viable Mycobacterium avium subsp. paratuberculosis cells. Scientific Reports 12.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	100	50	100

Reslova, N., Michna, V., Kasny, M., Mikel, P., Kralik, P., 2017. xMAP Technology: Applications in Detection of Pathogens. FRONTIERS IN MICROBIOLOGY 8.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	50	30	100

Reslova, N., Huvarova, V., Hrdy, J., Kasny, M., Kralik, P., 2019. A novel perspective on MOL-PCR optimization and MAGPIX analysis of in-house multiplex foodborne pathogens detection assay. SCIENTIFIC REPORTS 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	50	20	100

Jelinkova, P., Hrdy, J., Markova, J., Dresler, J., Pajer, P., Pavlis, O., Branich, P., Borilova, G., Reichelova, M., Babak, V., Reslova, N., Kralik, P., 2021. Development and Inter-Laboratory Validation of Diagnostics Panel for Detection of Biothreat Bacteria Based on MOL-PCR Assay. MICROORGANISMS 9.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	30	20	100

Hrdy, J., Vasickova, P., Nesvadbova, M., Novotny, J., Mati, T., Kralik, P., 2021. MOL-PCR and xMAP Technology A Multiplex System for Fast Detection of Food- and Waterborne Viruses. JOURNAL OF MOLECULAR DIAGNOSTICS 23, 765-776.

Experimental work (%)	Supervision (%)	Manuscript (%)	Research direction (%)
-	20	20	40

5. Aspect 1: Determination of DNA quantity by quantitative PCR

Quantitative PCR (qPCR), also known as real-time PCR, is a widely adopted technique in molecular biology for the quantification of DNA. The fundamental concept of Polymerase Chain Reaction (PCR) was first introduced by Kary Mullis in the 1980s, providing a revolutionary method for amplifying specific DNA sequences (Saiki et al., 1985). Initially, PCR was limited by the use of thermolabile enzymes that required frequent replenishment, rendering the process labor-intensive and impractical. The introduction of the thermostable DNA polymerase from Thermus aquaticus (Taq polymerase) significantly advanced the technique by enabling continuous cycling without enzyme degradation (Saiki et al., 1988).

The concept of monitoring DNA amplification in real-time, an essential feature distinguishing qPCR from conventional PCR, emerged in the early 1990s with the development of fluorescence-based detection methods (Higuchi et al., 1992; Holland et al., 1991). This advancement allowed researchers not only to detect the presence of target DNA but also to quantify it precisely through the measurement of fluorescence after each amplification cycle. The fluorescence signal, generated either by non-specific intercalating dyes like SYBR Green or highly specific fluorescently labeled probes, proportionally corresponds to the initial amount of PCR product (Kubista et al., 2006).

The capacity of qPCR to provide rapid, sensitive, and accurate quantification of nucleic acids has facilitated its widespread use across various fields, including clinical diagnostics, microbial detection, genetic research, and forensic sciences (Bustin et al., 2009). The probe-based chemistry, most commonly utilizing TaqMan probes, prevails in the routine diagnostics nowadays. Over the years, significant enhancements in instrumentation, reagents, and data analysis software have further established qPCR as a robust and essential tool in modern molecular diagnostic laboratories.

Besides the qualitative analysis, the qPCR itself allows the determination of the starting amount of DNA in the sample. Quantification by qPCR can be performed either relatively or absolutely. Relative quantification is commonly used in research and gene expression studies, comparing the expression levels of target genes to reference or housekeeping genes. Absolute quantification, on the other hand, involves determining the exact copy number of the target DNA in a sample, and it is predominantly used in microbial diagnostics. Several types of

standards are commonly employed in qPCR assays, each offering specific advantages and limitations. These include purified PCR products, recombinant plasmids containing the target sequences, and genomic DNA standards extracted directly from target organisms. PCR product standards are easy to produce but less stable over time, whereas plasmid standards provide higher stability and convenience for long-term storage (Beinhauerova et al., 2021). Genomic DNA standards most closely mimic the natural DNA targets present in samples, thus potentially offering higher accuracy, but their preparation can be more complex and can change batch-to-batch over time (Kralik and Ricchi, 2017).

When qPCR is applied routinely for diagnostic purposes, its reliability and consistency depend heavily on rigorous standardization. Several critical performance characteristics must be established, including analytical specificity and sensitivity (Limit of Detection, LOD), precision, trueness, and diagnostic sensitivity and specificity (Kralik and Ricchi, 2017). Analytical specificity pertains to the assay's ability to exclusively detect the intended DNA target, without cross-reacting with non-target DNA, while analytical sensitivity or LOD refers to the smallest concentration of DNA reliably detectable by the assay. Diagnostic sensitivity and specificity measure the accuracy of the qPCR assay in correctly identifying positive and negative samples, respectively.

One of the most critical parameters for quantification of DNA by qPCR assays is the limit of quantification (LOQ). LOQ is defined as the lowest concentration of DNA that can be reliably quantified with acceptable precision and accuracy. Determining the LOQ is challenging due to the absence of standardized criteria or universally accepted guidelines. Various strategies have been proposed for its determination, including a statistical approach suggested by Kralik and Ricchi (2017). This approach involves using a series of dilutions of the target DNA and defining LOQ based on the concentration at which precision (typically represented by the coefficient of variation) remains within an acceptable threshold, commonly below 25% (Kralik and Ricchi, 2017). Despite such suggestions, the establishment of LOQ remains laboratory-specific and can vary depending on the target DNA, assay conditions, and reagents used.

Moreover, quantitative data derived from qPCR assays can be potentially misleading due to their reliance on accurate quantification of standards. Standards employed in qPCR are typically precisely defined DNA molecules whose concentrations are measured mainly spectrophotometrically. However, spectrophotometric quantification of all types of the

standards (genomic DNA, purified PCR products and plasmids) can be problematic due to inherent variability in DNA purity and potential contamination, leading to inaccurate quantifications over time (Kralik and Ricchi, 2017). Additionally, the physical state of plasmid standards, specifically their superhelical conformation, can significantly influence amplification efficiency (Beinhauerova et al., 2020). Supercoiled plasmids can amplify differently compared to linear or relaxed circular plasmids, potentially leading to substantial deviations in calculated DNA quantities (Beinhauerova et al., 2020, Kralik and Ricchi, 2017).

The development in PCR technologies in the past 15 years led to the introduction of digital PCR (dPCR). Unlike qPCR, dPCR partitions the DNA sample into thousands of individual reaction compartments, such as droplets in droplet digital PCR (ddPCR) or microfluidic chambers in chip-based digital PCR (cdPCR). Each partition ideally contains one or several or no target DNA molecules (dependent on the input DNA concentration), which undergo PCR amplification independently. After amplification, partitions are evaluated for fluorescent signals, allowing direct counting of positive partitions containing amplified DNA.

In droplet digital PCR (ddPCR), the partitioning of DNA occurs within thousands of uniform oil droplets generated by specialized equipment. These droplets function as individual reaction vessels, where each droplet is analyzed separately after PCR. Conversely, chip-based digital PCR (cdPCR) partitions samples into numerous isolated chambers etched into a microfluidic chip. These chambers provide stable and consistent environments for amplification, allowing precise and reproducible quantification of target molecules (Beinhauerova et al., 2020).

The concentration of target DNA is calculated based on the fraction of positive partitions using Poisson statistics, providing absolute quantification independent of standard curves. Despite its inherent accuracy and precision, dPCR is not yet widespread in routine diagnostic laboratories due to several practical limitations. The specialized instrumentation required for dPCR is still costly and less accessible than conventional qPCR platforms, restricting its broader adoption. Additionally, dPCR procedures are typically more labor-intensive, involving complex sample preparation and careful handling during partitioning, which increases manual workload and processing time compared to qPCR. Due to these logistical and economic constraints, dPCR is often relegated to research laboratories.

Nevertheless, dPCR's unique ability to provide highly accurate quantification without external standards has driven proposals for its integration as a standardized procedure for the

quantification of qPCR standards. Employing dPCR to quantify DNA standards used in qPCR ensures accuracy, reproducibility, and consistency across different batches and laboratories. This integration allows routine control and monitoring of standard quality and stability over time, enhancing the reliability of quantitative diagnostics and research outcomes (Beinhauerova et al., 2020).

Experiences gained from the use and development of quantitative PCR (qPCR) and digital PCR (dPCR) techniques have significantly contributed to the establishment of a standardized reference method for quantifying MAP. This reference standardization initiative parallels the approach commonly adopted in clinical diagnostics, where international reference standards provide reliable quantitative benchmarks independent of laboratory-specific factors (Beinhauerova et al., 2021).

MAP is the causative agent of Johne's disease, a chronic infectious disease primarily affecting cattle and other ruminants. Johne's disease leads to significant economic losses due to decreased productivity, weight loss, reduced milk production, infertility, and eventual death of infected animals. Identifying infected animals accurately and differentiating them from passive shedders—animals that merely excrete ingested MAP without actual infection—is crucial. Passive shedders pose no real threat of spreading the infection, whereas infected animals actively propagate MAP and contaminate the environment, perpetuating the disease cycle (Slana et al., 2008a; Kralik et al., 2014).

The precise quantification of MAP in feces is essential to distinguish truly infected animals from passive shedders. Quantification is particularly challenging because MAP is often present in low concentrations, complicating detection and quantification (Kralik et al., 2011). To address this challenge, it was essential to solve two major problems. The first problem entailed establishing a quantitative threshold between passive shedders and infected animals, with the quantity of DNA being determined by qPCR. The term "passive shedder" was initially characterized based on culture data, and it was imperative to refine its definition for the interpretation of qPCR data. To this end, a predictive mathematical-statistical model was developed to assist in identifying this threshold. This model integrated quantitative qPCR data with clinical and epidemiological observations to define a reliable cutoff value. Through extensive validation and statistical analysis, the model identified 1,000 MAP cells per gram of feces as a critical threshold (Kralik et al., 2011). Animals shedding below this level were

classified as passive shedders, while those above were considered truly infected. This distinction is crucial for informing management decisions and limiting the spread of MAP within and between herds (Kralik et al., 2014; Beinhauerova et al., 2021).

The second problem to be resolved was the development and implementation of a universal reference standard that would address discrepancies in the quantification of MAP in different laboratories by their own methods. The objective of this standard was to provide laboratories worldwide with a robust framework to harmonize their quantification practices. The main idea was not to push for a universal reference qPCR method with a standardized quantification protocol. Such an initiative would rather be vain because the majority of laboratories performing MAP diagnostics would not adopt a prescribed method and would rely on their own protocols. An alternative solution was the preparation of an internationally recognized reference standard. In this scenario, laboratories can use the reference standard directly as a calibration point or to validate their internal qPCR standards against this reference. Such harmonization is expected to facilitate uniform reporting and interpretation of qPCR data worldwide, significantly improving disease control strategies (Beinhauerova et al., 2021).

The reference standard for MAP quantification was rigorously evaluated through an international ring trial involving 6 laboratories worldwide. This collaborative effort demonstrated the reliability and robustness of the reference standard across diverse laboratory environments and methodologies. Results confirmed the effectiveness and reproducibility of the reference standard, supporting its adoption as a global benchmark for MAP quantification (Beinhauerova et al., 2021).

The publication of a small-scale preparation and evaluation of the reference standard initiated the commercial application of the idea. One of the partners involved in the testing, the French company BioSellal, in collaboration with its partner Labocea, recently introduced a commercial version of the international MAP reference standard. Presently, the reference standard is undergoing laboratory evaluation in French research facilities and is utilized as a benchmark to differentiate passive shedders from animals that are likely infected. Following the evaluation, the reference standard will be incorporated into the French national paratuberculosis control program, which is currently being developed.

6. Aspect 2: Determination of microbial viability by qPCR

The assessment of microbial viability constitutes a critical component of microbiological diagnostics, particularly within the domains of clinical, food, and environmental or forensic microbiology. Conventionally, the prevailing standard method for determining the viability of microorganisms, particularly bacteria, has been culture-based techniques. These methods depend on the capacity of viable cells to proliferate on suitable nutrient media under controlled laboratory conditions. The presence of viable bacteria in a sample is thus inferred from the formation of visible colonies, which remains a foundational criterion for proving bacterial presence and potential pathogenicity. It should also be noted that the isolation of bacteria from the original material is critical for determining the antimicrobial resistance phenotype or the synthesis of specific molecules, such as toxins (Nocker et al., 2007).

However, bacterial culture is associated with several significant limitations. One major challenge is the vast diversity of bacterial species, each with unique and often demanding nutritional and environmental requirements for growth. Many species require specific temperature ranges, pH levels, oxygen tensions, or growth factors that may not be easily replicable in vitro. As a result, the successful cultivation of certain bacteria may necessitate complex and time-consuming optimization of culture conditions (Nocker et al., 2006).

Furthermore, a substantial fraction of environmental and host-associated bacterial populations comprises so-called “viable but non-culturable” (VBNC) cells or bacteria that are inherently unculturable using standard laboratory media. These bacteria, although alive and potentially metabolically active, do not grow on conventional media and hence escape detection by traditional culture methods. This is particularly problematic in ecological studies and diagnostics where the accurate detection of all viable cells is essential (Soejima et al., 2015).

The limitations of culture-based methods thus underscore the need for alternative approaches capable of reliably identifying viable microorganisms without dependence on growth. These alternatives, including those based on molecular biology and chemical viability markers, offer more comprehensive insights into microbial presence and activity, particularly in samples with fastidious or unculturable bacteria.

The development of viability PCR methods using intercalating dyes has significantly advanced microbial research, particularly through the use of ethidium monoazide (EMA) and later propidium monoazide (PMA). EMA was first introduced as a tool for discriminating viable from dead microbial cells in combination with PCR. It is a DNA-intercalating dye carrying an azide group that allows covalent crosslinking to DNA upon photoactivation by visible light. EMA preferentially penetrates cells with compromised membranes—typically dead cells—and binds to DNA, which, once crosslinked, is no longer amplifiable by PCR. This enables selective suppression of PCR signals from non-viable cells, allowing the detection of only viable organisms (Nogva et al., 2003).

However, EMA demonstrated limitations in selectivity, as it could also penetrate viable cells of certain bacterial species, particularly those with naturally more permeable membranes or in sub-lethally injured states. This resulted in the unintended inhibition of amplification from viable cell DNA, prompting the search for more selective alternatives (Nocker et al., 2006).

PMA, a structural analogue of EMA, was developed to address these limitations. It features a higher charge, which significantly reduces its ability to pass through intact cell membranes, thereby enhancing its selectivity for dead cells. Like EMA, PMA binds to DNA of membrane-compromised cells and, upon light activation, forms covalent bonds that prevent amplification during qPCR (Nocker et al., 2007).

These methods have also been extended to the detection of viral infectivity. Although viruses do not possess membranes in the bacterial sense, viability PCR using EMA or PMA has been applied based on capsid integrity. Only viruses with damaged capsids allow dye penetration and subsequent genome inactivation. For example, PMA has been successfully used to discriminate between infectious and heat-inactivated norovirus and enteric adenoviruses (Randazzo et al., 2018; Fraisse et al., 2018). However, efficacy varies by virus type and treatment conditions, underscoring the need for continued optimization.

In response to the limitations of EMA and PMA, particularly their partial permeability to viable cells and the need for light activation, alternative approaches have been suggested using metal-based compounds—most notably platinum (Pt) and palladium (Pd) salts. These compounds offer several advantages over monoazide dyes. Due to their smaller molecular size and lack of requirement for photoinactivation, Pt and Pd salts streamline the viability PCR

workflow and reduce the risk of introducing variability during light exposure steps (Soejima et al., 2015; Soejima and Iwatsuki, 2016).

The mechanism of action of these metal salts draws inspiration from platinum-based anticancer drugs, such as cisplatin. These compounds bind covalently to nucleic acids via coordination with nitrogen atoms in purine bases, forming stable DNA adducts that inhibit polymerase activity. Importantly, Pt and Pd compounds selectively penetrate compromised microbial cells. The results interpretation is identical for the EMA- or PMA-treated samples. As viable cells those with intact undamaged membranes are considered (Soejima et al., 2015; Soejima and Iwatsuki, 2016).

Applications of these compounds have been demonstrated not only for bacteria, including Enterobacteriaceae (Soejima and Iwatsuki, 2016), but also for viruses such as norovirus (Fraisse et al., 2018) and hepatitis E virus (Randazzo et al., 2018), showing improved selectivity and sensitivity over EMA and PMA in viability PCR assays.

The application of viability PCR methods using intercalating dyes and metal salts has proven particularly valuable in the study of MAP. As it was mentioned in the previous chapter, a central challenge in the microbiological detection of MAP is its notoriously slow growth rate and complex cultivation requirements, often taking up to 16 weeks for colonies to develop on solid media. This significantly hampers timely diagnostics and research into viable MAP detection. While the viability of MAP is not a primary concern when it is detected in its primary source, i.e., feces, it becomes a significant issue when MAP must be detected in milk. The presence of MAP in milk is a matter of serious concern, as evidence has emerged linking its development with Crohn's disease in human subjects; however, definitive proof of this connection remains elusive. The culture of MAP from milk poses significant challenges due to the low number of MAP cells present (up to 100 cells per milliliter) and the necessity of decontaminating the sample prior to culture. However, the detection of viable MAP cells in milk is of significant concern, as it has been documented that MAP can withstand the conventional pasteurization process (Slana et al., 2008b). Conventional qPCR methods demonstrate challenges in detecting MAP in milk. The scarcity of MAP in milk, compounded by its challenging nature due to its high protein and fat content, renders DNA isolation and subsequent qPCR detection particularly intricate processes (Slana et al., 2008a). Consequently, there is an imperative for methods that can provide insights into the viability of MAP. Consequently, alternative methods capable of

distinguishing between viable and non-viable cells without reliance on culture are imperative. Among these, PMA-qPCR was one of the first approaches evaluated (Kralik et al., 2010).

Initial experiments demonstrated that standard PMA treatment conditions, as used for Gram-negative or less hydrophobic bacteria, were insufficient to achieve effective discrimination between live and dead MAP cells. The dye penetration into dead MAP cells was suboptimal, leading to residual amplification from non-viable DNA and, consequently, overestimation of viability (Kralik et al., 2010).

However, a systematic optimization of PMA treatment parameters, including not only adjustments in dye concentration, incubation time, light exposure duration, and importantly, the inclusion of mechanical sample pre-treatments such as vortexing with glass beads or the addition of surfactants (e.g., Triton X-100 or SDS), were shown to improve dye accessibility in the thick mycobacterial cell envelope. The most effective protocol for the optimized PMA-qPCR method was then applied to MAP cultures containing defined ratios of live and dead cells. The results demonstrated a substantial decrease in the qPCR signal from heat-killed MAP samples compared to live controls, thereby substantiating the efficacy of the protocol in suppressing DNA from dead cells (Kralik et al., 2010).

Following the successful optimization of PMA-qPCR for the selective detection of viable MAP, the method was applied in a series of experimental studies aimed at evaluating its utility across various in vitro and practical scenarios. One of the first applications was the study of MAP exposed to antibiotics. Pribylova et al. (2012) conducted experiments comparing traditional culture-based methods and PMA-qPCR in assessing MAP viability following short- and long-term exposure to various antimycobacterial drugs, including vancomycin, amphotericin B, and nalidixic acid. The results showed high agreement between culture and PMA-qPCR data, with both methods indicating similar trends in viability decline. These findings led to the conclusion that PMA-qPCR could serve as a viable replacement for culture in controlled in vitro viability experiments on MAP (Pribylova et al., 2012).

Building upon this foundation, the PMA-qPCR method solely was next applied to evaluate the effect of commercially available disinfection agents, including those containing quaternary ammonium compounds and oxidizing agents and UV light on MAP cells of three different isolates. Crucially, the study introduced repeated cycles of disinfection (up to three cycles) to simulate real-world exposure scenarios in food processing or environmental settings. The

results showed that certain MAP isolates developed increased resistance to chlorine and UV treatment over successive cycles, as indicated by persistent viability signals in PMA-qPCR. However, peracetic acid remained the only disinfectant capable of completely inactivating MAP regardless of the number of disinfection cycles applied. PMA-qPCR proved particularly useful in detecting viable cells in some intermediate disinfection stages, thereby revealing its enhanced sensitivity in viability monitoring. This application demonstrated that repeated sub-lethal exposure to disinfectants can lead to increased tolerance in MAP populations and highlighted the importance of using robust and accurate methods like PMA-qPCR for evaluating long-term biocide efficacy (Kralik et al., 2014).

Another innovative application was the exposure of MAP to supernatants from lactic acid bacteria (LAB) cultures, which are known to contain natural antimicrobial compounds such as bacteriocins, organic acids, and hydrogen peroxide. Three MAP strains were treated with filtered cell-free supernatants prepared with the commercially available milk cultures, commercial nisin, and lactic acid. PMA-qPCR revealed significant reductions in viability, especially after treatment with nisin and specific LAB-derived supernatants. The study confirmed that PMA-qPCR could sensitively detect changes in MAP viability in response to complex biological treatments and could support screening of natural antimicrobial candidates (Kralik et al., 2018).

Lastly, the application of PMA-qPCR to raw milk samples was demonstrated as a crucial step in evaluating the method's practical usability. Ricchi et al. (2014) adapted the technique to milk spiked with MAP, applying peptide-mediated magnetic separation (PMS) prior to PMA treatment and qPCR detection. The results demonstrated the ability of the assay to accurately differentiate viable from dead MAP cells even in this complex matrix. Furthermore, when the method was tested on pasteurized milk, it was able to detect residual viability, confirming previous concerns that MAP may survive standard pasteurization processes. These results highlight the potential for PMA-qPCR as a rapid and accurate tool for MAP detection in dairy food safety surveillance (Ricchi et al., 2014).

The application of Pt and Pd salts in viability PCR represents an advanced alternative to PMA for the detection of viable MAP. Building upon the groundwork established by PMA-qPCR, studies by Beinhauerova et al. (2021) and Cechova et al. (2022) explored the effectiveness of

Pt and Pd compounds, respectively, in selectively inhibiting amplification from DNA originating from dead MAP cells.

In the study by Beinhauerova et al. (2021), various Pt salts were evaluated for their ability to discriminate viable and non-viable MAP cells. Among them, platinum(IV) chloride ($PtCl_4$) was identified as the most effective compound. The optimal treatment protocol involved incubation of MAP cells with $PtCl_4$ in phosphate-buffered saline, without the need for any light activation. The method showed a clear reduction of qPCR signal from heat-treated MAP cells, while amplification from viable cells remained unaffected. $PtCl_4$ treatment resulted in a Ct shift of up to 10 cycles for dead cells, highlighting its high efficiency in blocking amplification of non-viable MAP (Beinhauerova et al., 2021).

A parallel study by Cechova et al. (2022) investigated several Pd-based compounds for their potential in MAP viability assessment. Palladium acetate ($Pd(OAc)_2$) demonstrated the highest selectivity and effectiveness. Similar to $PtCl_4$, Pd salts did not require photoinitiation and performed well at room temperature with short incubation times. Pd-treated dead MAP cells showed substantial suppression of qPCR signals, with results comparable to Pt treatment. Importantly, both Pt and Pd salts exhibited minimal inhibitory effects on viable cell DNA, ensuring accurate detection of living bacteria. Pd salts also offered advantages in terms of lower toxicity and better solubility in aqueous solutions compared to Pt salts (Cechova et al., 2022).

When compared to the previously established PMA-based method described by Kralik et al. (2010), both Pt and Pd treatments provided similar or improved discrimination between viable and non-viable MAP cells. PMA treatment required a more complex workflow involving dark incubation, precise light activation, and sometimes additional mechanical treatment to facilitate dye entry through MAP's lipid-rich cell wall. These steps increased the overall complexity and potential variability of the method. In contrast, Pt and Pd salts offered simplified handling, faster processing, and reduced reliance on specialized equipment such as photoactivation systems.

However, each approach has its own limitations. PMA has been extensively validated across a wide range of bacterial species and matrices, and its photoactivation mechanism ensures permanent DNA inactivation. Pt and Pd salts, although promising, may present matrix-specific challenges. For example, complex biological fluids or environmental inhibitors could interfere

with metal ion binding or reduce penetration efficiency. Moreover, concerns related to heavy metal handling, disposal, and potential interference with downstream reactions still require comprehensive evaluation in routine diagnostic workflows (Beinhauerova et al., 2021; Cechova et al., 2022).

In conclusion, both Pt and Pd salts represent powerful and efficient alternatives to PMA for viability PCR targeting MAP. While PMA remains a well-established standard, metal-based approaches offer practical advantages in terms of workflow and selectivity. Future applications could benefit from a combined strategy or further optimization depending on the sample matrix and diagnostic requirements (Beinhauerova et al., 2021; Cechova et al., 2022).

In summary, culture remains the conventional gold standard for determining microbial viability. However, it can be inadequate due to its limitations in terms of speed, specificity, and its inability to recover viable but non-culturable organisms. However, modern molecular methods employing qPCR in conjunction with viability markers have been optimized for a multitude of bacterial, viral, and parasitic pathogens. These methods provide researchers with a powerful tool to distinguish between live and dead cells based on membrane integrity and nucleic acid accessibility. This is particularly advantageous in the context of viral and parasitic agents, where the *in vitro* propagation systems are scarce or unavailable for the majority of these organisms.

Despite their broad potential, viability qPCR methods remain largely confined to the research domain. Barriers to routine diagnostic adoption include the added complexity of sample processing, a lack of standardized interpretation criteria, and a general skepticism within regulatory and diagnostic laboratories toward new, non-validated technologies.

However, the example of MAP as a difficult-to-grow *in vitro* organism with a significant impact on animal health demonstrates the significant potential of these methods. Studies employing PMA and Pt/Pd-based viability PCR have demonstrated their efficacy in facilitating the transition from experimental research to practical diagnostic applications. This is particularly evident in the context of detecting viable MAP cells in milk, as it pertains to the survival of MAP cells after pasteurization and its association with the development of Crohn's disease. With further validation and simplification, these techniques hold promise to extend beyond research into routine practice, improving the accuracy and speed of microbial viability assessment across sectors.

7. Aspect 3: Multiplex detection of microorganisms

In the field of microbial diagnostics, detection approaches can broadly be divided into non-targeted and targeted strategies. Non-targeted methods, particularly high-throughput sequencing technologies, provide an unbiased overview of microbial communities or potential pathogens in a sample without prior knowledge of their presence. These approaches are powerful for metagenomic profiling and the discovery of novel agents, but they are often resource-intensive and limited in sensitivity when pathogen DNA is present in low abundance relative to host or background DNA.

In contrast, targeted detection methods focus on specific, known nucleic acid sequences associated with microbial agents. Among these, qPCR remains the gold standard due to its high sensitivity, specificity, and quantification capability. qPCR assays typically rely on sequence-specific primers and probes to amplify defined regions of microbial genomes. However, their main limitation lies in the restricted multiplexing capacity, usually only up to four or five targets can be reliably detected in a single reaction due to fluorescence channel availability.

To overcome these multiplexing limitations, alternative approaches have been developed. Notably, the xMAP (multi-analyte profiling) technology, developed by Luminex Corporation is a microsphere-based suspension array platform enabling simultaneous detection of tens to hundreds of targets in a single reaction by combining PCR with fluorescently labeled microspheres. This high-throughput system was originally designed for protein quantification and immunoassays, but its modularity has also facilitated adaptation for nucleic acid-based detection. Despite its broad success in immunodiagnostics, the use of xMAP for DNA-based assays remains relatively limited, though it offers significant multiplexing capabilities and flexibility for molecular diagnostics.

The cornerstone of the xMAP technology is a set of color-coded polystyrene or paramagnetic microspheres (beads), each internally dyed with two fluorescent dyes in varying ratios. This results in up to 100 distinct bead sets, each with a unique spectral identity, allowing parallel detection of up to 100 different targets in one sample (Reslová et al., 2017). Each bead set can be covalently coupled with a capture molecule—typically an oligonucleotide (in DNA assays) or an antibody (in immunoassays). There are two primary types of xMAP beads: polystyrene beads and magnetic beads (MagPlex®). MagPlex beads, composed of

paramagnetic materials, are preferred in many molecular applications because they enable efficient magnetic separation and washing, which enhances signal clarity and reduces background noise. Among the various instruments designed for xMAP analysis, the MagPix platform represents a compact and cost-effective solution particularly suitable for DNA assays. Unlike flow cytometry-based instruments such as Luminex 100/200, FLEXMAP 3D or IntelliFlex, which use lasers and fluidics to identify and interrogate beads individually, MagPix relies on CCD imaging and LED-based excitation.

In MagPix, the bead mixture is immobilized magnetically in a monolayer inside the detection chamber. The instrument sequentially excites the beads using red and green LEDs to determine the bead identity (based on internal dye ratios) and the bound reporter signal, respectively. Detection is performed via a CCD camera, which captures the fluorescent signal of up to 50 beads per bead set per well, ensuring statistically robust measurements. The use of magnetic beads streamlines the workflow by simplifying washing and transfer steps. The MagPix system supports up to 50-plex assays and offers advantages such as lower sample and reagent consumption, rapid run time (~60 minutes per 96-well plate), and user-friendly operation. Its compact format and affordability make it especially attractive for research and diagnostic laboratories implementing multiplex nucleic acid detection.

For DNA-based assays, the detection principle commonly relies on the xTAG strategy. xTAG refers to a universal tag/anti-tag hybridization system, in which target-specific oligonucleotide probes are extended or ligated with a short, unique nucleotide sequence (the "TAG"). This TAG sequence is complementary to an "anti-TAG" oligonucleotide immobilized on a specific magnetic bead set. Each TAG sequence is matched to a unique bead identity, enabling unambiguous assignment of the signal to the original DNA target.

The typical xMAP DNA assay involves three key steps: (1) target amplification or probe ligation, (2) hybridization of the amplified or labeled products to the bead-coupled anti-TAG sequences, and (3) fluorescence-based detection using a Luminex instrument. Several assay formats exist, including direct hybridization, allele-specific primer extension (ASPE) or single-base extension. These formats allow detection of gene presence/absence, point mutations, or species-specific polymorphisms.

In the direct hybridization format of xMAP DNA assays, fluorescently labeled PCR products are hybridized directly to beads coupled with sequence-specific anti-TAG oligonucleotides.

Each bead captures a unique target through complementary binding, enabling parallel detection of multiple sequences. This approach is relatively simple and well-suited for detecting presence or absence of specific genes but offers limited discrimination of closely related sequences.

Allele-Specific Primer Extension (ASPE) provides greater specificity, especially for SNP detection. In this format, target DNA is amplified, and allele-specific primers, each bearing a unique TAG sequence, are extended by DNA polymerase only if their 3' end is perfectly complementary to the target. Successful extension incorporates a biotin label, allowing hybridization of the extended product to the corresponding bead and subsequent fluorescence detection. This method enables precise genotyping by differentiating allelic variants at single nucleotide resolution.

Foundational insights and technological advances in multiplex DNA detection, especially in the context of pathogen diagnostics provided ligation-based DNA detection strategies exploiting the high specificity of DNA ligases to join oligonucleotides only when they are perfectly hybridized to a complementary target. One of the approaches was the design of modular ligation assays using linear oligonucleotides with complementary sequences to distinguish different Brucella species. In this approach, short oligonucleotide probes were ligated on target DNA and the ligation products were captured and detected via capillary electrophoresis. This method enabled simultaneous interrogation of the species (Wattiau et al., 2011).

Another ligation-based approach can be based on the use of padlock probes (PLPs), circularizable oligonucleotides that hybridize to adjacent sequences on a DNA template. Upon perfect complementarity, the 5' and 3' ends of the PLP are brought into proximity and ligated, forming a circular molecule. This structure can then be amplified via rolling circle amplification or detected through various hybridization strategies. PLPs were combined with universal microarray detection for multiplex detection of plant fungal pathogens, offering high specificity and flexibility due to the customizable probe design and ability to incorporate TAG sequences for downstream hybridization (Szemes et al., 2005).

Although effective, these earlier ligation assays were often complex, requiring probe design tailored to each target and sometimes involving labor-intensive detection steps. The development of as Multiplex Oligonucleotide Ligation-PCR (MOL-PCR) built upon these

concepts, streamlining the assay workflow through the use of universal primers, standardized ligation chemistry, and bead-based detection in xMAP assays, thereby increasing throughput, flexibility, and ease of implementation. This property makes MOL-PCR particularly suitable for applications demanding high specificity, such as SNP discrimination or pathogen genotyping.

In the context of MOL-PCR, the ligation step facilitates the system's modularity. The incorporation of an additional target into an existing MOL-PCR assay is permitted at any time without necessitating a complete optimization procedure. The method commences with the hybridization of two adjacent oligonucleotide probes (MOLigo1 and MOLigo2) to a target DNA region. MOLigo1 incorporates a 5’-phosphorylated universal primer binding site and a unique TAG sequence, while MOLigo2 comprises a complementary sequence and the reverse primer site. The ligation process, which results in the formation of a single DNA molecule, is initiated by a thermostable DNA ligase only if both probes are perfectly matched and aligned on the target (Desphande et al., 2010).

The ligated product then serves as a template in a universal PCR, using fluorescently labeled primers to amplify all ligated products in a single reaction. After amplification, the products are hybridized to anti-TAG-coupled beads, each corresponding to a specific target sequence. Detection is performed via fluorescence readout on a suspension array platform. Because only ligated products are amplified and detected, MOL-PCR minimizes background signal and non-specific amplification, a common limitation in highly multiplexed PCR assays.

MOL-PCR supports detection of up to 50 targets on MagPix instrument simultaneously (theoretically the number of targets can be higher when other higher capacity instruments would be used) and is compatible with both presence/absence detection and SNP genotyping. The universal PCR step significantly reduces assay complexity, as all targets are amplified using the same primer pair. This uniformity also mitigates amplification bias, a common challenge in conventional multiplex PCR. Furthermore, the use of magnetic beads allows for efficient washing and reduced background, contributing to high signal-to-noise ratios.

MOL-PCR has been successfully implemented in various applied settings, from microbial subtyping to detection of antimicrobial resistance and biothreat agents. Its modularity, high specificity, and compatibility with suspension arrays make it well-suited for complex diagnostic and surveillance tasks.

Reslová et al. (2019) presented a comprehensive optimization of an in-house MOL-PCR assay for the simultaneous detection of foodborne pathogens using a MAGPIX platform. The developed panel targeted bacteria (Salmonella spp., Listeria monocytogenes, Escherichia coli O157), as well as parasitic pathogens such as Cryptosporidium parvum and Giardia intestinalis. The protocol included considerations that are necessary to take into account during optimization of critical parameters such as probe concentration, thermal cycling conditions, and hybridization conditions. Importantly, the assay achieved detection sensitivity comparable to qPCR.

Hrdy et al. (2021) extended the MOL-PCR platform to viral diagnostics by designing a multiplex panel targeting major food- and waterborne viruses: adenovirus 40/41 (AdV), rotavirus A (RVA), norovirus genogroups I and II (NoV GI/GII), hepatitis A virus (HAV), and hepatitis E virus (HEV). This was a significant advancement, as virus detection often relies on RT-qPCR with limited multiplexing capacity. The assay achieved detection limits as low as 5×10^{o} genome equivalents per reaction for several targets, showing excellent analytical sensitivity and specificity when tested across multiple laboratories in an interlaboratory ring trial. Notably, it maintained stable performance even with diluted RNA/DNA samples and included an internal control to ensure sample integrity. The approach also demonstrated robustness against false positives, as no-template and irrelevant-pathogen controls consistently returned negative results.

In the study by Boland et al. (2018), a custom MOL-PCR-based bead array was developed to characterize monophasic variants of *Salmonella enterica* serovar Typhimurium (STMV), particularly those lacking expression of the phase 2 flagellin gene fljB. The assay was designed to address the diagnostic challenges posed by monophasic strains serotyped as 1,4,[5],12:i:-, which are phenotypically indistinguishable from other Salmonella serovars but genetically linked to S. Typhimurium. The array targeted 15 genetic markers and was applied to 240 STMV isolates collected in Belgium, confirming their monophasic character and enabling the identification of 10 distinct genetic subtypes. This MOL-PCR assay demonstrated the ability to resolve closely related bacterial lineages and detect microevolutionary events associated with gene disruptions. The method provided valuable epidemiological insights into the diversity and spread of STMV lineages in food-producing animals and contributed to public health surveillance.

Boland et al. (2022) further extended the MOL-PCR platform to antimicrobial resistance (AMR) profiling through the development of the AMR-ARRAY, a 53-plex bead-based assay targeting resistance determinants across five antimicrobial classes—β-lactams, aminoglycosides, macrolides, fluoroquinolones, and colistin. This assay was applied to 648 Enterobacteriaceae isolates (including *E. coli, Salmonella*, and *Shigella*) sourced from animals, food, and clinical settings. The AMR-ARRAY successfully detected resistance-associated genes and key SNPs with high concordance to phenotypic resistance profiles (94.7%) and whole-genome sequencing data (99.3% selectivity, 100% specificity).

MOL-PCR has also proven particularly valuable in the rapid detection and characterization of biothreat agents, where multiplex capacity, specificity, and portability are essential. A compelling demonstration of its utility comes from a study by Jelínková et al. (2021), who developed and validated a MOL-PCR-based panel for the detection of high-risk bacterial biothreat agents. Their assay targeted *Bacillus anthracis, Yersinia pestis, Francisella tularensis, and Brucella* spp., integrating 11 specific genetic markers associated with species identity and virulence. The panel was evaluated through an international interlaboratory trial involving four reference laboratories. A set of 16 blinded DNA samples representing relevant pathogens and near-neighbors was analyzed. All labs achieved correct identification of the target organisms, confirming the reproducibility, robustness, and diagnostic accuracy of the MOL-PCR platform. Importantly, the assay was shown to be compatible with standard molecular biology workflows and the Luminex MagPix instrument, making it highly adaptable for use in public health laboratories and field surveillance contexts.

These findings are consistent with earlier work by Thierry et al. (2013), who applied MOL-PCR to SNP typing of Bacillus anthracis. Their 13-plex assay enabled phylogenetic assignment of isolates into major lineages and subgroups based on canonical SNPs, with allele-specific MOLigo probes designed for each target. The assay was successfully validated across multiple laboratories, demonstrating high interlaboratory reproducibility and offering a scalable alternative to real-time PCR-based SNP typing.

Complementing these efforts, Deshpande et al. (2010) illustrated the versatility of MOL-PCR by detecting multiple biothreat agents—including B. anthracis, Y. pestis, and F. tularensis—in a single reaction. Their protocol, integrating ligation, amplification, and bead-based

detection in under four hours, highlighted the method’s speed and modularity for pathogen surveillance and emergency response.

Together, all these studies underscore MOL-PCR's suitability for high-throughput and multiplex detection, with excellent performance in both centralized laboratories and potentially decentralized, field-adaptable formats. The array's modular design allowed rapid updates and incorporation of new targets. It must be highlighted that MOL-PCR is compatible with both DNA and RNA samples and even mixed DNA/RNA, which further expands its application potential beyond the detection. Additionally, MOL-PCR has demonstrated besides the presence/absence analysis value in SNP genotyping.

To summarize, the MOL-PCR technology represents a powerful tool in the targeted multiplex microbial detection. The findings from the presented papers are now utilized and expanded in the development of the routine applications, like the integrated system for the simultaneous identification of the 27 probiotic bacteria in the food supplements, identification of 29 prevalent as well as rare causal agents of the urinary infections or identification of 25 pathogens transmitted by ticks.

8. Conclusions and Outlook

This habilitation thesis has addressed three advanced yet underutilized applications of PCR-based methods in microbiology: quantification of DNA using qPCR, assessment of microbial viability through modified PCR approaches, and high-throughput multiplex detection using technologies such as MOL-PCR. Each of these techniques represents a step forward in overcoming the certain limitations of conventional diagnostic microbiology, particularly culture-based methods. While these aspects are methodologically feasible and supported by robust scientific evidence, their broader application remains limited by a variety of practical and conceptual barriers. This thesis sought not only to explore their potential but also to document their real-world implementation through a series of studies and published data, primarily in the context of MAP, a challenging model organism for diagnostic development.

The ability to determine the amount of microbial DNA in a sample using qPCR has revolutionized microbial diagnostics. Moving beyond simple presence/absence results, quantification allows a nuanced understanding of microbial load, critical for interpreting the biological and clinical significance of findings. This thesis emphasized the rigorous requirements that accompany quantitative assays, including the need for robust, well-characterized standards, and defined analytical parameters such as the LOQ.

One of the major contributions of the presented work was the development and international validation of a reference standard for MAP quantification. The innovative idea that the variability associated with conventional spectrophotometric measurements could be minimized by using dPCR for qPCR standard calibration, increased the reliability and reproducibility of qPCR-based quantification. This effort culminated in the commercial application of a MAP reference standard, which is now undergoing evaluation for integration into the French national paratuberculosis control program. The ability to reliably quantify MAP cells has critical implications in veterinary diagnostics, particularly in distinguishing truly infected animals from passive shedders, thus informing more precise disease control strategies.

These findings underscore the value of quantification not only as a technical capability but as a tool that transforms data into actionable thresholds, improves inter-laboratory

comparability, and bridges the gap between molecular data and epidemiological decision-making.

The second key theme of this thesis explored the assessment of microbial viability using PCR techniques in combination with selective chemical treatments. Traditional microbiological diagnostics have long equated microbial viability with the ability to grow in culture. However, many organisms of clinical, environmental, or food safety relevance—including MAP—either grow very slowly or enter VBNC states, rendering culture-based approaches inadequate.

By integrating viability dyes such as PMA and EMA, and more recently, Pt and Pd compounds, into the PCR workflow, it became possible to selectively suppress signals from dead cells, offering a more accurate reflection of infectious potential. The application of this principle to MAP detection, particularly in milk, revealed that viable MAP cells may be routinely detected in their natural matrices.

The thesis presented an optimized PMA-qPCR protocol for MAP viability assessment and subsequently expanded by validating metal-based viability markers, which simplified workflows, eliminated the need for photoactivation, and demonstrated comparable performance in certain sample matrices. These studies provided a methodological foundation for replacing culture in specific contexts, especially for viability tracking after antimicrobial treatment, disinfection, or environmental exposure.

The broader implication is a shift in diagnostic thinking: from binary detection to biologically meaningful data that better reflects infectivity, treatment efficacy, and environmental persistence. Despite current barriers to routine implementation—including cost, complexity, and standardization—this work demonstrates that viability PCR holds strong potential as a complement or alternative to culture, particularly for slow-growing or unculturable organisms.

The final pillar of this thesis focused on multiplex detection of microbial targets, emphasizing the need for high-throughput solutions capable of detecting multiple pathogens, genotypes, or resistance markers within a single assay. Traditional qPCR platforms are inherently limited by the number of fluorescence channels and their susceptibility to cross-reactions in complex assays.

Through the development and optimization of MOL-PCR, a ligation-based multiplex assay combined with xMAP bead-based technology, the thesis showcased a scalable and modular approach for simultaneous detection of up to 50 targets—and potentially more with advanced platforms. These methods were validated in a range of applications, including foodborne and waterborne viruses and bacteria and biothreat agents. Notably, these MOL-PCR assays together with the thorough manual on optimization criteria enabled development of the advanced MOL-PCR multiplexes intended for the identification of common, but also rare urinary tract pathogens, and comprehensive profiling of probiotic species in dietary supplements.

A key strength of MOL-PCR lies in its modularity: new targets can be added without redesigning the entire assay. Furthermore, by combining universal primers, ligation-based specificity, and fluorescence-coded microspheres, the platform offers superior specificity, flexibility, and throughput compared to conventional multiplex PCR.

The implications are far-reaching: MOL-PCR provides a bridge between the depth of sequencing and the practicality of PCR, with potential utility in routine diagnostics, surveillance, and outbreak response.

The three areas explored—quantification, viability, and multiplexing—each contribute unique strengths to modern molecular diagnostics. However, their combined potential is greater than the sum of their parts. When used together, these tools allow for quantitative, biologically relevant, and broad-spectrum diagnostics that are faster, more informative, and increasingly aligned with real-world needs in clinical, veterinary, and environmental microbiology.

Throughout this thesis, MAP served as a model organism, illustrating both the challenges and opportunities inherent in applying advanced molecular tools to difficult diagnostic contexts. The work presented here not only advances the methodology but also demonstrates a roadmap for translating experimental innovation into applied diagnostics.

Looking forward, the challenge remains to streamline, standardize, and validate these approaches for broader adoption. As technology evelves and costs decline, the integration of these advanced PCR-based applications into routine laboratory workflows will become increasingly feasible. In doing so, molecular diagnostics will not only complement but often

surpass traditional microbiological methods, offering precision, speed, and insight across the microbial spectrum.

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On-farm spread of *Mycobacterium avium* subsp. *paratuberculosis* in raw milk studied by IS*900* and *F57* competitive real time quantitative PCR and culture examination

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ABSTRACT

A rapid, cheap and sensitive detection method of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in raw milk was needed for routine usage. We developed two duplex real time qPCR systems specific for MAP detection. These real time qPCR assays amplify the multicopy element IS*900* for qualitative analysis and the single copy element *F57* for quantitative analysis. Both assays incorporate an internal amplification control amplified with the same primers as the targets and the same probes are used in both assays. The specificity of the assays was confirmed by the testing of 6 different MAP isolates, 12 isolates of other mycobacteria or bacterial species and 4 different mammalian DNAs. The sensitivity of the developed assays and isolation efficiency were demonstrated through the analysis of raw milk samples artificially contaminated with MAP cells and with plasmids containing cloned fragments of the targets (IS*900* and *F57*). The developed assays for milk analysis were applied to samples from one farm with two faecal shedding cows. Three hundred and forty five individual milk samples were tested by real time qPCR assays and by cultivation. Hundred and eleven (32.5%) individual milk samples were positive by the real time qPCR, no milk sample was culture positive. The spread of MAP in individual, tank and bulk tank milk samples was also monitored.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) is a pathogen which causes a chronic granulomatous enteritis known as paratuberculosis or Johne's disease. Its primary hosts are domestic and wild ruminants. Clinical signs are intermittent diarrhoea, loss of weight, decreased milk production and ultimately death of the infected animal (Ayele et al., 2001; Chacon et al., 2004).

Similar symptoms to those of paratuberculosis in ruminants are characteristic of Crohn's disease in humans. This has led to the hypothesis that MAP could be involved in the development of this disease (Ayele et al., 2001; Behr and Kapur, 2008; Skovgaard, 2007; Uzoigwe et al., 2007). Several studies have proved that MAP is present in raw milk, which can be source of this organism for the possible public exposure (Bosshard et al., 2006; Corti and Stephan, 2002; Grant et al., 2002; Pillai and Jayarao, 2002). It has been documented that in cows in the clinical stage of paratuberculosis, the number of MAP cells shed into milk is lower than 100 CFU/ml (Giese and Ahrens, 2000; Taylor et al., 1981), while in infected cows without clinical signs, the total number of MAP ranges only from 2 to 8 CFU/50 ml (Sweeney et al., 1992). On the other hand, levels of MAP in faeces can exceed 10^8 CFU/g in clinically ill animals (Chiodini et al., 1984; Crossley et al., 2005).

Several authors have shown that MAP can survive the pasteurization process (Ayele et al., 2005; Grant et al., 1996, 2002). But it is assumed that current pasteurization protocols (used according to the European Union legislation recommendation) should be sufficient to inactivate MAP in milk (Gao et al., 2002). Because of the autoimmune basis of Crohn's disease, viable cells may not be required for the initiation of disease. It has been suggested that also dead or even disrupted MAP cells may trigger the manifestation of clinical signs of Crohn's disease, but evidence for this is lacking (Chamberlin and Naser, 2006).

For the routine direct detection of viable MAP, culture is considered to be the “gold standard” (Slana et al., 2008). However, the sensitivity of culture is low and may not identify all low and moderate shedders in a herd (Whitlock et al., 2000). The long incubation period required for MAP growth (at least 12 weeks for faeces and 18 to 52 weeks for milk) is a major disadvantage of this method. In addition, the decontamination steps before cultivation could inactivate MAP, which further reduces their diagnostic value (Chiodini et al., 1984) to consistently detect subclinically infected animals that shed mycobacteria in very low doses (Stabel, 1997).

With the aim of decreasing the time needed for the detection of MAP, molecular methods based on the polymerase chain reaction (PCR) were introduced. PCR methods are still quite expensive and are limited by the availability of DNA isolation procedures. Real time PCR, at present the most commonly employed modification of PCR, holds several advantages, compared to conventional PCR: (i) higher sensitivity; (ii) due to the use of specific probes confirmation of

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Table 1
Specificity of IS*900* and *F57* real time qPCR in different bacterial and vertebrate species

Species	Source
M. avium subsp. *paratuberculosis*	Collection of Animal Pathogenic Microorganisms 6381[a]
M. avium subsp. *paratuberculosis*	Cattle (*Bos taurus*), isolate N366/96, CZ[b]
M. avium subsp. *paratuberculosis*	Cattle (*Bos taurus*), isolate 12134, CZ[b]
M. avium subsp. *paratuberculosis*	Brown bear (*Ursus arctos*), isolate 580S, SK[c]
M. avium subsp. *paratuberculosis*	Fallow deer (*Dama dama*), isolate 2357S, CZ[b]
M. avium subsp. *paratuberculosis*	Moufflon (*Ovis musimon*), isolate 9161/S, CZ[b]
M. avium subsp. *avium*	Collection of Animal Pathogenic Microorganisms 5889
M. avium subsp. *avium*	Rabbit (*Oryctolagus cuniculus*), isolate 12214, CZ[b]
M. avium subsp. *hominissuis*	Pig (*Sus scrofa*), isolate 30/803, *CZ*[b]
M. avium subsp. *hominissuis*	Human (*Homo sapiens*) isolate 3127/04, CZ[b]
M. gordonae	The American Type Culture Collection 1447
M. kansasii	The American Type Culture Collection 12478
M. scrofulaceum	The American Type Culture Collection 19981
M. szulgai	The American Type Culture Collection 3799
M. intracellulare	Collection of Animal Pathogenic Microorganisms 5627[a]
M. porcinum	Environment, field isolate 39/607[a]
Escherichia coli Top 10F′	Competent cells (Invitrogen, Carlsbad, USA)
Salmonella enterica serovar Typhimurium	Collection of Animal Pathogenic Microorganisms 5438[a]
Sheep (*Ovis aries*)	Blood isolate O1VFU, clinical patient UVPS[d]
Goat (*Capra hircus*)	Blood isolate K1VFU, clinical patient UVPS[d]
Cattle (*Bos taurus*)	Blood isolate K1VFU, clinical patient UVPS[d]
Human (*Homo sapiens*)	Blood isolate

[a] Veterinary Research Institute, Brno, Czech Republic.
[b] Czech Republic.
[c] Slovakia.
[d] University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic.

specificity is not required; (iii) it allows quantification; (iv) large numbers of samples can be processed and (v) data handling is faster and easier (Rodriguez-Lazaro et al., 2005).

One of the important features that all real time PCR based systems must possess to be applicable in the routine diagnosis of paratuberculosis is an internal amplification control (IAC) that will distinguish between false-negative and truly negative results. It is usually based on the amplification of a different locus by a different set of primers (Herthnek and Bolske, 2006; Rodriguez-Lazaro et al., 2005; Schonenbrucher et al., 2008; Tasara and Stephan, 2005).

Several specific loci have been evaluated for the direct detection of MAP by PCR; the most common target is the multiple copy insertion sequence IS*900* (Green et al., 1989). This is in spite of several reports indicating the presence of IS*900*-like sequences in other closely related mycobacterial species, which could negatively affect the specificity of PCR tests targeting IS*900* (Cousins et al., 1999; Englund et al., 2002). IS*900* is present in the MAP genome in 12 to 18 copies (Bull et al., 2000; Pavlik et al., 1999). For this reason IS*900* is a better target for the sensitive detection of MAP compared to single copy elements like *F57* (Poupart et al., 1993). The advantage of *F57* lies predominantly in its strict uniqueness to MAP and its potential ability to quantify MAP DNA (Herthnek and Bolske, 2006; Tasara and Stephan, 2005).

The most common approach used for the detection of MAP in milk is based on the centrifugation of a large amount of milk and subsequently, only the pellet and/or cream fractions being used for further DNA isolation (Bosshard et al., 2006; O'Mahony and Hill, 2004; Rodriguez-Lazaro et al., 2005; Tasara and Stephan, 2005). There is a strong consensus that this procedure is the best suited to this microorganism as enrichment techniques are impractical due to the slow growth of MAP (Radstrom et al., 2004).

The aims of this study were as follows; (i) to develop and verify two independent duplex real time quantitative PCR (qPCR) assays with internal amplification controls based on the competitive principle of real time qPCR; (ii) to test an alternative technique for the estimation of the absolute number of MAP cells used for the artificial contamination of milk by *F57* real time qPCR assay; (iii) to develop a DNA isolation technique from milk and to determine the DNA isolation efficiency on artificially contaminated milk samples, and (iv) to determine the infection status in a herd infected with paratuberculosis by fecal culture and to assess the spread of MAP in individual, tank and bulk tank milk by the developed DNA isolation and real time qPCR assays.

2. Materials and methods

2.1. Bacterial and mammalian species

To evaluate the specificity of the developed real time qPCR assays, possible cross-reactions with 16 selected mycobacterial, non-mycobacterial and mammalian species were studied (Table 1). The bacterial species were selected with regard to their close genetic similarity to MAP or based on the probability of them being present in the same environment. The selection of mammalian species included those organisms which represented the most probable hosts for MAP. A single bacterial colony was resuspended in distilled water and DNA was isolated by the QIAamp DNA Tissue Mini Kit (Qiagen, Hilden, Germany) according to the modified protocol for G+ and G− bacteria listed in the manufacturer's handbook. Mammalian DNA was isolated from peripheral blood using the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's recommendations.

2.2. Design of primers and probes for IS900 and F57 real time qPCR

Specific real time qPCR primers for the IS*900* (X16293) and *F57* fragment (X70277) and all hydrolysis probes were designed using the Primer3 on-line tool (Rozen and Skaletsky, 2000; Table 2).

Table 2
Sequences of primers and probes used in IS*900* and *F57* competitive real time qPCR[a]

Target gene	Name	Type	Sequence 5′→3′	Location[b] (bp)	Length of the product (bp)
IS*900*	IS900qPCRF	Forward	GATGGCCGAAGGAGATTG	94–111	145
	IS900qPCRR	Reverse	CACAACCACCTCCGTAACC	238–220	
	IS900qPCRTM	Probe	6FAM–ATTGGATCGCTGTGTAAGGACACGT–BHQ	158–182	
F57	F57qPCRF	Forward	GCCCATTTCATCGATACCC	422–440	147
	F57qPCRR	Reverse	GTACCGAATGTTGTTGTCAC	568–549	
	F57qPCRTM	Probe	6FAM–CAATTCTCAGCTGCAACTCGAACACAC–BHQ	508–534	
IAC[c]–IS*900*	IACqPCRTM	Probe	Cy5–GGCTCTTCTATGTTCTGACCTTGTTGGA–BHQ	2712–2826[d]	152
IAC[c]–*F57*	IACqPCRTM	Probe	Cy5–GGCTCTTCTATGTTCTGACCTTGTTGGA–BHQ	2712–2826[d]	154

[a] All primers and probes are covered with the European patent application No. EP 08466007 (Czech patent No. PV2007-295) and were synthesized at VBC Biotech (Vienna, Austria).
[b] For the location of primers and probes, the sequences IS*900* (X16293) and *F57* (X70277) were used.
[c] Internal amplification control; sequences and location for IS*900* and *F57* target genes are identical.
[d] Location of IAC internal sequence without specific IS*900* and *F57* primers originating from the *St*TS1 gene (AF483209).

2.3. Preparation of plasmid standards and internal amplification controls

Plasmid standards for IS*900* and *F57* real time qPCR were prepared by cloning the desired real time qPCR product of IS*900* or *F57* into a pCR 2.1 cloning vector (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Plasmids containing the inserts of IS*900* and *F57* were then purified with the QIAprep Spin Miniprep Kit (Qiagen), eluted into Tris-EDTA (TE) buffer (Amresco, Solon, OH, USA) and sequenced to confirm that the sequence was accurate. The exact concentration at A_{260} was determined and subsequently the exact plasmid copy number was calculated. To increase the stability of the plasmids in TE buffer during storage at −20 °C and to decrease losses during manipulation, DNA from fish sperm (Serva, Heidelberg, Germany) was added as supplementing DNA to a final concentration of 50 ng/μl.

Internal amplification controls (IACs) for IS*900* and *F57* real time qPCRs were prepared in a similar way to plasmid standards. Part of the coding sequence of the *St*TS1 gene (AF483209) from the potato (*Solanum tuberosum*) was flanked with specific primers for IS*900* or *F57* and the PCR product was inserted in to a pCR 2.1 cloning vector. The IAC plasmids were processed in a similar way to the plasmid standards for IS*900* and *F57*, but the artificial DNA from fish sperm (Serva) was not added. The hydrolysis probes for both IACs were identical.

2.4. Real time qPCR conditions

The conditions for the IS*900* and *F57* competitive real time qPCR duplex assays were optimised until the best primer and probe concentrations, concentrations of both IACs, $MgCl_2$ concentration and cycling conditions were determined. The copy number of IS*900* and *F57* IAC plasmids that ensures optimal co-amplification with the target was determined by the titration of 5×10^3, 5×10^2, 5×10^1 and 5×10^0 copies of each IAC per real time qPCR reaction and respective plasmid gradient. The influence of the non-specific supplementing DNA from fish sperm present in the DNA template on the amplification of the target during both real time qPCR assays was evaluated by the addition of 0, 50, 100, 150, 200, 250, 500 and 1000 ng per real time qPCR reaction. The optimised reaction mixture was identical for both assays and contained 1× DyNAmo Probe qPCR Kit (Finnzyme, Espoo, Finland), 10 pmol of the primers IS900qPCRF or F57qPCRF and IS900qPCRR or F57qPCRR, 1 pmol of the IS900qPCRTM or F57qPCRTM probe labelled with FAM, 4 pmol of the IACqPCRTM probe labelled with Cy5, 0.2 U of Uracil DNA Glycosylase (Sigma, St. Louis, MO, USA), 5×10^1 copies of IS*900* or *F57* IAC plasmids and 5 μl of the DNA template, in a total reaction volume of 20 μl. Amplification and fluorescence detection, identical for both assays, was performed on the LightCycler 480 Instrument (Roche Molecular Diagnostic, Mannheim, Germany) using 96-well PCR plates under the following conditions: 37 °C for 10 min, followed by initial denaturation at 95 °C for 15 min and 47 cycles of 95 °C for 5 s and 60 °C for 40 s. Subsequent analysis was carried out using the "Fit point analysis" option of the LightCycler 480 software (version 1.2.0.0625).

The plasmid standards, IACs and primers for both assays as well as the real time qPCR protocol have been submitted for the patenting process (European patent application No. EP 08466007; Czech patent application No. PV2007-295).

2.5. Reproducibility of IS900 and F57 real time qPCR

The reproducibility of the IS*900* and *F57* real time qPCR assays was determined by 10-fold serial dilutions of the IS*900* and *F57* plasmid standards. Both plasmid gradients were diluted in a range from 5×10^5 to 5×10^0 copies per real time qPCR reaction. Dilutions were done in TE buffer (Amresco) supplemented with 50 ng/μl of DNA from fish sperm (Serva). Prepared plasmid gradients served for quantification, as positive controls in real time qPCR and for the calculation of the real time qPCR efficiency of the plasmid gradient in the actual real time qPCR run. The reproducibility of both real time qPCR assays was determined by 50 independent repeats with freshly prepared dilutions of the plasmid gradients in each run.

2.6. Sample processing and isolation of DNA from milk

For quantitative real time PCR, a total of 2×50 ml of milk was centrifuged at 4200 ×*g* for 45 min. The supernatant was poured off and the pellet was resuspended in what remained of the liquid and transferred to fresh 2-ml tubes. Subsequently, the samples were centrifuged again at 14,000 ×*g* for 10 min and the remainder of the supernatant discarded. Such "pre-processed" milk samples were then subjected to DNA isolation. The first 50 ml pellet was used for the isolation of DNA or stored at −70 °C until use while the second 50 ml pellet was stored at −70 °C and was used if the first DNA isolation failed.

DNA isolation from milk was based on a slightly modified QIAamp DNA Blood Mini Kit protocol (Qiagen). Briefly, increased volumes of Protease (100 μl) and AL buffer (500 μl), both provided with the kit, 6.25 μg DNA from fish sperm (Serva) and 350 mg of 0.1 mm zirconia/silica beads (Biospec, Bartlesville, OK, USA) were added to the tubes with the milk pellets prepared from 50 ml of milk. The pellets were homogenised in the MagNA Lyser instrument (Roche Molecular Diagnostic) at 6400 rpm for 60 s. The samples were than incubated at 56 °C for 10 min with shaking at 1400 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany). Subsequently, the samples were subjected to mechanical homogenisation in the MagNA Lyser instrument (Roche Molecular Diagnostic,) at 6400 rpm for 60 s. After the addition of 500 μl of 96% ethanol (Amresco), the supernatant was loaded onto spin columns in two steps leaving the beads in the tube. The DNA binding and washing steps were performed twice according to the manufacturer's manual. The DNA was eluted into 100 μl of preheated TE buffer (Amresco) after 5 min of on-column incubation. To improve the DNA yield, the filtrate was applied onto the spin column again and centrifuged. The isolated DNA was used as a template for the IS*900* and *F57* real time qPCRs assays described above. Each milk sample was analysed in duplicate by each real time qPCR assay and was considered to be positive only when both replicates gave a positive result. If this condition was not met, the real time qPCR or, alternatively the DNA isolation, was repeated.

2.7. Determination of the exact MAP cell number for the artificial contamination of milk

A MAP isolate (Strain 6381 from Collection of Animal Pathogenic Microorganisms) from Middlebrook 7H9 broth (Difco, Livonia, MI, USA), with Middlebrook ADC enrichment (Difco), supplemented with 2 mg Mycobactin J (Veterinary Research Institute, Brno, Czech Republic), was centrifuged at 11,000 ×*g* for 10 min, washed twice and resuspended in TE buffer (Amresco). To destroy clumps of MAP, twelve 1 mm zirconia/silica beads (Biospec) were added to 500 μl of the MAP suspension before mechanical homogenisation at 6400 rpm for 20 s in the MagNA Lyser instrument (Roche Molecular Diagnostic). The suspension was centrifuged at 100 ×*g* for 30 s to remove the biggest clumps and the supernatant was transferred to a fresh tube. From this tube, 10 aliquots of 500 μl were transferred into tubes containing 350 mg of 0.1 mm zirconia/silica beads (Biospec) and the MAP cells were mechanically disrupted in the MagNA Lyser (Roche Molecular Diagnostic) at 6400 rpm for 10, 20, 30, 40, 50, 60, 70, 80, 90 and 120 s in a total volume of 1000 μl TE buffer. Samples were centrifuged at 14,000 ×*g* for 10 min and the supernatant served as the DNA template for the *F57* real time qPCR. Each sample was run in triplicate. The total MAP cell number was determined from the *F57* serial plasmid dilutions included in the run. A prepared MAP

suspension with a known number of cells was used for the estimation of DNA isolation efficiency from artificially contaminated milk samples.

2.8. Evaluation of the DNA isolation procedure on artificially contaminated milk samples and the sensitivity of MAP detection in milk

For all of the following experiments, commercially available semi-skimmed milk was used. MAP negativity was confirmed by IS*900* real time qPCR before it was employed in further experiments. To determine the reliability of the total DNA isolation procedure from milk and the recovery of plasmids or MAP cells in the isolated DNA, six "pre-processed milk samples" were artificially contaminated with serial dilutions of the *F57* plasmid standard or MAP cells in a range from 1.25×10^7 to 1.25×10^2 plasmids or MAP cells per sample. All samples were analysed in 4 independent experiments in duplicates by *F57* real time qPCR. The limit of detection was determined as the lowest concentration of *F57* standard plasmid or MAP cells, which was possible to detect in all repeats by the *F57* real time qPCR assay. After total DNA isolation, the observed mean number of *F57* target sites in the eluted DNA was divided by the theoretical input. This ratio multiplied by 100 provided the overall efficiency of the DNA isolation.

2.9. Statistical evaluation

Crossing point values obtained from the real time qPCR were recalculated to the real copy number, according to the respective regression equation gained from the calibration curve included in the same run. The recalculated data from the respective IS*900* and *F57* plasmid standard serial dilutions were compared with each other by InStat software (GraphPad, San Diego, CA, USA). The data were tested for the homogeneity of variance by the Kolmogorov–Smirnov test and the variance was analysed by Student's *T*-test. Differences with $P<0.05$ were considered to be significant. A similar approach was used for the comparison of the artificially contaminated milk samples, where "pre-processed" samples were spiked with *F57* plasmid standard dilutions and defined numbers of MAP cells.

2.10. Cattle farm situation and collection of faeces and milk samples

Milk and faecal samples originated from one farm with a known history of paratuberculosis. The samples were taken between February and April 2007; at the beginning of the experiment there were 74 cows in two parallel fixed cow stalls: on the left were 37 cows (No. 1 to No. 37) and on the right were a further 37 (No. 38 to No. 74). At the beginning of the experiment two of these cows were in calf.

Faecal samples for cultivation were collected from all 74 cows at the beginning on day 1. Approximately 5 g of faeces were taken directly from the rectum and put in a sterile vial, using disposable gloves and transported on ice to the laboratory.

Seventy-two cows were in their lactation period and individual milk samples from these cows were collected. Mixed first milk samples from all four teats (taken after the first 1 or 2 squirts were discarded) were collected in a sterile 200 ml plastic container. The containers with milk were chilled and stored in an icebox at 4 °C until they were delivered to the laboratory where the samples were stored in the refrigerator and processed (cultured or pre-processed for DNA isolation) within 4 days of collection. Individual milk samples were taken from all cows on days 1, 5, 6 and 13. On days 1 and 6, continuous tank milk (TM) samples were collected during the milking process. On day 82, milk samples from only 53 animals were collected; the remaining previously examined cows had either been moved to other stables or had been culled. On each collection day, bulk tank milk (BTM) sample was also taken after 11 h of mixing at 6 °C.

2.11. Mycobacterial culture and PCR identification of colonies

Faecal samples were handled as previously described (Pavlik et al., 2000). Briefly, 1 g of faeces was added to 30 ml of sterile distilled water and mixed by horizontal shaking for 30 min. Debris and coarse material were allowed to settle for 30 min, and 5 ml of the supernatant was added to 25 ml of 0.75% hexadecylpyridinium chloride (HPC; Merck, Darmstadt, Germany). After 30 min of horizontal shaking, samples were incubated at room temperature for 72 h. From the sediment, 300 µl was inoculated into each of three vials containing Herrold's egg yolk medium (HEYM) with Mycobactin J (produced at the Veterinary Research Institute, Brno, Czech Republic) and incubated at 37 °C for 12 to 15 weeks.

Milk samples were processed according to a protocol described previously (Ayele et al., 2005). Briefly, 10 ml of raw milk were collected with a sterile pipette, transferred to a centrifuge tube, and centrifuged for 15 min at 2500 ×*g*. The resulting pellet was resuspended in 10 ml of 0.75% HPC and decontaminated for 5 h. After centrifugation for 15 min at 2500 ×*g*, the pellet was resuspended in 800 µl of sterile distilled water. A 250 µl portion of the resuspended pellet was inoculated onto three HEYM media with Mycobactin J and incubated at 37 °C for 18 to 30 weeks.

The grown colonies were stained by the Ziehl–Neelsen method for the presence of acid-fast bacilli, and identified by conventional multiplex PCR as described previously (Moravkova et al., 2008).

3. Results

3.1. Optimization of IS900 and F57 real time qPCR assays

The optimal copy number of IS*900* and *F57* IAC plasmids that ensured successful co-amplification with the target was identical for both assays. We determined that 50 copies of IS*900* and *F57* IAC plasmids per real time qPCR reaction did not influence the amplification of the relevant plasmid gradient even at the lowest concentrations and provided a sufficiently strong signal to be distinguished from the background.

In the next step, the influence of the non-specific supplementing DNA from fish sperm present in the DNA template on the amplification of the target during both real time qPCR assays was evaluated. Data comparison revealed that the crossing points of both plasmid gradients were not influenced even in the presence of 500 ng per real time qPCR reaction of the non-specific DNA. The concentration of 1000 ng per real time qPCR reaction significantly inhibited both real time qPCR assays. According to the results from these experiments, the amount of fish sperm DNA used for the dilutions of the plasmid standards was set at 250 ng per real time qPCR reaction, i.e. 50 ng/µl.

3.2. Specificity of IS900 and F57 real time qPCR assays

Both developed assays were tested for their capacity to selectively discriminate MAP from non-targeted bacteria and selected animal DNAs. No bacterial or mammalian DNAs other than MAP as seen in Table 1 were found positive by either the IS*900* assay or the *F57* assay, and all the negative samples gave a clear signal for the IAC.

3.3. Reproducibility of IS900 and F57 real time qPCR assays

It was possible to detect 5×10^1 copies of IS*900* and *F57* plasmid standard per real time qPCR reaction in all 50 independent repeats (Table 3). The lowest concentration of 5×10^0 copies per real time qPCR reaction was detected only in 86.0% (IS*900* real time qPCR assay) and 96.0% (*F57* real time qPCR assay) of reactions. The mean PCR efficiencies for the IS*900* and *F57* assays were 103.2% (minimum 87.9%; maximum 116.1%) and 95.2% (minimum 81.7%; maximum

Table 3
Evaluation of the reproducibility and sensitivity of the IS*900* and *F57* standard plasmid dilutions by the IS*900* and *F57* real time qPCR assays

Plasmid gradient	Plasmid copy number[a]	Recovered copy number[b]	
		Mean	SD[c]
IS*900* assay	500,000	520,829	73,514
	50,000	49,356	8,378
	5000	5099	1023
	500	528	167
	50	49	15
	5	5	3
F57 assay	500,000	512,147	95,445
	50,000	52,951	12,327
	5,000	4924	1066
	500	505	148
	50	55	18
	5	5	2

[a] Theoretical number of plasmid copies per 5 µl of real time qPCR reaction.
[b] Recovered copy number was calculated from the raw Crossing point values separately according to the respective regression equation from the actual run; subsequently mean and standard deviations were calculated.
[c] Standard deviation.

116.2%), respectively. The IS*900* and *F57* data for all dilutions were not significantly different ($P>0.05$).

3.4. Determination of the exact MAP cell number for the artificial contamination of milk

After analysis by the *F57* real time qPCR assay it was shown that the highest yield was achieved after 60 s of the treatment and this was constant at the subsequent time checkpoints (data not shown).

3.5. Evaluation of the total milk DNA isolation procedure and the experimental sensitivity of IS900 and F57 real time qPCR assays on the artificially contaminated milk samples

For the determination of the total DNA isolation efficiency, the yield of the "pre-processed" milk samples artificially contaminated with the gradients of *F57* plasmids and MAP cells were analysed by the *F57* real time qPCR assay and compared with each other (Table 4). The DNA isolation procedure was shown to be reliable for the milk samples artificially contaminated with the serially diluted *F57* plasmid standard. The mean yield was 95.8% with the minimum 70.6% and maximum 156.9%. It was possible to detect even the lowest theoretical concentration (5 copies per real time qPCR reaction) in all 8 repeats. The DNA isolation from the milk samples artificially contaminated with defined numbers of serially diluted MAP cells provided lower yields than the identical procedure with the *F57* plasmid standard dilutions. It was possible to detect all theoretical concentrations in all 8 repeats except the lowest concentration, which was successfully amplified in 4 repeats only. The overall MAP yield after DNA isolation was only 31.8% with a minimum of 23.5% and a maximum of 40.8%. Because we were able to consistently detect MAP in samples that theoretically contained 50 copies of MAP per real time qPCR reaction in all repeats, the detection limit was set to this level. Samples with lower numbers of MAP could also be determined as positive, but the probability of detection is lower. There was a significant difference ($P<0.05$) between identical dilutions of the *F57* standard plasmid and MAP cells.

Milk samples artificially contaminated with MAP cells were also analyzed by the IS*900* real time qPCR assay. All samples were positive in all repeats even at the lowest concentration. According to the parameters of the DNA isolation, the number of MAP cells obtained by the *F57* real time qPCR assay divided by two represents the number of MAP in the initial 1 ml of milk. If we also take into account that regardless of the number of MAP in the sample, the yield after DNA isolation was only 30%, the sensitivity of the *F57* real time qPCR assay is 83 MAP cells per 1 ml. The sensitivity of the IS*900* real time qPCR was not experimentally determined, because exact quantification in low concentrations is rather doubtful, but considering that there is on average 15 copies of IS*900* per single MAP cell, the sensitivity would be between 5 and 6 MAP cells per 1 ml of milk.

3.6. Farm situation and culture examination of faeces and milk

By culture, there were 2 cows from the 74 present in the stable that excreted viable MAP in faeces (Table 5). Colonies were confirmed by Ziehl–Neelsen staining for the presence of acid-fast bacilli and conventional multiplex PCR identified the colonies as MAP. Cow No. 11 was in calf during the experiment and after parturition was culled due to poor condition. However this cow shed moderate numbers of MAP in faeces (30 colonies). Cow No. 58 was emaciated and exhibited chronic enteritis over the course of the experiment. Culture examination for the presence of MAP in milk was negative.

3.7. Individual milk sample analysis

Altogether, 342 individual milk samples were analysed by the IS*900* and *F57* real time qPCR assays over 5 collection days. In total, 47 (13.7%) individual milk samples were positive by the *F57* assay and 64 (18.7%) were positive by the IS*900* real time qPCR assay only. Over the course of the whole experiment, 111 (32.5%) individual milk samples were positive by both real time qPCR assays (Table 5). None of the analyzed samples were simultaneously negative by the IS*900* qPCR assay and positive by the *F57* qPCR assay. Samples that were weakly positive for the presence of IS*900* and negative for the presence of *F57* were considered to be positive.

In each stall, there was 1 cow that excreted MAP in faeces (cow No. 11 on the left and No. 58 on the right). In both cases the cows in closest proximity to them were positive for MAP in milk on the first 4 collection days. The occurrence of MAP in the milk of cows not in the immediate neighbourhood of the faecal shedders was rather sporadic. After the removal of the infected cows from the farm (82nd collection

Table 4
Evaluation of the reliability and efficiency of the total DNA isolation procedure from milk by the *F57* real time qPCR assay

AMC[a]	Plasmid copy or MAP cells number[b]	Recovered copy number[c]		Mean DNA isolation efficiency (%)[e]
		Mean	SD[d]	
with *F57* plasmid	500,000	422,525	31,311	84.5
	50,000	47,832	19,918	95.7
	5000	4418	764	88.4
	500	353	60	70.6
	50	39	14	78.6
	5	8	3	156.9
with MAP cells	500,000	136,674	17,230	27.3
	50,000	20,422	10,004	40.8
	5000	1651	677	33.0
	500	184	90	36.8
	50	15	10	29.1
	5	2	1	23.5

[a] AMC: Artificial milk contamination, presented data are from the *F57* assay only.
[b] Theoretical number of plasmid copies or *Mycobacterium avium* subspecies *paratuberculosis* cells per 5 µl of real time qPCR reaction.
[c] Recovered copy number was calculated from the raw Crossing point values separately according to the respective regression equation from the actual run; subsequently mean and standard deviations were calculated.
[d] Standard deviation.
[e] Calculated as the mean obtained yield divided by the theoretical number of plasmid copies or *Mycobacterium avium* subspecies *paratuberculosis* cells added into the reaction multiplied by 100 (resulting values are in percent).

Table 5
Arrangement of cows in the stable and distribution of *Mycobacterium avium* subspecies *paratuberculosis* in faeces and milk

Anim. no.	Faecal culture[a]	Real time qPCR in milk (days)[b]					Anim. no.	Faecal culture[a]	Real time qPCR in milk (days)[b]				
		1	5	6	13	82			1	5	6	13	82
1	−	nt	nt	nt	−−	−−	38	−	−−	−−	−−	−−	−−
2	−	−−	−−	−−	−−	np	39	−	+−	+−	−−	−−	np
3	−	+−	−−	−−	−−	−−	40	−	−−	+−	−−	+−	−−
4	−	+−	−−	+−	−−	−−	41	−	−−	−−	+−	+−	−−
5	−	+−	−−	−−	−−	−−	42	−	−−	−−	−−	−−	−−
6	−	+−	−−	+−	−−	+−	43	−	−−	+−	−−	−−	−−
7	−	+−	−−	−−	++	−−	44	−	+−	+−	−−	−−	−−
8	−	+−	++	−−	+−	−−	45	−	−−	+−	−−	−−	−−
9	−	++	+−	+−	++	np	46	−	−−	++	−−	−−	np
10	−	+−	+−	+−	+−	−−	47	−	−−	+−	−−	−−	−−
11	30	nt	nt	nt	nt	np	48	−	−−	−−	++	−−	−−
12	−	−−	−−	++	+−	−−	49	−	+−	−−	−−	−−	−−
13	−	−−	−−	++	++	−−	50	−	+−	+−	++	−−	−−
14	−	−−	++	++	−−	−−	51	−	−−	+−	−−	−−	−−
15	−	++	+−	−−	−−	−−	52	−	−−	−−	−−	−−	−−
16	−	−−	−−	++	−−	np	53	−	+−	+−	++	+−	−−
17	−	−−	−−	++	+−	−−	54	−	++	−−	−−	−−	np
18	−	−−	−−	+−	−−	np	55	−	++	−−	++	++	−−
19	−	+−	−−	−−	−−	np	56	−	+−	+−	+−	++	np
20	−	+−	−−	−−	−−	np	57	−	+−	++	++	++	np
21	−	−−	−−	−−	−−	−−	58	TNTC	++	+−	++	++	np
22	−	++	−−	−−	−−	np	59	−	++	++	−−	++	np
23	−	−−	−−	−−	+−	−−	60	−	++	++	−−	−−	−−
24	−	++	++	−−	−−	−−	61	−	++	−−	−−	+−	−−
25	−	−−	−−	+−	−−	−−	62	−	++	−−	+−	−−	−−
26	−	−−	−−	−−	−−	−−	63	−	++	+−	+−	−−	−−
27	−	−−	−−	−−	−−	−−	64	−	+−	++	−−	−−	−−
28	−	−−	−−	−−	−−	np	65	−	+−	−−	−−	−−	−−
29	−	−−	−−	−−	−−	np	66	−	++	−−	−−	−−	−−
30	−	−−	−−	+−	−−	−−	67	−	+−	−−	−−	+−	−−
31	−	−−	−−	−−	−−	np	68	−	−−	−−	−−	+−	−−
32	−	−−	−−	++	−−	−−	69	−	++	−−	++	++	−−
33	−	+−	−−	−−	−−	−−	70	−	++	+−	−−	−−	−−
34	−	−−	−−	−−	−−	−−	71	−	−−	−−	−−	−−	−−
35	−	−−	−−	−−	+−	np	72	−	+−	−−	−−	++	np
36	−	−−	−−	−−	−−	++	73	−	+−	−−	−−	+−	np
37	−	−−	−−	−−	−−	−−	74	−	−−	−−	+−	−−	−−
Subtotal													
	Cows	35	35	35	36	25			37	37	37	37	28
	++	4	3	6	3	1			11	5	7	7	0
	+−	10	3	7	6	1			12	13	5	7	0
Total													
	Positive	14	6	13	9	2			23	18	12	14	0
	%	40.0	17.1	37.1	25.0	8.0			62.2	48.6	32.4	37.8	0.0

np: not present in the stable.
nt: not tested, but present in the stable (dried cows before parturition).
[a] No. of colony forming units; −: 0 CFU; 30: total number of CFU on the three culture media used per culture examination; TNTC: too-numerous-to-count.
[b] IS*900* and *F57* real time qPCR examination of samples from different collection days: −− negative result in both assays, +− IS900 assay positive and F57 assay negative results, ++ positive in both assays.

day), there were only 2 cows from 53 that were positive for MAP in their milk (Table 5).

Because both developed real time qPCR assays allow quantification of the MAP load in samples we have also calculated the absolute number of MAP in the positive individual milk samples. The highest detected number of MAP in milk was 560 cells/ml (cow No. 58; 1st collection day). The mean of the absolute number of MAP in milk in cows that were positive by both assays was in the several tens of MAP cells/ml. In milk samples that were positive by the IS*900* real time qPCR assay only, the absolute number of MAP cells varied between units and tens of organisms per ml.

3.8. TM and BTM samples analysis

To assess the dilution effect on contaminated milk in the tank, the occurrence of MAP in the tank during the progress of the milking procedure on 1st and 6th of collection days was monitored (TM samples, Table 6). Altogether 73 cows were milked on each of both collection days. At our request cow No. 58 was milked first. After this, the other cows were milked according to the standard order. Milk was stirred in the tank during the whole process of milking. TM samples were collected continuously according to the number of cows milked into the tank (Table 6). In TM samples, it was possible to detect MAP in the mixed milk of cow No. 58 (clinically infected) and two other non-infected cows on both collection days. Other positive TM samples were detected in the later phase of milking and their occurrence was rather sporadic.

MAP was detected in 4 of 5 BTM samples. These samples were collected after the 11 h mixing of all the milk collected each day. All 4 positive BTM samples included milk from the clinically infected cow No. 58. In the fifth BTM sample the milk of this cow and 20 others was not present and the BTM sample was negative (Table 6).

The mean number of MAP cells detected by the IS*900* and *F57* real time qPCR assays in TM and BTM samples was in units of bacteria/ml.

4. Discussion

To enable the use of our real time qPCR assays in routine diagnostics, separate plasmid IACs based on real time qPCR approach

Table 6
Mycobacterium avium subsp. *paratuberculosis* detection in tank and bulk tank milk samples determined by IS*900* and *F57* real time qPCR

Day	Tank milk samples[a,b]											BTM[b,c]
	1+1	1+2	1+4	1+9	1+14	1+19	1+23	1+33	1+43	1+53	1+63	
1	++	+ −	+ −	−−	−−	−−	−−	+ −	+ −	+ −	−−	+ −
5	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	++
6	+ −	++	−−	−−	−−	+ −	−−	+ −	++	−−	+ −	++
13	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	+ −
82	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	nt	−−

BTM: bulk tank milk.
nt: not tested.
[a] Dilutions of milk from cow No. 58 which shed *Mycobacterium avium* subspecies *paratuberculosis* in faeces plus the milk of cows which did not shed *Mycobacterium avium* subspecies *paratuberculosis* in faeces.
[b] IS*900* and *F57* real time qPCR examination: −− negative result in both assays, +− IS*900* assay positive and *F57* assay negative results, ++ positive in both assays.
[c] Bulk tank milk samples collected after 11 h of mixing at 6 °C.

were introduced to each assay. This means that only one primer set is present in the real time qPCR reaction thus allowing the easier optimization of the duplex system of real time qPCR. Financial considerations were also taken into account especially while running multiple sample experiments. The usage of competitive real time qPCR with an IAC reduces the risk of primer dimers and non-specific band formation, and allows the identification of false-negative results caused by incorrect isolation or human error.

During the optimization of both our real time qPCR assays, we have performed 50 independent repeats to determine if the reproducibility of our assays is sufficient for quantification. The main parameter used to estimate this was the calculation of the overall qPCR efficiency for both assays separately. Both values were very close to the theoretical value of 100%. However, in real experiments it is difficult to obtain 100% real time qPCR efficiency in each experiment and it usually deviates from this value. The mean real time qPCR efficiency can be then lower or even higher than 100% as it was in the case in the IS*900* real time qPCR assay in this study.

Our intention was to develop a routine diagnostic method. Due to this fact, we tried to avoid home-made DNA isolation methods that are laborious and time consuming. Due to the impracticality of carrying out any pre-enrichment because of the slow growth of MAP, we proceeded with an initial centrifugation of milk. Cream was not included in the DNA isolation (lipids can interfere with the chemicals used in protocol), although it has been observed that free MAP cells are strongly lipophilic and gather predominantly in the cream fraction of milk (Grant et al., 1998). However, we speculate that MAP, as an intracellular pathogen in naturally contaminated milk should be present inside the eukaryotic cells of the host and that the portion of free MAP cells is likely very low. This matter requires investigation.

Subsequent verification of the DNA isolation protocol was carried out on artificially contaminated milk samples. There is no reliable approach of determining the total number of MAP cells that are used for the artificial contamination. Conventional plating of MAP cells and the counting of colonies is not accurate, because the MAP cells form clusters and the number colonies does not reflect the real number of cells (O'Mahony and Hill, 2004). Another approach, the calculation of MAP cells number according to the amount of DNA, is also not accurate because of losses during the DNA isolation. Our approach allows determination of the MAP cells number directly by *F57* real time qPCR and also allows simple and effective method how to compare the input and recovered amount of MAP cells.

In this study 32.5% and 80.0% of the individual and BTM milk samples tested positive for MAP by real time qPCR. This prevalence is in agreement with other authors (Pillai and Jayarao, 2002; Stabel et al., 2002). The reason for this might be that milk does not constitute a natural environment (intestine) for MAP or that MAP cells in milk are present in not well cultivable, so called cell wall deficient forms (Beran et al., 2006; Hulten et al., 2000). A very important factor for the detection of MAP cells in milk by culture is the preparation of the inoculum and the level of sample contamination (Slana et al., 2008).

The infection in a selected herd was spread randomly among all the animals. Higher levels of positivity for MAP in milk were exhibited by cows in close proximity to the two clinically ill individuals. Other positive cows that were stabled quite far from the clinically ill ones had moderate or very low numbers of MAP in milk. These data suggest that MAP spreads mainly by the exogenous route via the contamination of milking equipment such as teat cup liners. Further study of this matter is required.

The described technique for DNA isolation from milk using a commercially available kit is fast, relatively cheap and allows the processing of large numbers of samples. It also allows partial automation, which increases the number of samples that can be processed. We believe that the described methods enlarge the spectrum of available MAP detection methods and will be a useful tool for the detection of MAP in not only food samples, but will also contribute to the analysis of veterinary samples from animals infected with *paratuberculosis* and to the investigation of the possible involvement of MAP in Crohn's disease.

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Research article

Development of a predictive model for detection of *Mycobacterium avium* subsp. *paratuberculosis* in faeces by quantitative real time PCR

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ABSTRACT

This study focused on the development of a reliable and cost-efficient DNA isolation procedure for the detection of *Mycobacterium avium* subsp. *paratuberculosis* (*MAP*) in faeces by previously developed IS*900* and *F57* quantitative real time PCR (qPCR) and their comparison with culture. The recovery of *MAP* DNA from the spiking experiments ranged from 29.1 to 102.4% of the input amount of *MAP* with median 37.9%. The limit of detection was determined to be 1.03×10^4 for *F57* qPCR and 6.87×10^2 *MAP* cells per gram of faeces for IS*900* qPCR, respectively. The developed technique for DNA isolation was coupled with IS*900* qPCR and compared to traditional *MAP* culture using a cohort of 1906 faecal samples examined from 12 dairy cattle farms in our laboratory. From those 1906 original faecal samples, 875 were positive by IS*900* qPCR and 169 by culture. None of the culture positive samples was negative by IS*900* qPCR. This data facilitated development of a predictive model capable of estimating the probability of being culture positive by estimating the absolute number of *MAP* per gram of faeces as determined IS*900* qPCR without performing the culture.

1. Introduction

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) the causative agent of the inflammatory disease called paratuberculosis (Johne's disease) primarily affects the gastrointestinal tract of domestic and wild ruminants (Ayele et al., 2001). Detection of *MAP* in faeces has been based on traditional culture of *MAP* on solid media and culture is nowadays considered as the "gold standard" test (Ayele et al., 2001). *MAP* culture requires a minimum 2, and up to 4 months to grow (the sheep strains even more than a year), which compromises the efficient and rapid removal of *MAP* infected animals from the herd.

The time-to-detection of *MAP* in faeces can be decreased by PCR based methods (Taddei et al., 2004; Bogli-Stuber et al., 2005; Herthnek and Bolske, 2006; Donaghy et al., 2008; Schonenbrucher et al., 2008; Slana et al., 2008). Widespread implementation of PCR has been limited by its relatively high expense in comparison with traditional culture or ELISA. Most PCR systems are based on the amplification of the specific insertion sequence IS*900* (Bogli-Stuber et al., 2005; Donaghy et al., 2008; Slana et al., 2008), which is now considered the "gold standard" in the molecular detection of *MAP* by PCR.

The advantage of the IS*900* as the diagnostic marker lies predominantly in its multicopy presence (15–20 copies) in the *MAP* genome (Pavlik et al., 1999; Bull et al., 2000). Other targets used for the detection of *MAP* are a single copy fragment *F57* and *hspX* gene (Herthnek and Bolske, 2006; Schonenbrucher et al., 2008; Slana et al., 2008) or the low copy element IS*Mav2* (Schonenbrucher et al., 2008).

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The first aim of this study was to optimise a rapid, reproducible and cost-efficient DNA isolation procedure for *MAP* detection from faeces using quantitative real time PCR (qPCR), as well as to determine the DNA isolation efficiency and limit of detection (LOD). The second aim was the coupling of the developed DNA isolation to IS*900* qPCR and application of this newly developed test to the routine detection of *MAP* in faecal samples and to compare the data with culture results. These data served as the source for the development of predictive model of *MAP* detection probability by culture determined according to the absolute numbers of *MAP* indicated by IS*900* qPCR.

2. Materials and methods

2.1. MAP strain used for spiking experiments

DNA isolation from faecal specimens was optimised by a series of *MAP* spiking experiments in faeces with the cattle reference strain CAPM 6381 (Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Brno, Czech Republic). The *MAP* cells were cultured in the liquid Middlebrook 7H9 broth (DIFCO, Livonia, MI, USA), with Middlebrook AODC enrichment (DIFCO), supplemented with 2 μg/ml Mycobactin J (Allied Monitor, Fayette, MO, USA) and with antibiotics (penicillin G, chloramphenicol and amphotericin B) at 37 °C for 6 weeks. The grown *MAP* suspension was used for the spiking of the faeces and as the reference for the determination of DNA isolation efficiency.

2.2. Faecal samples and culture examination

Altogether 1906 faecal samples from 12 dairy cattle farms involved in Czech National Control Programme were submitted to our laboratory during last 18 months. Individual faecal samples were collected directly from the rectum using a disposable glove. Samples were chilled and immediately transferred to our laboratory where they were processed within 3 days from the arrival. To prevent possible cross-contamination, the faecal aliquots for culture and qPCR were prepared in the separated laboratory. All faecal samples were examined by the routine culture protocol used in our laboratory (Pavlik et al., 2000). Briefly, 5 g of faeces were resuspended in 30 ml of sterile water. After sedimentation, 5 ml of the supernatant were added to 25 ml of 0.75% hexadecylpyridinium chloride (HPC) and decontaminated for 3 days with mild shaking. Culture was performed on two flasks with the Herrold's Egg Yolk Medium (HEYM) with 2 μg/ml of Mycobactin J (Allied Monitor) per sample and with antibiotics (penicillin G, chloramphenicol and amphotericin B) and incubated at 37 °C for 3 months.

2.3. Preparation of MAP suspension for spiking of faeces

MAP cells for the spiking of faeces were similarly prepared using a previously described method by Slana et al. (2008). Briefly, a *MAP* cell suspension in media was centrifuged and the pellet was twice washed and resuspended in Tris–EDTA (TE) buffer (Amresco, Solon, OH, USA). Twelve 1 mm zirconia–silica beads (Biospec, Bartlesville, OK, USA) were added to the *MAP* suspension and homogenised in the MagNA Lyser (Roche Molecular Diagnostic, Mannheim, Germany) at 6400 × *g* for 10 s. The *MAP* suspension was centrifuged at 100 × *g* for 30 s, which is intended to remove big clumps that could cause problems in the subsequent optimisation experiments. The supernatant was serially diluted (10-fold) and the amount of *MAP* cells in each dilution was quantified by *F57* qPCR assay with internal amplification control (Slana et al., 2008). Absolute quantification of *MAP* cells was performed according to the calibration curve derived from 10-fold diluted plasmid standard with known copy number. The calibration curve was included in each qPCR run. Eight serial dilutions of *MAP* cells with the number of *MAP* cells in range approximately 10^8–10^1 were used for the spiking of cattle faeces.

2.4. DNA isolation procedure from faeces

The total DNA from faeces was isolated by a modified protocol from the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany). Initially, 0.5 g of faeces was divided into two separate 2 ml screw cap tubes and 350 mg of 0.1 zirconia–silica beads (Biospec) and 1.6 ml of ASL buffer were added to each tube. The samples were then incubated at 100 °C for 5 min with shaking at 1400 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany) and subsequently subjected to mechanical homogenisation in the MagNA Lyser (Roche) at 7000 rpm for 60 s. The samples were then centrifuged at 14,000 × *g* for 1 min and 0.75 ml of the liquid phase from each duplicate tube was put together to a single tube containing the InhibitEX tablet. The InhibitEX tablet was dissolved in the suspension in the MagNA Lyser (Roche) at 6400 rpm for 10 s. In order to remove all traces of the InhibitEX tablet, the samples were spun twice at 14,000 × *g* for 3 min and the supernatant was transferred to a new tube. The on-column purifications were done according to the manufacturer's protocol whereas the input amount of the supernatant (600 μl), proteinase K (100 μl), AL buffer (600 μl) and ethanol (600 μl) was increased. The whole volume of the mixture was applied on the column in three steps. Washing with AW1 and AW2 buffers was done twice. The resulting DNA was eluted to 100 μl of TE buffer (Amresco) preheated to 70 °C. To increase elution yield, the filtrate containing DNA in TE buffer was applied on the column again. The *MAP* presence in the 5 μl of eluate was detected and quantified by well established IS*900* and *F57* duplex competitive qPCRs with internal amplification controls in duplicate as described previously by Slana et al. (2008). DNA isolated from the sample was analysed in duplicate by both qPCR assays. Samples were considered positive if at least one sample from the duplicate was positive.

2.5. Determination of the DNA isolation efficiency and limit of detection (LOD)

LOD for both qPCR assays used was established as described previously (Slana et al., 2008). Cattle faeces that were proved negative for the presence of *MAP* by IS*900*

qPCR were spiked with 8 prepared dilutions containing known amounts of *MAP* cells quantified by *F57* qPCR. To each of the two tubes with 0.25 g of faeces 25 μl of respective 10-fold diluted *MAP* suspension was added and without subsequent mixing DNA isolation described above was performed. For the purposes of statistical evaluation, the DNA isolation was performed 6 times and each sample was analysed in duplicate by IS*900* and *F57* qPCR. The DNA isolation efficiency for each dilution was calculated as the quotient of the recovered amount of *MAP* after DNA isolation and theoretical input multiplied by 100. The theoretical and experimental number of *MAP* cells was separately calculated from the raw crossing point values according to the respective regression equation from the actual run; subsequently mean and standard deviations were calculated. From all 8 dilutions the mean value of DNA isolation efficiency for respective *MAP* dilution was calculated. In order to obtain the "recalculation coefficient" for the subsequent *MAP* quantification in unknown samples, median of the mean DNA isolation efficiency values was determined. The LOD for the IS*900* and *F57* qPCR was determined as the lowest theoretical amount of *MAP* per gram of faeces that was possible to detect in all replicates regardless the absolute quantity. For the IS*900* qPCR, the LOD was determined experimentally and by calculation dividing *F57* qPCR data by 15, which corresponds to the average amount of IS*900* element in the *MAP* genome (Slana et al., 2008).

2.6. Statistical analysis of culture and qPCR data and construction of predictive model

Statistical analysis of data obtained from 1906 faecal samples was performed by a statistical software Statistica 9 (StatSoft, Inc., Tulsa, OK, USA) and GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). The samples and corresponding data were divided into four groups according to the level of a total number of CFU in two culture flasks: (i) negative, (ii) 1–10 CFU, (iii) 11–100 CFU and (iv) too numerous to count (TNTC). To compare the absolute numbers of *MAP* as determined by IS*900* qPCR with number of CFU linear regression analysis was applied. Normality of the IS*900* qPCR data was tested by D'Agostino & Pearson omnibus normality test. For the construction of model for the prediction of culture results based on the *MAP* absolute numbers gained by IS*900* qPCR binary logistic regression analysis was performed. *P* value differences lower than 0.05 were considered statistically significant.

3. Results

3.1. Efficiency of the MAP DNA detection and determination of LOD

Faecal samples were spiked with a known number of *MAP* cells and compared with the recovered number (Table 1). DNA isolation efficiency ranged from 29.1 to 101.4% of the input amount of *MAP*, with a median of 39.8%. This coefficient was used in the subsequent calculations of "real" *MAP* number in unknown samples. The LOD for *F57* qPCR was determined at 1.03×10^4 and for IS*900* qPCR at 1.10×10^3 *MAP* cells per gram of faeces (Table 1). By calculation, the LOD for IS*900* qPCR from *F57* qPCR data was determined to be 6.87×10^2 *MAP* cells per gram of faeces.

3.2. Comparison of culture and qPCR on faecal samples and construction of predictive model for the probability of MAP detection by culture or qPCR

From all 1906 faecal samples analysed 875 were shown to be positive by IS*900* qPCR and 169 by culture (Table 2). Diagnostic sensitivity of IS*900* qPCR calculated from the raw data was determined to be 100% whereas specificity was only 59.4% (Table 2). The raw IS*900* qPCR data did not

Table 1
Evaluation of the reliability and efficiency of the total *Mycobacterium avium* subsp. *paratuberculosis* DNA isolation procedure from cattle faeces using the IS*900* and *F57* real time quantitative PCR (qPCR) assay.

Theoretical input of *MAP* cells[a]		Experimental output of *MAP* cells by *F57* qPCR[b]			Experimental output of *MAP* cells by IS*900* qPCR[e]			Mean *MAP* DNA isolation efficiency (%)[f]
Mean[c]	SD	Mean[c]	SD	Signal ratio[d]	Mean[c]	SD	Signal ratio[d]	
1.78×10^8	8.89×10^7	5.68×10^7	2.00×10^7	6/6	5.56×10^7	2.45×10^7	6/6	31.8
1.52×10^7	6.42×10^6	5.45×10^6	2.42×10^6	6/6	5.54×10^6	2.98×10^6	6/6	35.9
1.34×10^6	6.44×10^5	3.89×10^5	1.64×10^5	6/6	4.62×10^5	2.75×10^5	6/6	29.1
1.20×10^5	6.17×10^4	4.78×10^4	2.46×10^4	6/6	4.65×10^4	2.85×10^4	6/6	39.8
1.03×10^4	6.04×10^3	5.46×10^3	3.64×10^3	6/6	5.74×10^3	5.47×10^3	6/6	53.1
1.10×10^3	7.52×10^2	1.13×10^3	7.45×10^2	2/6	7.56×10^2	2.45×10^2	6/6	76.8
1.24×10^2	5.65×10^1	–	–	0/6	2.15×10^2	3.23×10^1	3/6	101.4
1.59×10^1	7.02×10^0	–	–	0/6	–	–	0/6	–

MAP: *Mycobacterium avium* subspecies *paratuberculosis*.
[a] Number of *MAP* cells used for the spiking of cattle faeces.
[b] Number of *MAP* cells recovered after DNA isolation by *F57* qPCR.
[c] The mean values correspond to the absolute amount of *MAP* cells in gram of cattle faeces.
[d] Number of positive replicates/total number of replicates.
[e] Number of *MAP* cells recovered after DNA isolation by IS*900* qPCR, the value was divided by 15 (average number of IS*900* copies in *MAP* genome) to obtain real number of *MAP* cells.
[f] Calculated as the quotient of the mean experimental and theoretical yield of *MAP* cells from IS*900* and *F57* qPCR added to and gained from the DNA isolation multiplied by 100 (resulting values are in percents).

Table 2
Results of culture and IS*900* qPCR examination of faecal samples.

	IS*900* qPCR positive	IS*900* qPCR negative	Σ	Diagnostic sensitivity	Diagnostic specificity
Culture positive	169	0	169	100%	59.4%
Culture negative	706	1031	1737		
Σ	875	1031	1906		

Table 3
Descriptive statistics of data positive by IS*900* qPCR.

	Culture[a]			
	Negative	1–10	11–100	TNTC[b]
Number of IS*900* qPCR positive samples	706	92	23	54
Minimum	1.00×10^0	1.11×10^2	9.44×10^2	2.27×10^2
Maximum	3.72×10^7	3.56×10^7	5.55×10^5	2.30×10^8
Median	5.65×10^2	2.28×10^3	5.71×10^4	2.87×10^6
Lower quartile ($Q_{0.25}$)	2.38×10^2	8.20×10^2	5.51×10^3	1.95×10^5
Upper quartile ($Q_{0.75}$)	1.38×10^3	7.06×10^3	1.76×10^5	8.09×10^6
Interquartile range ($Q_{0.75}$–$Q_{0.25}$)	1.15×10^3	6.24×10^3	1.71×10^5	7.90×10^6

[a] Total number of *Mycobacterium avium* subspecies *paratuberculosis* CFU on two flasks with Herrold Egg Yolk Medium with Mycobactin J.
[b] TNTC: too numerous to count.

pass through D'Agostino & Pearson normality test ($P < 0.01$) and thus the logarithmic transformation was performed. Despite this transformation, the data still did not show normal Gaussian distribution except for 11–100 CFU selective group. Thus, non-parametric methods were used for the statistical description of logarithmically transformed data.

Kruskal–Wallis test for IS*900* qPCR positive data showed that the level of CFU is a statistically significant source of variability ($P < 0.01$). Subsequent comparison of medians of respective groups using the Dunn's multiple comparison test showed that all differences (except for difference between groups 11–100 CFU and TNTC) were statistically significant ($P < 0.01$). The linearity trend post-test showed that medians of IS*900* qPCR values statistically significantly increase ($P < 0.01$) when numbers of CFU increase (Table 3).

For the modelling of a probability of *MAP* detection by culture based on the IS*900* qPCR data all samples were taken into consideration. Culture results were considered as a dichotomised variable that can take value "0" (negative culture) or "1" (positive culture) despite of the actual number of CFU. The binary logistic regression in a common form with two-parametric logit function was used. Suitability of the model was confirmed by the Hosmer–Lemeshow goodness-of-fit test ($P > 0.05$) and its statistical significance proven for both, the whole model (likelihood ratio test; $P < 0.01$) and parameters of a model (Wald statistic; $P < 0.01$). According to the model it is possible to estimate the probability of positive culture using absolute number of *MAP* as derived from IS*900* qPCR. For example, in samples with 10^5 *MAP* cells per gram of faeces there is approximately 79% probability that these will be positive by culture (Fig. 1). The odds ratio value

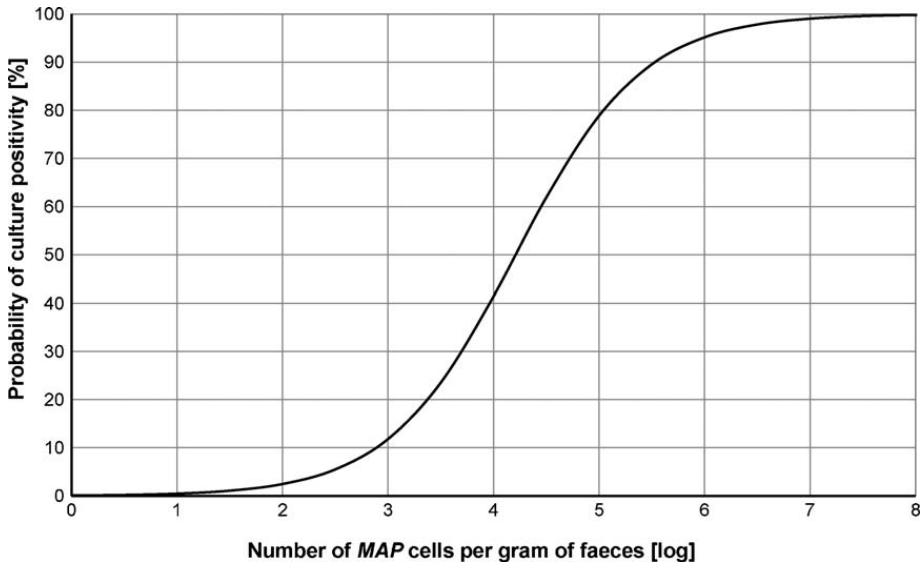

Fig. 1. Scatter plot representing the dependence of absolute numbers of *Mycobacterium avium* subsp. *paratuberculosis* (log scale) versus probability of culture positivity (in %).

Table 4
Diagnostic sensitivity and specificity at optimal cut-off value derived for the predictive model.

	IS*900* qPCR positive[a]	IS*900* qPCR negative[b]	Σ	Diagnostic sensitivity	Diagnostic specificity
Culture positive	144	25	169	85.2%	85.3%
Culture negative	255	1482	1737		
Σ	399	1507	1906		

MAP: *Mycobacterium avium* subsp. *paratuberculosis*.
[a] When IS*900* qPCR value is higher than 8.77×10^2 *MAP* cells.
[b] When IS*900* qPCR value is lower or equal to 8.77×10^2 *MAP* cells.

derived from the model used in this study was 5.28. This shows that odd of the culture positivity increases more than 5 times when an IS*900* qPCR value increases by one order of magnitude.

The cut-off value derived from the model was determined to be 8.77×10^2 *MAP* cells as measured by the IS*900* qPCR. After application of this cut-off value on the raw data we are able to correctly identify 85.2% of culture positive (diagnostic sensitivity) and 85.3% of culture negative samples (diagnostic specificity; Table 4).

4. Discussion

To monitor *MAP* presence in faeces by qPCR, we have adapted a previously published DNA isolation procedure based on a commercially available kit intended for the efficient and quick isolation of *MAP* DNA (Taddei et al., 2004; Herthnek and Bolske, 2006; Schonenbrucher et al., 2008). The original protocol was modified to increase the probability of *MAP* detection. For the determination of the DNA isolation efficiency from faeces necessary for the quantification of *MAP* in the sample we have used approach based on spiking of faeces as described previously for milk (Slana et al., 2008).

In milk overall DNA isolation efficiency was determined as the mean of the overall DNA isolation efficiencies for the respective input of *MAP* cells. The mean DNA isolation efficiency was set on 31.8% (Slana et al., 2008). In present study, we have used similar approach, but mean of the overall DNA efficiencies (48.7%) was replaced by median (37.9%) due to the high fluctuation of the overall values in lower concentrations of spiked faecal samples. This may occur when samples are at or under the LOD (Slana et al., 2008). Employment of the median should ensure that the DNA isolation efficiency would reflect the "universal" DNA yield regardless the *MAP* cells number present on the sample. The LOD value is theoretical and positive samples below LOD should be considered as positive as well, but the probability of their successful detection in repeated analysis is lower.

Published correlation between culture and conventional PCR or real time PCR is strongly dependant on type of matrix and protocols used. For example, milk was referred to be very difficult template for the *MAP* culture and number of milk samples positive for *MAP* by real time PCR was significantly higher than culture (Donaghy et al., 2008; Slana et al., 2008, 2009). Conversely, some studies reported higher percentage of positive samples by culture rather than conventional PCR (Shankar et al., 2010).

When faeces are used as the template for the *MAP* detection, differences between culture and PCR results are not as high as in the case of milk. It was referred that PCR techniques for *MAP* detection from faeces are comparable with culture on solid or liquid media (Bogli-Stuber et al., 2005; Kawaji et al., 2007; Scott et al., 2007; Vansnick et al., 2007; Schonenbrucher et al., 2008; Pinedo et al., 2008; Irenge et al., 2009; Soumya et al., 2009). Majority of these papers dealt with quite limited number of *MAP* positive faecal samples. On the contrary, in this study we compared culture and qPCR results from 875 qPCR positive faecal samples. Only 19.3% (169) of these samples were positive by culture. Thus, we suggest IS*900* qPCR to be used as an alternative reference method for the determination of positive animals.

High number of IS*900* qPCR positive samples that were negative by culture can be explained by several factors. Perhaps the most obvious is that PCR detects total *MAP* DNA whereas culture measures only viable *MAP*. At the present technique employing propidium monoazide dye that selectively inactivate DNA of bacterial cells with compromised or disrupted cell wall was introduced (Kralik et al., 2010). Such techniques could represent significant improvement in *MAP* viability detection by PCR in future as direct alternative to culture.

The next major factor is the reduction in viable *MAP* due to HPC decontamination. Reddacliff et al. (2003) reported 2.7 $\log_{10}$ decrease in *MAP* recovery from faeces after HPC decontamination. In addition, other factors like incubation with antibiotics after HPC decontamination and prior to the seeding *MAP* on HEYM were also referred to reduce the *MAP* recovery (Whittington, 2009). It was found that vancomycin is less inhibitory to C strains compared with S strains (Gumber and Whittington, 2007). Moreover, vancomycin prevents growth of contaminating microflora from faeces more efficiently than other antibiotics (Whittington, 2009).

It must be noted that the median of qPCR values for culture negative faecal samples lies under the LOD of the method. However, animals with low numbers of *MAP* in their faeces should not represent an imminent threat for other animals. These animals should be marked as "suspected" and monitored in the future as the disease progresses (Slana et al., 2008).

The model for the prediction of *MAP* detection probability by culture from faeces based on the qPCR results (absolute numbers of *MAP* cells per gram of faeces) can be used in the future studies as the tool for the determination of infectious status of an animal with respect to the culture examination results classification (heavy, moderate and low shedders) described previously (Whitlock et al., 2000). This approach can speed up the determination of paratuberculosis prevalence and could help to choose suitable control or eradication procedure.

The presented technique for faecal DNA isolation is fast, relatively inexpensive and allows automation, which makes it suitable for the routine diagnostic laboratories. The isolated DNA is sufficiently pure for real time qPCR analysis, allowing successful amplification. Its applicability for the routine diagnosis was proven on field samples collected from 12 infected herds. The DNA isolation with subsequent qPCR analysis is sufficiently sensitive and results are provided within a single day. Developed technique can be employed in the paratuberculosis control programmes because it can be performed in each laboratory with standard molecular biology equipment.

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Evidence of passive faecal shedding of *Mycobacterium avium* subsp. *paratuberculosis* in a Limousin cattle herd

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ABSTRACT

It has been suggested that passive shedding of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) in faeces may occur, but reliable data are missing. Passive shedding assumes the ingestion of MAP in contaminated feed and passive passage through the gastrointestinal tract without causing infection. In this study the presence of MAP in faeces in a closed herd of Limousin cattle was monitored for 53 months using quantitative real time PCR (qPCR) and culture. The initial prevalence of MAP in the herd was determined to be 63.4% and 4.9% using qPCR and culture, respectively. After the removal of two culture- and qPCR-positive (>10^4 MAP cells/g) cows, the prevalence of MAP using qPCR decreased to 42.1% and later to 15.6% and 6.7%. The continuous removal of suspected animals from the herd during the monitoring period minimised the presence of MAP in faeces to sporadic, which may have resulted from a decrease in the environmental infectious pressure. The findings suggest that the presence of low numbers of MAP in bovine faeces may not necessarily be caused by real infection, but rather by passive passage of MAP. This phenomenon should therefore be considered when interpreting MAP qPCR data.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease (Manning and Collins, 2001) and the main route of transmission in ruminants is generally faecal-oral. Animals shedding high numbers of MAP in their faeces can maintain good physical condition over a long period and as they do not show any clinical signs of disease (Crossley et al., 2005) they remain a significant risk for other animals in the same environment, especially young stock. Animals with clinical signs of Johne's disease may therefore represent only the tip of the iceberg of infected individuals in the herd (Whitlock and Buergelt, 1996).

Current control programmes for Johne's disease are based solely on faecal culture and ELISA tests (Bihrmann et al., 2012; Espejo et al., 2012). Detection of MAP using culture on solid media is time-consuming and lacks sensitivity (Kralik et al., 2011). Modern methods based on the detection of molecular markers specific for MAP (IS*900* or F57) by PCR are more sensitive and faster than culture (Slana et al., 2008; Soumya et al., 2009), although PCR does not distinguish between viable and dead cells. In spite of having some limitations (Cousins et al., 1999), the IS*900* sequence is the most commonly used target for detection, as it is present in the MAP genome in approximately 15 copies (Pavlik et al., 1999; Bull et al., 2000) and therefore provides increased sensitivity compared with low copy and single copy loci (Slana et al., 2008).

On the basis of the shedding of MAP in faeces, animals are termed as passive or active shedders. In active shedders, shedding of MAP occurs as a result of real infection of their intestinal tract. After ingestion of contaminated feed or water, MAP enters intestinal tissue using M-cells associated with Peyer's patches. After being transported by M-cells through the intestinal epithelial layer, MAP is ingested by macrophages that assure their distribution to other tissues and organs (Woo and Czuprynski, 2008). In contrast, in passive shedders positive culture results occur due to the simple ingestion of contaminated feed rather than ongoing infection (Pradhan et al., 2011). This phenomenon was first described in cows fed with faeces from paratuberculosis infected cattle (Sweeney et al., 1992), and has been addressed in several studies (Sweeney et al., 2006; Moloney and Whittington, 2008; Whitlock et al., 2008; Pradhan et al., 2011), all of which have used faecal culture. No study has evaluated the passive shedding of using real time quantitative PCR (qPCR).

Passive shedding of MAP in faeces could complicate attempts to distinguish between infected and non-infected cows using qPCR techniques. The aims of the present study were to assess, in a closed herd of Limousin cows with a long history of paratuberculosis infection, the potential occurrence of passive faecal shedding

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in cows after the removal of heavily shedding animals from the herd and to evaluate the influence of passive shedding on the interpretation of data obtained using faecal qPCR.

Materials and methods

Herd status and sample collection

The herd of Limousin beef cattle comprised 40 cows and one breeding bull. Previously, the herd had taken part in a paratuberculosis control programme and, based on faecal culture, was considered to be free of paratuberculosis. The median animal age at the beginning of the screening was 5 years (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). The herd was housed in a shed during winter and kept at pasture during summer. Calves remained with their dams until they were 8–9 months of age, when heifers were moved to another location to prevent inbreeding and young bulls were transferred to a feedlot for fattening. After three negative faecal culture examinations of all animals older than 18 months during the previous 2 years, a fourth faecal culture was performed in parallel with qPCR examination of faecal samples and ELISA examination of serum (month 0 in this study).

Individual faecal sample were collected directly from the rectum using a disposable glove, chilled, transported to the laboratory and analysed within 3 days. To prevent possible cross contamination, separate faecal aliquots for culture and qPCR were prepared in a separate MAP-free laboratory. Samples were collected again 4, 9, 12, 17, 24, 30, 36, 43, 48 and 53 months after the first examination (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). Tissue samples (intestine and mesenteric lymph nodes) from slaughtered animals were collected after culling at the slaughterhouse by a trained pathologist. At the laboratory, samples were processed under sterile conditions and samples from the inside part of the tissue were taken. Samples were considered positive when at least one tissue (intestine or mesenteric lymph node) was positive.

Culture examination of faeces and tissues

All samples were analysed by culture as previously described (Pavlik et al., 2000). Briefly, 5 g of faeces were resuspended in sterile water and decontaminated in 0.75% hexadecylpyridinium chloride (HPC) for 3 days with mild shaking. For tissues, 1 g was homogenised in a stomacher with 0.75% HPC and decontaminated overnight. Culture was performed in three flasks using Herrold's egg yolk medium supplemented with 2 μg/mL of Mycobactin J per sample (HEYM; Allied Monitor) and antibiotics (penicillin G, chloramphenicol and amphotericin B). Samples were incubated at 37 °C. The reading of slants was performed continuously for 3 months.

Examination of faeces by IS900 qPCR

DNA from faeces was isolated using a modified protocol of the QIAamp DNA Stool Mini Kit (Qiagen). The introduced modifications were aimed at increasing the yield of bacterial DNA (Kralik et al., 2011). Briefly, 0.5 g of faeces were divided into two separate tubes and twice homogenised with 0.1 mm zirconia-silica beads (Biospec) in ASL buffer in a MagNA Lyser (Roche Molecular Diagnostic). Possible PCR inhibitors from faeces were removed using an InhibitEX tablet supplied with the kit. The on-column purification and washing of DNA were undertaken according to the manufacturer's protocol. The detection and quantification of the MAP DNA was performed using an IS*900* qPCR assay (Slana et al., 2008). Each qPCR assay contained its own plasmid internal amplification control to allow identification of false negative samples. Quantification was performed according to a plasmid gradient with a known number of copies. The recalculation to 1 g of faeces was done according to an established yield of DNA isolation from faeces (Kralik et al., 2011).

ELISA examination of serum samples

Blood from all available animals was collected at the study start, after 12 months and at the end of the qPCR monitoring. Blood samples were collected using a Hemos set (Gama Group) from the coccygeal vein, and the sera was harvested and frozen at –80 °C until testing for the presence of antibodies against MAP using the ID Screen Paratuberculosis Indirect ELISA kit (ID Vet).

Results

Real time qPCR and culture examination

In the first examination of faeces (month 0), 26 out of 41 examined cows (63.4%) tested positive using IS*900* qPCR; two animals (4.9%) were culture positive (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). The two cows (Cows 1 and 2) with the highest number of MAP in faeces, based on the qPCR (>10^4 MAP cells/g of faeces), were removed from the herd immediately. Subsequent post-mortem examination of the intestinal tract showed gross lesions, and the ileal mucosa and mesenteric lymph nodes of both cows were positive. One cow (Cow 6) was faecal culture-positive, but qPCR negative. This cow remained in the herd.

Four months later (2 months after the removal of both heavy shedders), faecal examination showed that the number of qPCR-positive cows dropped to 16/38 (42.1%), and no cow was culture positive (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). Thirteen cows positive in the first qPCR were negative, while five cows which were negative became positive (including Cow 6). Nine months after the beginning of the study, the total number of cows in the herd had been reduced to 33 due to culling for animal husbandry reasons, health reasons (this included Cow 6) or death after parturition problems. Of the remaining 33 animals demonstrated only two (6.1%) cows (10 and 11) were qPCR-positive. These cows had been positive in both of the previous two qPCR tests. None of the animals slaughtered between the 4th and 12th month had gross lesions, and culture of mesenteric lymph nodes and ileum was routinely negative (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011).

Twelve months after the beginning of the study, the percentage of MAP-positive cows increased slightly. Cow 11 was culture and qPCR-positive and was thus culled immediately, along with Cow 10 which was qPCR-positive for the fourth consecutive test. In both culled cows, multiple tuberculous lesions in organs and the presence of viable MAP in the intestines were confirmed by pathological and culture examination. During the two next examinations (months 17 and 24), the qPCR positivity in the herd decreased to 7.4% (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011).

In month 30, 10/25 animals (40%) were qPCR-positive, however, none were culture positive. During four subsequent examinations, MAP qPCR positivity decreased to 5.6% (month 36), and fell further down to zero positivity (months 43 and 48), and finished at 6.7% (month 53). No animal was found to be culture positive from month 17 onwards, nor were any lesions found in any slaughtered animal (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011).

ELISA examination

Six cows had a positive ELISA test (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). Three culled animals (Cows 1, 2 and 10) were proven to be infected by MAP; the remaining three animals (Cows 18, 27 and 32) tested positive using qPCR only at the beginning of the monitoring, and remained negative for all subsequent tests.

Discussion

This study presents evidence for passive shedding using quantitative real time IS*900* PCR, faecal culture and histopathological changes. Passive shedding, where MAP is present in the faeces as a result of the oral ingestion of organisms, is not connected with current infection of the host (Sweeney et al., 1992, 2006). In previous studies, the infection status of animals suspected of being passive shedders was tested using the culture of faeces. However, as demonstrated in this study, assaying for the presence of the multiple loci IS*900* sequence results in a significantly higher percentage of positive cows compared to culture (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011); these data are consistent with the finding by Kralik et al. (2011) that the sensitivity of culture is approximately 10^3 MAP cells/g of faeces.

Heavy shedders can shed >10^4 MAP bacilli/g of faeces into the farm environment. Compared to low shedders, such animals have a significant impact on the prevalence of MAP-infected individuals in the herd (Mitchell et al., 2008). In this study, removal of the two heavy shedders (Cows 1 and 2) dropped the overall qPCR-positive rate, supporting the findings of Whitlock et al. (2008) who associated a higher rate of passive shedding with cows being in a herd with just one massively shedding cow, and the observation by Crossley et al. (2005), who noted that higher prevalence herds had more low MAP shedders which might be attributable to 'pass-through' passive shedding.

Our findings indicate that the removal of heavy shedders decreases the amount of MAP in the environment and thus the infection pressure on the other 'sensitive' animals. This is supported by our finding that out of the 16 qPCR-positive animals found in month 0 which were culled, 12 had negative faecal and lymph node culture and no pathological changes (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011).

The decreasing trend in the detection of IS*900*-positive animals continued until month 24. In month 30, 10 individuals that were negative in previous months tested positive in qPCR, however, none of these were culture-positive (Appendix S1 in the online version at doi:10.1016/j.tvjl.2014.02.011). The reason for this laid in the import of 19 heifers which were previously separated from the herd as calves. The import of the heifers was arranged by the owner of the herd during month 24 after the beginning of monitoring and despite the paratuberculosis control programme recommendations. Subsequent testing showed 7/19 heifers were qPCR-positive (10^2–10^3 MAP cells/g) and one was a culture-positive animal (data not shown). This observation strongly supports the theory of passive shedding and the occurrence of 'temporarily contaminated animals' as after the immediate removal of the heifers, the decreasing trend in prevalence of qPCR-positive animals continued until the end of the monitoring.

Whitlock et al. (2008, 2009) defined passive shedders as cows with low to moderate counts of MAP on the surface of culture media, with at least two subsequent negative faecal cultures and with no MAP bacilli in their tissues or which had negative ELISA tests. Cows 18 (10 years old), 27 and 32 (both 3 years old at the beginning of the testing) would be automatically assessed as positive. The interpretation of ELISA-positive results is, inter alia, based on an assumption that older animals can better cope with the infection compared to animals that are ≤ 3 years of age (Larsen et al., 1975). The assessment of samples from the qPCR point of view with combination with others methods does not give such weight to the age of animals. Therefore, on the basis of these qPCR results, all three animals would be assessed as 'suspicious' and their future monitoring would be recommended.

Recently, a locus for tolerance to paratuberculosis in cattle has been identified. Animals with this locus shed low levels or no bacteria in faeces and are considered as tolerant; selecting such animals could contribute to reducing MAP infection in the environment (Zanella et al., 2011; McSpadden et al., 2013).

The findings support the existence of passive shedding, and that such shedding and can be demonstrated using qPCR. The data also suggest that the sporadic detection of MAP in 'contaminated' animals is basically unavoidable in infected herds, and that this phenomenon must be considered during the interpretation of qPCR data. If qPCR data are to be used, the term 'heavy shedder' should be employed with caution or modified. In our study, animals shedding >10^4 MAP cells/g of faeces were identified using qPCR. However, these animals were either culture-negative or only slightly positive, and were therefore not heavy shedders using faecal culture criteria. We propose that studies using qPCR should use the terms 'qPCR heavy shedder' or 'qPCR low shedder' for individuals that shed more or less than 10^4 MAP cells/g of faeces, respectively.

Conclusions

In a herd with heavy shedders there are 'susceptible' cows that are 'temporarily contaminated', with MAP detectable in their faeces using qPCR, but not by culture. The results from this study suggest that concentrations of MAP in qPCR-positive faeces ≤10^3 MAP cells/g do not automatically mean that the animal is truly infected, regardless of its physical condition. Repeating the PCR examination after 2–4 months is likely to be useful to reliably remove all heavy shedders (>10^4 MAP cells/g) from the herd. Additionally, three consecutive positive qPCR tests will identify an infected animal, which should then be culled. In contrast, repeated non-consecutive low MAP concentrations identified by qPCR indicate a 'temporarily contaminated' passive shedder which could remain within the herd. The simultaneous occurrence of irregular qPCR positivity with positive ELISA tests denotes a 'suspect' animal which should be observed but not necessarily culled immediately, particularly if they are ≤3 years of age. These results show how using qPCR testing can provide another dimension to our understanding of the distribution of MAP in cattle.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.tvjl.2014.02.011.

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A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything

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Real time PCR (quantitative PCR, qPCR) is now a well-established method for the detection, quantification, and typing of different microbial agents in the areas of clinical and veterinary diagnostics and food safety. Although the concept of PCR is relatively simple, there are specific issues in qPCR that developers and users of this technology must bear in mind. These include the use of correct terminology and definitions, understanding of the principle of PCR, difficulties with interpretation and presentation of data, the limitations of qPCR in different areas of microbial diagnostics and parameters important for the description of qPCR performance. It is not our intention in this review to describe every single aspect of qPCR design, optimization, and validation; however, it is our hope that this basic guide will help to orient beginners and users of qPCR in the use of this powerful technique.

Keywords: quantitative PCR, limit of detection, limit of quantification, accuracy, precision, trueness

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INTRODUCTION

The phrase "Polymerase chain reaction" (PCR) was first used more than 30 years ago in a paper describing a novel enzymatic amplification of DNA (Saiki et al., 1985). The first applications of PCR were rather unpractical due to the usage of thermolabile Klenow fragment for amplification, which needed to be added to the reaction after each denaturation step. The crucial innovation which enabled routine usage of PCR was utilization of thermostable polymerase from *Thermus aquaticus* (Saiki et al., 1988). This improvement, together with the availability of PCR cyclers and chemical components, led to the worldwide recognition of PCR as the tool of choice for the specific enzymatic amplification of DNA *in vitro*. It must be noted that the general concept of PCR, which includes primers, DNA polymerase, nucleotides, specific ions, and DNA template, and consisting of cycles that comprise steps of DNA denaturation, primer annealing, and extension, have not been changed since 1985. The invention of PCR has greatly boosted research in various areas of biology and this technology has significantly contributed to the current level of human knowledge in many spheres of research.

The most substantial milestone in PCR utilization was the introduction of the concept of monitoring DNA amplification in real time through monitoring of fluorescence (Holland et al., 1991; Higuchi et al., 1992). In real time PCR (also denoted as quantitative PCR—qPCR; usage of RT-PCR is inappropriate as this abbreviation is dedicated to reverse transcription PCR), fluorescence is measured after each cycle and the intensity of the fluorescent signal reflects the momentary amount of DNA amplicons in the sample at that specific time. In initial cycles the fluorescence is too low to be distinguishable from the background. However, the point at which the fluorescence

intensity increases above the detectable level corresponds proportionally to the initial number of template DNA molecules in the sample. This point is called the quantification cycle (C_q; different manufactures of qPCR instruments use their own terminology, but since 2009, the term C_q is used exclusively) and allows determination of the absolute quantity of target DNA in the sample according to a calibration curve constructed of serially diluted standard samples (usually decimal dilutions) with known concentrations or copy numbers (Yang and Rothman, 2004; Kubista et al., 2006; Bustin et al., 2009).

Moreover, qPCR can also provide semi-quantitative results without standards but with controls used as a reference material. It this case, the observed results can be expressed as higher or lower multiples with reference to control. This application of qPCR has been extensively used for gene expressions studies (Bustin et al., 2009), but did not obtain the same success in microbiology quantification since it is unable to produce absolute quantitative values.

There are two strategies for the real time visualization of amplified DNA fragments—non-specific fluorescent DNA dyes and fluorescently labeled oligonucleotide probes. These two approaches were developed in parallel (Holland et al., 1991; Higuchi et al., 1992) and are used in pathogen detection; however, probe-based chemistry prevails. This is due to its higher specificity mediated by the additional oligonucleotide—the probe—and the lower susceptibility to visualize non-specific PCR products, e.g., primer dimers (Bustin, 2000; Kubista et al., 2006).

To fully understand the possibilities of qPCR in detecting and quantifying target DNA in samples it is essential to describe the mathematical principle of this method. The PCR is an exponential process where the number of DNA molecules theoretically doubles after each cycle (if the efficiency of the reaction is 100%). More generally, the amplification reaction follows this equation:

$$N_n = N_0 \times (1 + E)^n \quad (1)$$

where N_n is the number of PCR amplicons after n cycles, N_0 is the initial number of template copies in the sample, E is the PCR efficiency that can assume values in the range from 0 to 1 (0–100%) and n is number of cycles. In a scenario where there is initially one copy of the template in the reaction and PCR efficiency is 100%, it is possible to simplify the equation as follows:

$$N_n = 2^n \quad (2)$$

If a calibration curve is run, usually 10-fold serial dilutions are used. The difference in C_q values between two 10-fold serial dilutions could be expressed as

$$10 = 2^n \quad (3)$$

Then $n = 3.322$. When E should be determined the (1) is starting point and the equation is

$$E = 10^{-\left(\frac{1}{n}\right)} - 1 \quad (4)$$

If n is taken to be 3.322, then $E = 1$, i.e., 100%.

The PCR efficiency is therefore a significant factor for the quantification of the target DNA in unknown samples. The reliability of the calibration curve in enabling quantification is then determined by the spacing of the serial dilutions. If the Log_{10} of the concentration or copy number of each standard is plotted against its C_q value (**Figure 1**), the E can be derived from the regression equation describing the linear function:

$$y = kx + c \quad (5)$$

Where x and y, the concentration/amount of target and C_q values respectively, characterize the coordinates in the plot, k is the regression coefficient or slope and c is the intercept. Taking the model regression equation from **Figure 1**, the slope is −3.322, which mean that $E = 100\%$ according to (4). The intercept shows the C_q value when one copy would be theoretically detected (Kubista et al., 2006; Johnson et al., 2013). The concentration or amount of target nucleic acid in unknown samples is then calculated according to the C_q value through Equation (5).

From the definitions above it is evident that C_q values are instrumental readings, and must be recalculated to values with specific units, e.g., copies of organism, ng of DNA, various concentrations, etc., (Bustin et al., 2009; Johnson et al., 2013). However, referral to C_q values in scientific papers is widespread and interpretations based on C_q values can lead to misleading conclusions. Concentrations in qPCR are expressed in the logarithmic scale (**Figure 1**) and C_q differences between 10-fold serial dilutions are theoretically always 3.322 cycles. Therefore, although the numerical difference between C_q 20 and 35 is rather negligible, the difference in real numbers (copies, ng) is almost five orders of magnitude (Log_{10}).

This feature must be reflected in the subsequent calculations. For example, the coefficient of variation (CV, ratio between standard deviation and mean) calculated from the C_q values and real numbers results in profoundly different results. The same applies for any statistical tests where C_q values are used, even for cases where the logarithm of C_q values is used for

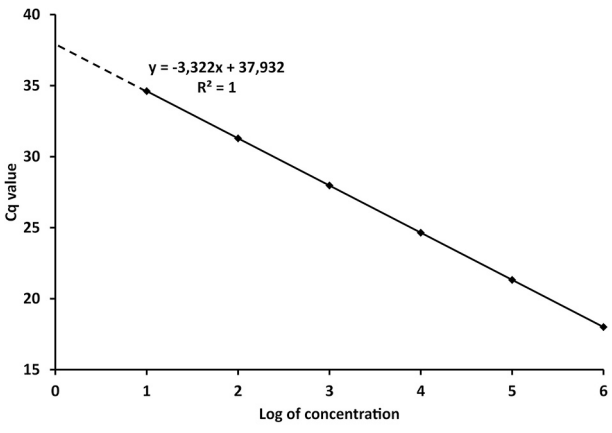

FIGURE 1 | Model calibration curve with the regression equation (characterized by the slope and intercept) and regression coefficient.

the normalization of data before the statistical evaluation. The correct procedure should include initial recalculation to real numbers followed by logarithmic transformation.

PROS AND CONS OF USING qPCR IN DETECTION AND QUANTIFICATION OF PATHOGENS

Since PCR is capable of amplifying a specific fragment of DNA, it has been used in pathogen diagnostics. With the increasing amount of sequencing data available, it is literally possible to design qPCR assays for every microorganism (groups and subgroups of microorganisms, etc.) of interest. The main advantages of qPCR are that it provides fast and high-throughput detection and quantification of target DNA sequences in different matrices. The lower time of amplification is facilitated by the simultaneous amplification and visualization of newly formed DNA amplicons. Moreover, qPCR is safer in terms of avoiding cross contaminations because no further manipulation with samples is required after the amplification. Other advantages of qPCR include a wide dynamic range for quantification (7–8 Log_{10}) and the multiplexing of amplification of several targets into a single reaction (Klein, 2002). The multiplexing option is essential for detection and quantification in diagnostic qPCR assays that rely on the inclusion of internal amplification controls (Yang and Rothman, 2004; Kubista et al., 2006; Bustin et al., 2009).

qPCR assays are used not only for the detection, but also to determine the presence of specific genes and alleles, e.g., typing of strains and isolates, antimicrobial resistance profiling, toxin production, etc., However, the mere presence of genes responsible for resistance to antimicrobial compounds or fungal toxin production does not automatically mean their expression or production. Therefore, although qPCR-based typing tests are faster, their results should be correlated with phenotypic and biochemical tests (Levin, 2012; Osei Sekyere et al., 2015).

As for the microbial diagnostics, there are different considerations in detecting and quantifying viral, bacterial, and parasitic agents. These considerations are based on the target (DNA or RNA), cultivability, interpretation of results, and clinical significance of qPCR results.

qPCR plays an important role in the detection, quantification, and typing of viral pathogens. This is because detection of important clinical and veterinary viruses using culture methods is time-consuming or impossible, while ELISA tests are not universally available and suffer from comparatively low sensitivity and specificity. qPCR (with the inclusion of reverse transcription for the diagnostics of RNA viruses) provides the appropriate sensitivity and specificity (Hoffmann et al., 2009). Moreover, determination of the viral load by (RT)-qPCR is used as an indicator of the response to antiviral therapies (Watzinger et al., 2006). For these reasons (RT)-qPCR has become an indispensable tool in virus diagnostics (Yang and Rothman, 2004).

The situation is similar in the case of intestinal protozoan diagnostics (Rijsman et al., 2016). The gold standard technique for the detection of protozoan agents, the microscopic examination of feces, is laborious, time-consuming, and requires specifically trained personnel. Similarly, ELISA testing suffers from low sensitivity and specificity (Rijsman et al., 2016). Therefore, qPCR is now emerging as a powerful tool in the routine detection, quantification, and typing of intestinal parasitic protozoa.

In contrast to viral and protozoan detection and quantification, many bacteria of clinical, veterinary, and food safety significance, can be cultured. For this reason, culture is considered as the gold standard in bacterial detection and quantification. However, in cases when critical and timely intervention for infectious disease is required, the traditional, slow, and multistep culture techniques cannot provide results in a reasonable time. This limitation is compounded by the necessity of culturing fastidious pathogens and additional testing (species determination, identification of virulence factors, and antimicrobial resistance). qPCR is capable of providing the required information in a short time; however, the phenotypic and biochemical features must be confirmed from bacterial isolates (Yang and Rothman, 2004).

In food safety, all international standards for food quality rely on the determination of pathogenic microorganisms using traditional culture methods. qPCR techniques represent an excellent alternative to existing standard culture methods as they enable reliable detection and quantification (for several pathogens) and harbor many other advantages as discussed above. However, there are limitations with respect to the sensitivity of assays based on qPCR. As culture methods rely on the multiplication of bacteria during the pre-culture steps (pre-enrichment), samples for DNA isolation usually initially contain very low numbers of target bacteria (Rodriguez-Lazaro et al., 2013). This limitation leads to the most important disadvantage of qPCR, which is its inherent incapability of distinguishing between live and dead cells. The usage of qPCR itself is therefore limited to the typing of bacterial strains, identification of antimicrobial resistance, detection, and possibly quantification in non-processed and raw food. It is important to note that processed food can still contain amplifiable DNA even if all the potentially pathogenic bacteria in food are devitalized and the foodstuff is microbiologically safe for consumption (Rodriguez-Lazaro et al., 2013). To overcome this problem, a pre-enrichment of sample in culture media could be placed prior to the qPCR. This step may include non-selective enrichment in buffered peptone water or specific selective media for the respective bacterium. This procedure is primarily intended to allow resuscitation/recovery and subsequent multiplication of the bacteria for the downstream qPCR detection; the second advantage is dilution and elimination of possible PCR inhibitors present into the sample (presence of salts, conservation substances, etc.). The extraction of the DNA from the culture media is easier than that from the food samples, which are much more heterogeneous in terms of composition (Margot et al., 2015).

Although qPCR itself cannot distinguish among viable and dead cells attempts have been made to adapt qPCR for viability detection. It was shown that RNA has low stability and should be

degraded in dead cells within minutes. However, the correlation of cell viability with the persistence of nucleic acid species must be well characterized for a particular situation before an appropriate amplification-based analytical method can be adopted as a surrogate for more traditional culture techniques (Birch et al., 2001). Moreover, difficulties connected with RNA isolation from samples like food, feces or environmental samples can provide false-negative results especially when low numbers of target cells are expected.

Another option for determination of viability using qPCR is the deployment of intercalating fluorescent dyes like propidium monoazide (PMA) and ethidium monoazide (EMA; Nocker and Camper, 2009). In these methods, the criterion for viability determination is membrane integrity. Metabolically active cells (regardless of their cultivability) with full membrane integrity keep the dyes outside the cells and are therefore considered as viable. However, if plasma membrane integrity is compromised, the dyes penetrate the cells, or react with the DNA outside of dead cells. The labeled DNA is then not available for the amplification by qPCR and the difference between treated and untreated cells provides information about the proportion of viable cells in the sample. The limitation of this method is the necessity to have the cells in a light-transparent matrix, e.g., water samples, cell cultures, etc., as the intercalation of the dye to DNA requires exposure to light. Therefore, samples of insufficient light transparency do not permit the application of these dyes. There is a preference for PMA over EMA, as it was shown that EMA penetrates the membranes of live bacterial cells (Nocker et al., 2006).

Moreover, another topic we want to just to mention here is the generation and use of standards required for the calibration curves. In general, two are the most diffused approaches for the generation of calibration curves. One employs dilutions of target genomic nucleic acid and the other plasmid standards. Both strategies can lead to a final quantification of the target, but plasmids containing specific target sequences offer the advantages of easy production, stability, and cheapness. On the other hand, in principle, PCR efficiency obtained by plasmid standards sometimes could differ compared to the efficiency obtained using genomic standard, which instead, for organisms fastidious to growth, could be isolated only starting from a given matrix, and thus susceptible to degradation and losses (Chaouachi et al., 2013). Finally, the production and validation of international quantification standards for qPCR assays is technically demanding and these standards are currently available only for a few targets (Pavšič et al., 2015).

qPCR PARAMETERS IN MICROBIAL DETECTION AND QUANTIFICATION

Analytical Specificity (Selectivity)

This parameter in qPCR refers to the specificity of primers for target of interest. Analytical specificity consists of two concepts: inclusivity describes the ability of the method to detect a wide range of targets with defined relatedness e.g., taxonomic, immunological, genetic composition (Anonymous, 2009, 2015a). Another definition describes inclusivity as the strains or isolates of the target analyte(s) that the method can detect (Anonymous, 2012). ISO 16140 and other standards recommend that inclusivity should be determined on 20–50 well-defined (certified) strains of the target organism (Anonymous, 2009, 2011, 2012, 2015a; Broeders et al., 2014), or for *Salmonella*, it is recommended that 100 serovars should be included for inclusivity testing (Anonymous, 2012).

On the other hand, exclusivity describes the ability of the method to distinguish the target from similar but genetically distinct non-targets. In other words, exclusivity can also be defined as the lack of interference from a relevant range of non-target strains, which are potentially cross-reactive (Anonymous, 2009, 2011, 2012, 2015a). The desirable number of positive samples in exclusivity testing is zero (Johnson et al., 2013).

Analytical Sensitivity (Limit of Detection, LOD)

Many official documents have discussed theories and procedures for the correct definition of the LOD for different methods. A general consensus was reached around the definition of the LOD as the lowest amount of analyte, which can be detected with more than a stated percentage of confidence, but, not necessarily quantified as an exact value (Anonymous, 2011, 2013, 2014). In this regard, the confidence level obtained or requested for the definition of LOD can reflect the number of replicates (both technical and experimental) needed by the assay in order to reach the requested level of confidence (e.g., 95%). It is clear that the more replicates are tested, the narrower will be the interval of confidence. Another definition describes the LOD as the lowest concentration level that can be determined as statistically different from a blank at a specified level of confidence. This value should be determined from the analysis of sample blanks and samples at levels near the expected LOD (Anonymous, 2015a). However, it should be noted that LOD definitions described above were reported for chemical methods, and are not perfectly suited for PCR methods (Burns and Valdivia, 2008). This is because, for limited concentrations of analyte (nucleic acids), the output of the reaction can be a success (amplification), or a failure (no amplification at all), without any blank, or critical level at which it is possible to set a cut-off value over which the sample can be considered as positive one. Moreover, it should be remembered here that, by definition, a blank sample should never be positive in PCR.

Since the definitions reported above are not practicable for PCRs, other approaches have been proposed. A conservative approach is to consider the LOD value as the minimum concentration of nucleic acid or number of cells, which always gives a positive PCR result in all replicates tested, or in the major part (over 95%) of them (Nutz et al., 2011). In practice, multiple aliquots of a specific matrix are spiked with serial dilutions of the target organism and undergo the whole process of nucleic acid isolation and qPCR. The LOD is then defined as the spike amount of target organism in dilution that could be detected in 95% of replicates. For example, 10 replicates of milk samples were spiked with serial dilutions of *Campylobacter jejuni* in amounts of

10^5–10^0 cells per 1 ml of milk. The experimentally determined LOD of the method for the detection of *C. jejuni* in milk is approximately 1.56×10^3 cells/ml of milk (**Figure 2**). In order to better define the most precise value, more dilutions can be tested before reaching a final LOD value as close as possible to the real one. The number of replicates tested should be at least six (Slana et al., 2008; Kralik et al., 2011); however, the more replicates (10 or 15 and more; Ricchi et al., 2016) performed, the higher level of confidence of the LOD that can be achieved (Anonymous, 2015b).

According to the Poisson distribution, it was concluded that the LOD for PCR cannot be lower than at least three copies of the nucleic acid targets (Bustin et al., 2009; Johnson et al., 2013). However, this value refers to the theoretical LOD of the qPCR methodology, which is capable of detecting a single target DNA molecule in the sample. Assuming this, such LOD for all optimized qPCR assays will be similar. Therefore, as stated above, the LOD must be related to the whole method that includes nucleic acid preparation and qPCR. Only under these conditions can it represent a valid parameter that describes the features of the respective qPCR method (Anonymous, 2015a).

However, sometimes it is not possible to obtain large numbers of replicates, for both financial and technical reasons. To overcome these problems, an increasing number of reports utilize Probit or Logit approaches for determining the LOD for PCR methods (Burns and Valdivia, 2008; Anonymous, 2014; Pavšic et al., 2016; Ricchi et al., 2016). Briefly, both mathematical functions are regressions used to analyse binomial response variables (positive or negative) and are able to transform the sigmoid dose-response curve, typical of a binomial variable, to a straight line that can then be analyzed by regression either through least squares or maximum likelihood methods. The final end-point of the analysis is a concentration (coupled with relative intervals of confidence), associated to a probability (e.g., 95%) to detect the nucleic acid. Moreover, Probit regression is exploitable only for normally distributed data, while Logit function can also be used for data not normally distributed; however, in this context, both functions have the same meaning.

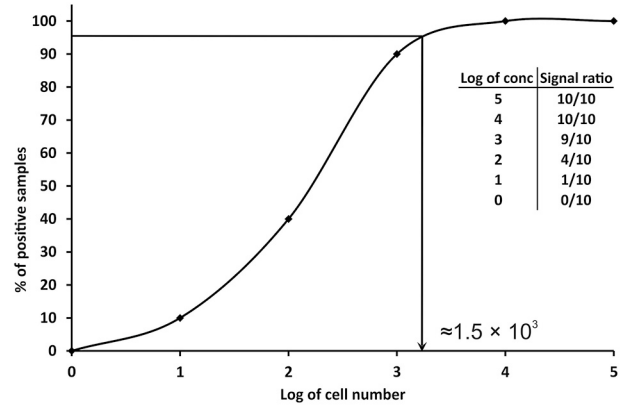

FIGURE 2 | Graphical representation of the determination of limit of detection (LOD) in qPCR. The data in the table show the number of positive samples/all analyzed samples (signal ratio).

Finally, it must be noted that LOD is not a limiting value and therefore, that C_q values below the LOD cannot automatically be considered as negative. From the definition of LOD, it is evident that values below the LOD are absolutely valid in terms of microorganism presence; however, the probability of their repeated detection is lower than 95%. This feature is connected with the Poisson distribution when working with small numbers.

Limit of Quantification (LOQ)

The documents already cited for the LOD definitions also contain analog definitions for the LOQ. The LOQ was defined as the smallest amount of analyte, which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation (Armbruster and Pry, 2008; Anonymous, 2011, 2013). An alternative definition is that the LOQ is the lowest amount or concentration of analyte that can be quantitatively determined with an acceptable level of uncertainty (Anonymous, 2015a). It is clear that, according to the previous definitions, the LOQ can never be lower than the LOD.

In practice, the LOQ is determined as is the LOD, on replicates of spiked samples, but the assessment of results is quantitative. Numerically, the LOQ is defined as the lowest concentration of analyte, which gives a predefined variability, generally reported as the coefficient of variation (CV). For qPCR, this value has been proposed to be fixed under 25% (Broeders et al., 2014; Dreo et al., 2014; Anonymous, 2015b; Pavšic et al., 2016), bearing in mind that the C_q values must be recalculated to copies or g of nucleic acids before performing the evaluation of the CV (Bustin et al., 2009; Johnson et al., 2013). Hoverer, this value was proposed based on the experience accrued in GMO detection laboratories (Broeders et al., 2014; Anonymous, 2015b), and there is no general agreement regarding any technical standards for molecular methods in microbiology. Therefore, we propose here to define the LOQ in the molecular diagnosis of microorganisms as the lowest concentration, amount, or number of analytes with a $CV < 25\%$.

Another approach for the determination of the LOQ of qPCR is based on the use of the Youden index (J) and receiver operating characteristic curves (Nutz et al., 2011). This last index was defined as $J =$ sensitivity $+$ specificity -1 (Fluss et al., 2005). A series of spiked samples with different concentrations of target DNA were analyzed and the *J*-values were calculated for each PCR cycle. The LOQ was then fixed as the concentrations of DNA where the *J*-values were highest (Nutz et al., 2011).

Finally, an issue that should be addressed for the determination of the LOQ as well as LOD is the efficiency of recovery of target molecules during the nucleic acid extraction phases. Generally, nucleic acids are extracted from different complex matrices, like food, feces, or other samples using different procedures. The efficiency of DNA recovery is usually around 30% and lower (Slana et al., 2008; Kralik et al., 2011; Ricchi et al., 2016) and neglecting this parameter leads to underestimation of the true number of target microorganisms

in the original sample, which is then reflected by the lower LOD and LOQ values. Therefore, determination of DNA isolation efficiency should be part of the LOD and LOQ. DNA isolation efficiency is a quotient between the number of microorganisms recovered after the entire procedure (nucleic acid extraction + qPCR) and the number of microorganisms used for spiking the negative matrices (Slana et al., 2008; Kralik et al., 2011; Ricchi et al., 2016). Due to the fact that these data are provided during the determination of the LOD and LOQ, it is not necessary to perform additional experiments. It is recommended that the median of mean DNA isolation values from different dilutions is used as the practical overall DNA isolation efficiency (Kralik et al., 2011).

Similarly to the LOD, quantity can also be assessed in samples with numbers of organisms or concentrations of DNA lower than the LOQ, but the confidence of such quantification will be lower than that declared by the definition of LOQ. Moreover, there are possibilities of how to refer to such quantities in terms of semi-quantitative interpretation, e.g., range of values (10^2–10^1 cells/g).

Amplification Efficiency of qPCR (E)

This parameter was mentioned above in the section dedicated to the mathematical description of qPCR (Equation 4). PCR efficiency should be in the range of 0–1 (0–100%); when $E = 1$ this means that the number of newly formed DNA amplicons is doubled in each cycle. This is difficult to reach repeatedly over time. In practice, this parameter is likely to be in the range 90–105% (Johnson et al., 2013). This parameter can be estimated from the slope of the calibration curve.

In connection to this issue, the lowest and highest concentrations of the standard included in the calibration curve, which can be truly quantified, should be determined according to the linear dynamic range of over at least 6 Log_{10}. The dynamic range is defined by the MIQE guidelines as the range over which a reaction is linear (Bustin et al., 2009).

The determination of PCR efficiency by the standard curve actually provides two pieces of information. If an inhibitor would be present in the most concentrated sample, there would be a visible increase in C_q values in these and therefore a diminishment of the 3.322 C_q span at higher concentrations. However, this is not a frequent phenomenon, as standards are usually well-characterized and therefore, any inhibition is rather unlikely. If there would be a similar situation in lower concentration samples, this suggests a possible pipetting error rather than the presence of inhibitors. An important function to assess this is the coefficient of determination (R^2 value), that should be higher than 0.98 (Johnson et al., 2013). In reality, it is much more important to determine the PCR inhibition and subsequent diminishment of the PCR efficiency in analyzed samples. There are approaches based on the analysis of the fluorescent curve of each sample by specific software (LinRegPCR), which can calculate the PCR efficiency of each sample without the series of dilutions. However, this approach is not flawless as it does not take into account all possible variables that can affect the analysis (Ruijter et al., 2009).

Accuracy of PCR

The following parameters of qPCR deal with ways of how to compare novel qPCR methods with reference methods or materials. Accuracy is defined as a measure of the degree of conformity of a value generated by a specific procedure to the assumed or accepted true value (Anonymous, 2015a). In other words, accuracy describes the level of agreement between reference and measured values. There are several aspects that need to be considered in terms of defining accuracy. In binary classification tests (qualitative detection), the samples analyzed by a novel (alternative) test that needs to be verified (typically a novel qPCR assay) are categorized according to their concordance with the reference method in four basic categories (**Table 1**). This division originates from the statistical classification known as error matrix and allows determination of several parameters that describe the diagnostic potential of the qPCR method.

Diagnostic sensitivity, which is described as TP/(TP + FN), refers to the ability of the new test to correctly identify samples identified by the reference method as positive. The lower the diagnostic sensitivity, the poorer will be the inclusivity of the tested qPCR. Another explanation could be that the analytical sensitivity (LOD) of the reference method is higher than the tested qPCR.

Diagnostic specificity is defined as the TN/(TN + FP) and refers to the ability of the test to correctly identify samples that were found to be negative by the reference method. The lower the diagnostic specificity, the poorer will be the exclusivity of the tested qPCR. Another explanation could be that the sensitivity of the reference method is quite bad, and the new qPCR method is capable of identifying more positive samples than the reference method.

Relative accuracy is defined as the (TP + TN)/(TP + TN + FP + FN) and describes the proportion of all correctly identified samples among all samples (Anonymous, 2009). If no FN and FP are detected, then it is 100%. In all other cases, this value is lower than 100%.

In quantitative determination, the accuracy numerically describes the distance of the value from the novel tested qPCR and some reference (true) value. For this reason, accuracy is referred to as trueness in quantitative classification (Anonymous, 1994). Trueness is defined as the degree of agreement of the expected value with the true value or accepted reference value. This is related to systematic error (Anonymous, 2015a,b). In

TABLE 1 | Parameters for comparison of qPCR results with a reference method in a 2×2 error matrix contingency table.

		Reference method	
		Positive	**Negative**
Alternative method	Positive	TP	FP
	Negative	FN	TN

TP—True positive—Positive sample correctly identified as positive.
TN—True negative—Negative sample correctly identified as negative.
FP—False positive—Negative sample wrongly identified as positive.
FN—False negative—Positive sample wrongly identified as negative.

GMO testing the trueness must be within 25% of the accepted reference value (Anonymous, 2015b). There are no fixed values of trueness that the novel tested qPCR method must meet in microbiological diagnostics. This might be caused by the fact that the trueness in qPCR can be determined by the comparison with some certified reference material, with the reference method or by proficiency testing. Certified reference material with a quantified number of target organisms is available only for a limited number of organisms (especially viruses like HIV, HBV, HCV, HAV, HPV, CMV, EBV), while for the remainder of clinically significant organisms, these materials are often available only for the qualitative analysis, and are therefore not suitable for trueness determination. Reference methods usually have varying diagnostic sensitivities and specificities and often they do not fit for the purposes of the quantitative assessment of novel qPCR methods. Moreover, the organization of proficiency testing via ring trials is expensive and requires a supplier of the reference material (like QCMD). These are the main reasons why determination of trueness in qPCR methods for the microbial detection in clinical, and especially in veterinary food safety areas, is rather limited.

Precision of qPCR

Precision is defined as the degree of agreement of measurements under specified conditions. The precision is described by statistical methods such as SD or confidence limit (Anonymous, 2015a). From the definition of precision, it is evident that this qPCR parameter is quantitative. For practical determination of precision, two conditions termed repeatability, and reproducibility were introduced (Anonymous, 1994). These two parameters are used to describe the variability of measurements introduced by the operator, equipment, and its calibration, environmental factors that can influence the measurement like temperature, humidity etc., and time between measurements (Anonymous, 1994). Repeatability is described as the closeness of agreement between successive and independent results obtained by the same method on identical test material under the same conditions (apparatus, operator, laboratory, and short intervals of time) and expresses within-laboratory variations (Anonymous, 1994, 2009, 2015a). Repeatability consists of two different variables: intra- and inter-assay variation. The intra-assay variation describes the variability of the replicates conducted in the same experiment; the inter-assay variation describes the variability between different experiments conducted on different days. Numerically, the repeatability is characterized as the SD of replicates at each concentration of each matrix for each method (Anonymous, 2012). The interval characterized by the SD of the replicates is called the repeatability limit (r) and is defined as the value less than or equal to the expected absolute difference, with a probability of 95%, between two tests results obtained under repeatability conditions (Anonymous, 1994, 2009, 2015b; Broeders et al., 2014). If the measured value lies outside the SD, it should be considered as suspect (Anonymous, 2009). It is necessary to perform the estimation of repeatability on 15 repeats at least (Anonymous, 1994, 2015b). Testing of repeatability requires analysis of the spiked relevant matrix at least at four levels—high, medium, low (near to the LOD) and negative in at least duplicates (Anonymous, 2009). For more rigorous testing the use of five replicates and the addition of one more sample spiked with a competitor strain that gives similar results in the given detection system is recommended. Natural background microflora can fulfill this requirement as long as they are present in the matrix at a level 1 Log_{10} greater than the target analyte (Anonymous, 2015a). In clinical, veterinary and food microbial detection, there are no specific recommendations for the repeatability *SD* value in terms of its proportion with respect to the mean. In GMO detection the repeatability *SD* must be $\leq$25% established on samples containing 0.1% GM related to the mass fraction of GM material (Broeders et al., 2014; Anonymous, 2015b).

On the other hand, reproducibility is the closeness of agreement between single test results on identical test material using the same method, obtained in different laboratories using different equipment and expresses the variation between laboratories (Anonymous, 1994, 2009, 2015a). Numerically, the reproducibility is characterized as the *SD* replicates at each concentration for each matrix across all laboratories (Anonymous, 2012). Similarly to repeatability, the reproducibility limit (R), as the interval characterized by the *SD* of the replicates, is defined as a value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions is expected to have a probability of 95% (Anonymous, 1994, 2009, 2015b). If the difference between two results from different laboratories exceeds R, the results must be considered suspect (Anonymous, 2009). The reproducibility is usually defined by collaborative studies, which determine the variability of the results obtained by the given method in different laboratories using identical samples (Anonymous, 2009, 2012; Molenaar-de Backer et al., 2016). The number of laboratories with valid results which should be included in the collaborative study is at least eight. Therefore, it is advisable to select 10–12 labs (Anonymous, 2009, 2015a). The requirements for the minimal number of testing samples are identical to the repeatability determination (Anonymous, 2009, 2015a). Similarly, there are no specific recommendations for *SD* values of reproducibility with regard to the mean in clinical, veterinary, and food microbial detection. Again, in GMO testing the *SD* of reproducibility should be <35% over the whole dynamic range. However, at relative concentrations <0.2% or at an amount <100 copies *SD* values <50% are deemed acceptable (Broeders et al., 2014; Anonymous, 2015b).

Although determination of qPCR precision requires quantitative data, there is also the possibility of determining the precision of the method qualitatively. The mechanism of precision determination remains identical as for the quantitative estimation, including the validation within collaborative studies. However, the results are evaluated only qualitatively (positive/negative). This approach can be used for the validation of the specific new qPCR method in different laboratories, but it is preferably used for the validation and routine control of various qPCR methods in different laboratories on a set of reference samples. Such samples are provided by certain authorities (reference laboratories) or private companies (QCMD), which collect data from different laboratories and in

the case of success, provide certificates regarding participation in such testing.

CONCLUSION

qPCR technology represents a powerful tool in microbial diagnostics. In viral and parasitical detection, quantification and typing, the suitability of this technique is beyond doubt; in the area of bacterial diagnostics it can replace culture techniques, especially when rapid and sensitive diagnostic assays are required. The spread of qPCR to different areas of routine microbial diagnostics together with the lack of standard procedures for the determination of basic functional parameters of qPCR has led to a scenario in which standardization of methods is performed according to different rules by different laboratories. This issue was partially solved by the publication of MIQE guidelines (Bustin et al., 2009); however, there are differences in attitude to validation and standardization of qPCR assays across clinical, veterinary and food safety areas. Any contribution to the unification of standardization and validation procedures will improve the quality of qPCR assays in microbial detection, quantification and typing.

AUTHOR CONTRIBUTIONS

Both authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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Utilization of Digital PCR in Quantity Verification of Plasmid Standards Used in Quantitative PCR

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Quantitative PCR (qPCR) is a widely used method for nucleic acid quantification of various pathogenic microorganisms. For absolute quantification of microbial load by qPCR, it is essential to create a calibration curve from accurately quantified quantification standards, from which the number of pathogens in a sample is derived. Spectrophotometric measurement of absorbance is a routine method for estimating nucleic acid concentration, however, it may be affected by presence of other potentially contaminating nucleic acids or proteins and salts. Therefore, absorbance measurement is not reliable for estimating the concentration of stock solutions of quantification standards, based on which they are subsequently diluted. In this study, we utilized digital PCR (dPCR) for absolute quantification of qPCR plasmid standards and thus detecting possible discrepancies in the determination of the plasmid DNA number of standards derived from UV spectrophotometry. The concept of dPCR utilization for quantification of standards was applied on 45 qPCR assays using droplet-based and chip-based dPCR platforms. Using dPCR, we found that spectrophotometry overestimated the concentrations of standard stock solutions in the majority of cases. Furthermore, batch-to-batch variation in standard quantity was revealed, as well as quantitative changes in standards over time. Finally, it was demonstrated that droplet-based dPCR is a suitable tool for achieving defined quantity of quantification plasmid standards and ensuring the quantity over time, which is crucial for acquiring homogenous, reproducible and comparable quantitative data by qPCR.

Keywords: digital PCR, absolute quantification, quantity verification, quantification plasmid standard, qPCR, real time PCR

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INTRODUCTION

Quantitative PCR (qPCR) is currently the method of choice for nucleic acid detection and quantification of various microbial pathogens due to its high sensitivity, specificity, reproducibility, and wide dynamic range. Its theoretical limit of detection was set at three copies per qPCR reaction, assuming its ability to detect a single copy of the target nucleic acid (Bustin et al., 2009). qPCR is used in various applications in a wide range of areas including food safety and healthcare

(Kralik et al., 2011; Alidjinou et al., 2017; Walker et al., 2017). In order to determine the absolute number of microbial pathogens in a sample using qPCR, it is necessary to create a calibration curve derived from a serially diluted quantification standard containing a known amount of copies or concentration of plasmids, genomic DNA or other nucleic acid molecules carrying target genes. Therefore, the exact assessment of the amount of nucleic acid copies or concentration of standards is essential for the correct quantification of pathogens in a sample. Typically, the concentration of nucleic acid of a standard is determined based on spectrophotometric or fluorometric measurements of nucleic acid absorbance or fluorescence, respectively (Kline et al., 2009), subsequently the number of DNA copies can be calculated according to the molecular weight of DNA material. However, low purity may influence the results of absorbance measurements. The concentration of nucleic acid measured may be altered by residues of DNA or RNA, proteins, and salts, which may lead to production of the standards containing incorrect amount of nucleic acid copies (Sanders et al., 2011). Currently, there is no standardized protocol for independent quantity verification of desired DNA in qPCR standards and ensuring measurement accuracy in qPCR. Moreover, since quantification standards are not uniform among laboratories performing quantification of pathogenic microorganisms using qPCR, the determination of the number of pathogens in various laboratories using different qPCR standards is not comparable, which is then reflected in differing interpretations of results (Pavsic et al., 2015).

In addition to accurately quantified qPCR standards, a number of other factors that may affect the qPCR quantification of target genes cannot be omitted. The choice of nucleic acid isolation method has a distinctive effect on subsequent qPCR quantification of pathogens in sample and thus the use of different methods may lead to gene number variability (Smith et al., 2006). Furthermore, possibility of additional variability arising from the diverse reagents and instruments used and different calibration curves should be taken into account in absolute qPCR quantification (Bustin et al., 2009). Therefore, direct comparison of the absolute gene copy numbers determined in different qPCR assays and different standard curves should be made with caution (Smith et al., 2006). Besides, another disadvantage of qPCR-based methods is the need for sequence information about a specific target gene enabling the design of primers and probes, therefore it can only be applied on already known genes (Smith and Osborn, 2009).

Digital PCR (dPCR) nowadays represents one of the most powerful tools for absolute nucleic acid quantification, which does not require the creation of a standard curve (Hindson et al., 2011). dPCR has become a widely used method that offers a number of advantages for detection and quantification of nucleic acids (Gerdes et al., 2016). It is used for molecular analyses in clinical as well as research applications, such as for the detection of microRNAs associated with cancer (Ma et al., 2013), chromosomal abnormalities (Zimmermann et al., 2008), quantification of pathogenic bacteria (Porcellato et al., 2016; Talarico et al., 2016), viral load (Lui and Tan, 2014; Nicot et al., 2016), testing of genetically modified organisms (Morisset et al., 2013), and next-generation sequencing (White et al., 2009). The principle of dPCR utilizes a limiting dilution and random sample distribution into hundreds to millions of uniformly sized nanoliter or picoliter separate reaction partitions, in which the target nucleic acid sequence is amplified. Currently, there are several available dPCR platforms that differ mainly in the arrangement of reaction partitions. The reaction partitions may be either microfluidic chambers or microwells placed on a microchip (chip-based digital PCR, cdPCR) or water-in-oil emulsion droplets (droplet-based digital PCR, ddPCR). Quantification of target nucleic acid sequence is based on counting the number of positive (sequence detected) and negative (sequence not detected) reaction partitions after previous amplification with the correction to real numbers utilizing Poisson distribution (Hindson et al., 2011). The advantages of dPCR are high sensitivity and precision, tolerance to inhibitors (Huggett et al., 2013; Lui and Tan, 2014) and increased signal-to-noise ratio due to partitioning of the sample, thereby the background signal is diluted out and it is thus possible to detect low-abundance targets (Sanders et al., 2011).

The main aim of this study was to evaluate the suitability of dPCR utilization for independent quantity verification of quantification plasmid standards used in qPCR, more specifically, for absolute quantification of the standards and thus the estimation of discrepancies in the determination of the plasmid DNA number of standards derived from spectrophotometric absorbance measurements. The concept of dPCR as a promising technique for accurate quantification of standards was applied on a panel of 45 qPCR assays, comparing the performance of ddPCR and cdPCR platforms. Batch-to-batch variation in standard quantity was investigated, as well as possible quantitative changes in standards over time when stored at −20°C. Furthermore, the effect of conformation structure of the quantification plasmid standards (circular and linear form of plasmid DNA) on the quantity estimation by dPCR and differences with qPCR amplification was examined. Several plasmid isolation kits commercially available were tested for their ability to remove possible contamination affecting absorbance measurement of plasmid standard stock solutions. Finally, this study demonstrated that ddPCR could be a suitable tool for achieving defined quantity of quantification plasmid standards and ensuring the quantity over time, which is essential to obtain homogenous, reproducible, and comparable quantitative data by qPCR in various commercial and research laboratories.

MATERIALS AND METHODS

Preparation of Quantification Standards

Quantification plasmid standards of 45 qPCR assays utilized for the detection and quantification of various bacterial, viral, and parasitical agents (**Table 1** and **Supplementary Table 1**) were prepared by the cloning of a specific nucleotide sequence of a particular microbial pathogen to pDRIVE plasmid vector and transformed to chemocompetent *Escherichia coli* (*E. coli*) TOP10 cells (both supplied by Qiagen, Germany). Clones carrying specific plasmids were propagated 16 h in Luria-Bertani broth (Sigma-Aldrich, USA) containing 50 μg/ml of

TABLE 1 | The list of quantification standards of 45 qPCR assays utilized in this study.

Microorganism	Target locus	Abbreviation
Bacillus anthracis	BA5357	BA5357
	pagA	BA pag
Brucella spp.	BCSP31	BCSP31
	omp2	B omp
Campylobacter coli	glyA	Camp col
Campylobacter jejuni	hipO	Camp jej
Campylobacter lari	bipA	Camp lar
Campylobacter upsaliensis	bipA	Camp ups
Clostridium botulinum	16S rDNA	CB 16S
Clostridium difficile	Tpi	CD tpi
Clostridium perfringens	Cpa	CP cpa
Clostridium spp.	16S rDNA	CP 16S
Clostridium tetani	Tetox	Clos tet
Cronobacter sakazakii	rpsU gene 3′ end and the primase (dnaG) gene 5′ end	Crono rps
	rpoB	Crono rpo
Cryptosporidium spp.	hsp70	Cryp par
Erysipelothrix rhusiopathiae	ERH 1059	ERH 1059
	Soda	ERH sod
Escherichia coli	uidA	EC uid
	rfbE	EC rfbe
Giardia lamblia	β-giardin	GL
Human adenovirus	Hexon	AdV hex
Human adenovirus (serotype 40 and 41)	Fiber	AdV fib
Listeria monocytogenes	hlyIII	LM hly
Listeria spp.	23S rDNA	LM 23S
Mycobacterium avium complex	IS1311	MAC IS1311
Mycobacterium avium ssp. *avium*	IS901	MAA IS901
Mycobacterium avium ssp. *hominissuis* and *Mycobacterium avium* ssp. *avium*	IS1245	MAHA IS1245
Mycobacterium avium ssp. *paratuberculosis*	IS900	MAP IS900
	F57	MAP F57
Mycobacterium spp.	ITS	Myco ITS
Mycobacterium tuberculosis	devR	Myco dev
Pseudomonas aeruginosa	gyrB	PA gyr
	ecfX	PA ecf
Pseudorabies virus	gB	PRV
Salmonella enterica	Ttr	SE ttr
Staphylococcus aureus	SA442	SA442
	Nuc	SA nuc
Toxoplasma gondii	B1 gene	T gon
Verotoxigenic *Escherichia coli*	stx 1, 2	VTEC stx
	Eae	VTEC eae
Yersinia enterocolitica	Ail	YE ail
Yersinia pestis	caf1	YP caf
	Pla	YP pla
Yersinia spp.	ompF	YE omp

kanamycin (Sigma-Aldrich) at 37°C with shaking. The culture was centrifuged at 6,800 × g for 3 min at room temperature and plasmid DNA was isolated from the prepared pellet using QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions.

Isolated plasmids were divided into two aliquots and one was linearized using *Bam*HI restriction endonuclease (New England Biolabs, USA). The absence of the *Bam*HI restriction enzyme site within all the plasmid inserts was checked by the Webcutter online tool (http://www.firstmarket.com/cutter/cut2.html) prior to experimental work. The restriction enzyme digest reaction was composed of 10 μg plasmid DNA, 5 μl NEBuffer 3.1 (New England Biolabs), and 100 U *Bam*HI in a final volume of 50 μl. The enzymatic reaction was carried out for 2 h at 37°C, subsequently, the linearized plasmid DNA was purified using QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. Linearization was verified by agarose gel electrophoresis (1%) and staining with ethidium bromide. The second non-linearized aliquot of plasmids underwent the same procedure of linearization and purification with the only exception that the restriction enzyme was replaced with an identical volume of water in the digestion reaction.

The concentration of the purified plasmid DNA was determined by spectrophotometric measurement of nucleic acid absorbance using NanoDrop 2000c (Thermo Scientific, USA). Then both linearized and non-linearized plasmid DNA stock solutions were ten-fold serially diluted in Tris-EDTA buffer (Amresco, USA) with Carrier DNA solution (salmon sperm DNA, 50 ng/μl; Serva, Germany) to a gradient of the standards with expected concentrations in a range of 10^5–10^0 copies/μl and stored at −20°C. This was followed by quantification of diluted plasmid standards using qPCR and dPCR assays.

To investigate a possible effect of the plasmid purification kit on the quality of the isolated plasmids in terms of presence of contaminating DNA affecting absorbance measurement of stock solution and subsequent dilution of the standards, six different kits for the plasmid DNA purification commercially available—QIAprep Spin Miniprep Kit already mentioned above, NucleoSpin Plasmid (Macherey-Nagel, Germany), Monarch Plasmid Miniprep Kit (New England BioLabs, USA), GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA), High Pure Plasmid Isolation Kit (Sigma-Aldrich, USA), and GenElute Plasmid Miniprep Kit (Sigma-Aldrich, USA)—were compared on the standards used for detection of human adenovirus (AdV fib) and *Staphylococcus aureus* (SA442), selected from a list of pathogens (**Table 1**). A single batch of the propagated *E. coli* cells with the inserted plasmids was split into six aliquots and each underwent plasmid purification by the respective kit according to the manufacturer's recommendations. The subsequent procedure was the same as mentioned above, isolated plasmid standards were linearized, then purified by QIAquick PCR Purification Kit, diluted based on spectrophotometric estimation to concentrations 10^4, 10^3, and 10^2 copies/μl and quantified using ddPCR assay.

Comparison of the Performance of the Circular and Linear Forms of Plasmids in qPCR Assays

To evaluate whether there is a difference in amplification of linear and circular plasmid standards, four plasmid standards containing specific sequence from pathogen (**Table 1**) were

selected—*Campylobacter jejuni* (Camp jej), *Campylobacter lari* (Camp lar), human adenovirus (AdV fib), and *S. aureus* (SA442). The quantity of the circular and linear form of these four plasmid standards, which were ten-fold serially diluted based on spectrophotometric estimation in a range of 10^5–10^0 copies/μl was determined using qPCR. The experiment was run in independent biological duplicates comprising the whole procedure of transformed cells propagation, plasmid purification, and spectrophotometric determination (two batches of each of the four standards differing in preparation time). The linear plasmids quantified using ddPCR were utilized for construction of standard curves in qPCR.

qPCR reaction mixtures used in this study consisted of 10 μl of LightCycler 480 Probes Master (Roche, Czech Republic), 1 U of Uracil DNA Glycosylase (Roche), 50 nM of TaqMan probe, 500 nM of each of primers, and 5 μl of plasmid DNA in a total volume of 20 μl. The qPCR assays were run in duplicate for each analyzed sample (each dilution) using the LightCycler 480 instrument (Roche) under the following reaction conditions: initial denaturation at 95°C for 7 min, followed by 45 amplification cycles at 95°C for 10 s, 60°C for 30 s, and 72°C for 1 s. The subsequent analysis of the results was performed using the "Fit point analysis" option of the LightCycler 480 Software (1.5.0.39).

Comparison of the Performance of the Circular and Linear Forms of Plasmids in ddPCR Assays

Similarly to qPCR, linear and circular forms of plasmid standards (again two batches of each standard) were investigated on the four model assays. The manner of the testing was identical to qPCR, but the range of concentrations tested by ddPCR was 10^4, 10^3, and 10^2 copies/μl as expected based on spectrophotometric estimation.

ddPCR was carried out using the QX200 droplet digital PCR system (Bio-Rad, USA) according to the manufacturer's instructions. The assays were run in duplicate for each analyzed sample (each dilution) in a total volume of 22 μl. The reaction mix contained 11 μl of ddPCR Supermix for Probes (No UTP), 5 μl of plasmid DNA and the same probes and primers at the same concentrations, 50 and 500 nM, respectively, as in qPCR. The reaction mix and 70 μl of droplet generation oil were loaded into wells of the DG8 cartridge and placed into the QX200 Droplet generator. Forty microliters of generated droplets were transferred into 96-well PCR plate, which was then sealed using foil heat seal in PX1 PCR plate sealer (Bio-Rad, USA) and placed in a T100 Thermal Cycler (Bio-Rad, USA) for PCR. Cycling conditions were enzyme activation at 95°C for 10 min, followed by 40 cycles of a two step thermal profile at 94°C for 30 s and 60°C for 1 min and a final enzyme inactivation at 98°C for 10 min while maintaining ramp rate 2°C/s. After thermal cycling, the 96-well plate was placed in the QX200 Droplet Reader, where droplets were read and analyzed using QuantaSoft Software (1.7.4.0917). From the values measured by ddPCR, a maximum likelihood estimate (MLE) of the plasmid concentration was calculated according to Equation (1) and plotted against the theoretical quantity according to Equation (2) as mentioned below (Statistical and Mathematical Analysis).

Comparison of the ddPCR and cdPCR Plasmid Quantification on 45 qPCR Assays Intended for the Detection and Quantification of Various Viral, Bacterial, and Parasitical Agents

Both dPCR platforms, ddPCR and cdPCR, were used for the quantification of plasmid standards diluted based on spectrophotometric estimation of concentrations at 10^4, 10^3, and 10^2 copies/μl. ddPCR assays were performed as stated above.

cdPCR was performed with the QuantStudio 3D Digital PCR System (Applied Biosystems, USA). cdPCR assays were run in duplicate for each dilution. The reaction mix at a total volume of 16 μl was composed of 8 μl of QuantStudio 3D Digital PCR Master Mix v2, 5 μl of plasmid DNA and again the same probes and primers at the same concentrations as in qPCR and ddPCR. Then, 14.5 μl of the reaction mixture was loaded on a QuantStudio 3D Digital PCR Chip v2 using QuantStudio 3D Digital PCR Chip Loader. The chips were placed in ProFlex 2x Flat PCR System for thermal cycling under the conditions: 96°C for 10 min, followed by 39 cycles of a two step thermal profile at 60°C for 2 min, and 98°C for 30 s and the final extension step at 60°C for 2 min. Thereafter, the chips were read with the QuantStudio 3D Digital PCR Instrument and analyzed with the QuantStudio 3D AnalysisSuite Software web application. As with ddPCR, MLE of concentration was calculated and plotted against the theoretical quantity according to Equations (1) and (2), respectively.

Comparison of the Concentrations of 5 Selected Linear Plasmid Standards Diluted Based on Spectrophotometric Estimation and Re-diluted Based on Value (MLE) Determined by ddPCR

After quantification of 45 linear plasmid standards diluted based on spectrophotometric estimation using dPCR, five standards were selected—AdV fib, Camp lar, Crono rps, EC uid, and MAP F57—to be re-diluted based on the MLE of the concentration calculated from values measured by ddPCR (Equation 1). After re-dilution, the concentrations of plasmid standards were again measured by ddPCR and plotted against the theoretical quantity (Equation 2).

Evaluation of Stability of Linear Plasmid Standards During Long-Term Storage

To evaluate whether the quantity of linear plasmid standards changes during long-term storage, stock solutions of purified linearized plasmids from the initial experiment with four qPCR systems comparing circular and linear plasmid form were reassessed after 19 (first batch) or 14 (second batch) months of storage at −20°C. Each solution was again diluted based on previously determined spectrophotometric estimation and copy number of the plasmids was determined by ddPCR.

Subsequently, calculated MLE (Equation 1) of the plasmid concentration from the values measured by the second ddPCR assay was considered a reference value and served for the preparation of the newly diluted plasmid standards, which were again measured by ddPCR and MLE calculated. The values obtained were plotted against the theoretical quantity (Equation 2).

Statistical and Mathematical Analysis

Calculation of MLE of the Plasmid Concentration

The measured concentrations of ten-fold serially diluted standards should follow Poisson's distribution. Assuming that the concentration of the plasmid standard in the initial sample is equal to λ, then the sample on the i-th ten-fold dilution ($i = 1, 2, 3, \ldots$) has a Poisson's distribution Po ($\lambda/10^i$) with an expected value $\lambda/10^i$. For example, the measured values x, y, and z corresponding to ten-fold serial dilution four, five, and six, respectively, should be approximately equal to the expected values $\lambda/10^4$, $\lambda/10^5$, and $\lambda/10^6$, from which three estimates of the initial concentration λ can be made: $x \times 10^4$, $y \times 10^5$, and $z \times 10^6$. The maximum likelihood estimate (MLE) of concentration λ is the arithmetic average of the individual estimations, which can be calculated as follows:

$$\lambda = (x \times 10^4 + y \times 10^5 + z \times 10^6)/3 \quad (1)$$

As the value y corresponds to a dilution five, it should be recalculated to $\lambda/10^5$.

Comparison of the Concentration Estimates With the Required Concentration of Plasmid Standards

MLE of the concentration calculated from the values measured by dPCR according to Equation (1) was compared to the theoretical quantity according to this quotient:

$$x\,\% = \frac{\text{MLE of the concentration}}{\text{theoretical quantity}} \times 100 \quad (2)$$

The theoretical quantity represents the individual concentrations of the standard dilution series expected to be obtained after dilution of the standard stock solution, i.e., ten-fold serial dilutions in the range of 10^5–10^0 copies/μl.

Statistical analysis of ddPCR and cdPCR was performed by a statistical software GraphPad Prism 5.04 (GraphPad Software, Inc., San Diego, CA, USA). *P*-value differences lower than 0.05 were considered statistically significant.

RESULTS

Quantification of Circular and Linear Plasmid Standards Using ddPCR

Comparison of the circular and linear plasmid standards using ddPCR failed. The concentration of circular plasmid standards could not be accurately determined by ddPCR due to the so-called rain effect, which is characterized by droplets exhibiting fluorescence ranging between those of explicit negative and positive droplets, which makes it difficult to set the threshold correctly (**Figure 1**). In order to set the threshold correctly, the plasmid standards need to be linearized, which corresponds with the manufacturer's recommendation, and therefore further analyzes using dPCR were performed only with linearized plasmid standards.

MLE of the linear plasmid concentration was calculated according to Equation (1), and then compared to the theoretical quantity according to Equation (2). E.g., for Camp jej, calculation of MLE of plasmid concentration based on three estimates of the initial concentration λ is as follows: $\lambda_{MLE} = (5{,}080 \times 10^4 + 555 \times 10^5 + 64.6 \times 10^6)/3 = 5.697 \times 10^7$ (**Table 2**). As for the value 555, which corresponds to a dilution five, it should be recalculated to $\lambda/10^5$, i.e., $5.697 \times 10^7/10^5 = 569.7$. Similarly, values for dilutions four and six were calculated. By percentage comparison of MLE of the concentration with the theoretical quantity, a value of 57% was obtained.

Using ddPCR, it was found that the concentrations of four selected linear plasmid standards—Camp jej, Camp lar, AdV fib, and SA442—diluted based on spectrophotometric estimation corresponded to a theoretical quantity of 57, 91, 90, and 71%, respectively, in the first batch, and of 74, 53, 51, and 55%, respectively, in the second batch (**Table 2**).

Comparison of Circular and Linear Plasmid Standards by qPCR

The concentration of the circular form of the plasmid standards determined by qPCR was lower compared to the linear form in all cases (**Table 3**). The percentage of circular plasmid concentration to linear plasmid concentration was in the range of about 20–30%.

Comparison of Six Different Kits for Plasmid DNA Isolation by ddPCR

Differences were found in the concentrations of linear plasmid standards diluted based on spectrophotometric estimation after prior isolation by six different isolation kits, as well as between two standards isolated by the same kit (**Figure 2**). The concentrations of linear plasmid standards differed from a theoretical quantity in the range of 24–61% and individual plasmid standards when using various kits differed from each other by up to approximately 50%. The removal of contamination affecting the absorbance measurements of standard stock solutions was apparently not achieved using any of the kits.

Quantification of 45 Linear Plasmid Standards Using ddPCR and cdPCR Assays

Linear plasmid standard concentrations of 45 qPCR assays diluted based on spectrophotometric estimation in a range of 10^4, 10^3, and 10^2 copies/μl were determined using ddPCR and cdPCR, MLEs of concentration were calculated and plotted against the theoretical quantity (**Figure 3**).

Percentage comparison of measured concentration values with the required concentrations, median, mean and confidence intervals of gained data indicated that the concentration values measured by both dPCR assays were mostly lower than the

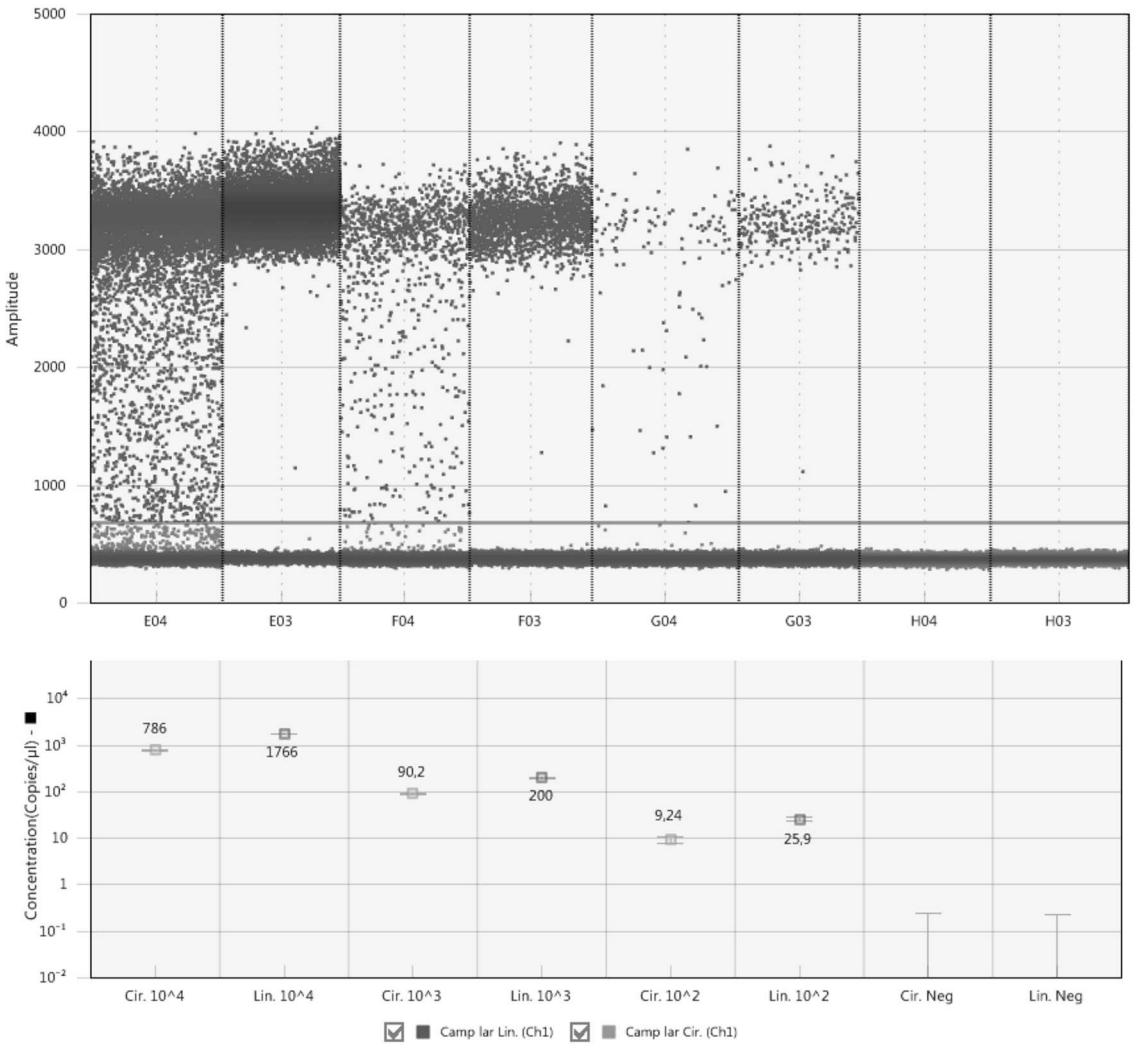

FIGURE 1 | Comparison of circular and linear form of plasmid standards by ddPCR. The circular plasmids are show in columns E04, F04, and G04, the linear ones in columns E03, F03, and G03. H04 and H03 are corresponding negative controls. The line across all columns indicates a threshold line separating positive and negative droplets situated above and below the line, respectively.

required concentrations, i.e., 100% (**Tables 4**, **5**). The mean deviation from 100% were −14.06 percentage points for the ddPCR assay, and −25.81 percentage points for the cdPCR assay. The medians of both dPCR assays differed statistically significantly from 100% ($P < 0.01$; Wilcoxon one-sample test).

Regarding the difference, in which the concentrations of standards determined by cdPCR were subtracted from those determined by ddPCR, the mean of these values was 11.41 and the median was 11.72 (**Table 4**). The difference was positive for 32 samples (76.19%), suggesting that the values measured by ddPCR assay were mostly higher than those measured by the cdPCR assay, an average of 11.41 percentage points. The results of both dPCR assays on the same samples differed statistically significantly ($P < 0.01$; Wilcoxon paired test).

Comparison of the Concentrations of Five Selected Linear Plasmid Standards Diluted Based on Spectrophotometric Estimation and Re-diluted Based on MLE Value Determined by ddPCR

The measured concentrations of selected plasmid standards (AdV Fib, Camp lar, Crono rps, EC uid, and MAP F57) diluted based on spectrophotometric estimation corresponded to a range of 2–120% of the theoretical quantity and showed a deviation of 9–98% from the theoretical quantity. After re-dilution based on value (MLE) measured by ddPCR, the concentrations of the standards differed from the theoretical quantity in the range of 5–12% (**Figure 4**).

TABLE 2 | The quantity value of the linearized plasmid standards diluted based on spectrophotometric estimation determined by ddPCR.

Standard	Theoretical quantity (copies/μL)	1st batch			2nd batch		
		Measured conc. (copies/μL)	MLE of conc. (copies/μL)[a]	Ratio (%)[b]	Measured conc. (copies/μL)	MLE of conc. (copies/μL)[a]	Ratio (%)[b]
Camp jej	10^4	5.08×10^3	5.70×10^3	57	6.93×10^3	7.39×10^3	74
	10^3	5.55×10^2	5.70×10^2		7.20×10^2	7.39×10^2	
	10^2	6.46×10^1	5.70×10^1		8.04×10^1	7.39×10^1	
Camp lar	10^4	7.78×10^3	9.14×10^3	91	5.06×10^3	5.30×10^3	53
	10^3	8.64×10^2	9.14×10^2		5.35×10^2	5.30×10^2	
	10^2	1.10×10^2	9.14×10^1		5.49×10^1	5.30×10^1	
AdV fib	10^4	8.35×10^3	9.00×10^3	90	5.16×10^3	5.12×10^3	51
	10^3	9.29×10^2	9.00×10^2		5.10×10^2	5.12×10^2	
	10^2	9.36×10^1	9.00×10^1		5.12×10^1	5.12×10^1	
SA442	10^4	6.58×10^3	7.13×10^3	71	5.10×10^3	5.48×10^3	55
	10^3	7.07×10^2	7.13×10^2		5.57×10^2	5.48×10^2	
	10^2	7.74×10^1	7.13×10^1		5.77×10^1	5.48×10^1	

[a] *MLE (maximum likelihood estimate) of concentration was calculated as arithmetic average of the measured concentration values of the three standard dilutions converted to the same order.*
[b] *The ratio expresses the percentage of MLE of plasmid standard concentration to the theoretical quantity.*

TABLE 3 | Comparison of quantity value of circular and linear form of the plasmid standards by qPCR.

Standard	Theoretical quantity (copies/μL)	1st batch				2nd batch			
		Linear (copies/μL)	Circular (copies/μL)	Ratio (%)[a]	Mean ratio (%)	Linear (copies/μL)	Circular (copies/μL)	Ratio (%)[a]	Mean ratio (%)
Camp jej	10^5	5.29×10^4	1.21×10^4	23	24	6.69×10^4	1.22×10^4	18	18
	10^4	4.60×10^3	1.05×10^3	23		7.04×10^3	1.24×10^3	18	
	10^3	4.70×10^2	1.09×10^2	23		7.11×10^2	1.17×10^2	16	
	10^2	5.55×10^1	1.14×10^1	21		7.48×10^1	1.36×10^1	18	
	10^1	5.38×10^0	1.28×10^0	24		7.33×10^0	1.27×10^0	17	
	10^0	5.01×10^{-1}	1.61×10^{-1}	32		6.51×10^{-1}	-	-	
Camp lar	10^5	8.23×10^4	1.69×10^4	21	25	5.25×10^4	9.26×10^3	18	16
	10^4	8.62×10^3	2.00×10^3	23		4.60×10^3	7.31×10^2	16	
	10^3	8.20×10^2	2.10×10^2	26		5.32×10^2	6.77×10^1	13	
	10^2	6.84×10^1	2.20×10^1	32		5.03×10^1	7.57×10^0	15	
	10^1	7.06×10^0	2.68×10^0	38		7.04×10^0	1.36×10^0	19	
	10^0	9.83×10^{-1}	1.30×10^{-1}	13		4.17×10^{-1}	-	-	
AdV fib	10^5	8.71×10^4	1.80×10^4	21	26	5.49×10^4	1.36×10^4	25	24
	10^4	8.12×10^3	1.79×10^3	22		5.39×10^3	1.39×10^3	24	
	10^3	9.04×10^2	1.48×10^2	16		4.18×10^2	1.14×10^2	27	
	10^2	8.77×10^1	1.86×10^1	21		5.11×10^1	1.12×10^1	22	
	10^1	8.46×10^0	1.24×10^0	15		5.84×10^0	1.39×10^0	24	
	10^0	9.80×10^{-1}	6.16×10^{-1}	63		5.19×10^{-1}	-	-	
SA442	10^5	6.95×10^4	1.21×10^4	17	19	5.20×10^4	1.15×10^4	22	20
	10^4	6.73×10^3	1.22×10^3	18		5.06×10^3	9.18×10^2	18	
	10^3	6.18×10^2	1.16×10^2	19		5.38×10^2	8.79×10^1	16	
	10^2	6.76×10^1	9.93×10^0	15		5.40×10^1	9.77×10^0	18	
	10^1	6.99×10^0	1.35×10^0	19		5.04×10^0	1.22×10^0	24	
	10^0	6.68×10^{-1}	1.91×10^{-1}	29		7.56×10^{-1}	1.73×10^{-1}	23	

The quantity value of the linear plasmids was determined by ddPCR and these linear plasmids served as the standards for qPCR quantification.
[a] *The ratio expresses the percentage of circular plasmid concentration to linear plasmid concentration of the standards.*

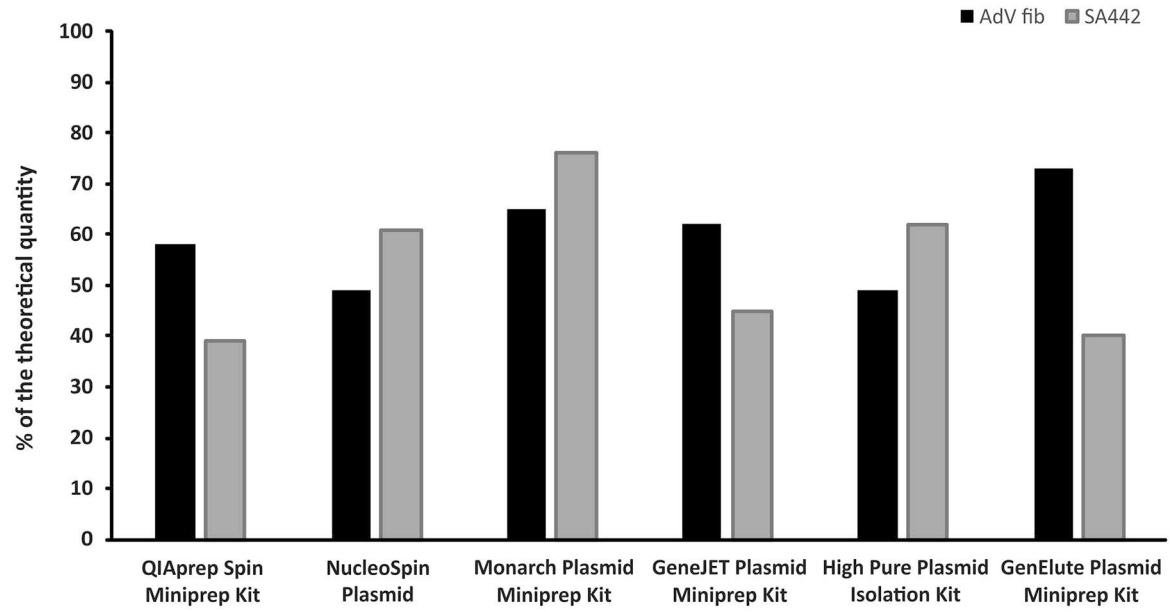

FIGURE 2 | Comparison of six different kits for plasmid DNA isolation. The bars represent the calculated MLE of plasmid standard concentration compared to the theoretical concentration (the values are given in percent).

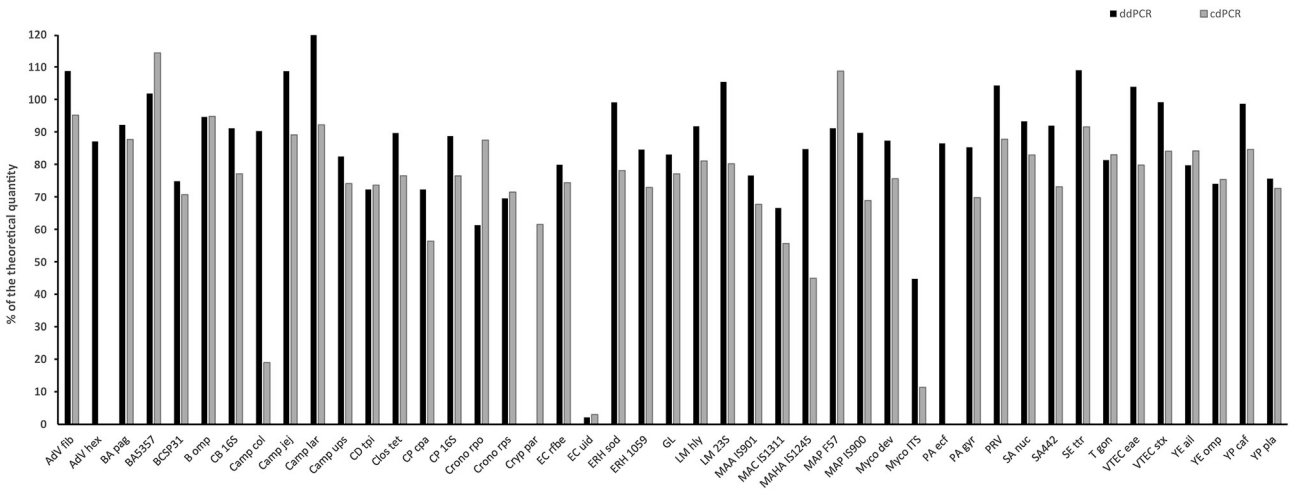

FIGURE 3 | Comparison of quantity value of 45 linear plasmid standards using ddPCR and cdPCR. The bars represent the calculated MLE of plasmid standard concentration compared to the theoretical concentration (the values are given in percent).

Evaluation of Stability of Linear Plasmid Standards During Long-Term Storage

The linear plasmid standards stored at −20°C for 19 months (first batch) showed a noticeable decrease in the concentration measured by ddPCR. In the case of storage for 14 months (second batch) at −20°C, changes in concentrations were also observed except for one standard whose concentration was not significantly altered. However, after recalibration (re-dilution) of these linear plasmid standards based on values from ddPCR, their concentrations again corresponded to a range of 92–107% of the theoretical quantity (**Figure 5**).

DISCUSSION

Nowadays, qPCR is a widely used method for the quantification of nucleic acids of various pathogenic microorganisms. For absolute quantification using qPCR, it is necessary to construct a calibration curve from accurately quantified quantification standards, from which the amount of the microbial pathogens in the sample is derived. However, currently, there is no established procedure for standard verification of these quantification standards. Laboratories quantifying microbial pathogens in the same samples by qPCR using quantification standards estimated

TABLE 4 | Descriptive statistics regarding ddPCR and cdPCR assays.

Statistics	ddPCR	cdPCR	Difference
N	44	43	42
Minimum	2.17	2.98	−26.17
Maximum	120.36	114.45	71.43
Lower quartile (Q_1)	77.52	70.78	2.24
Median	88.16	76.61	11.72
Upper quartile (Q_3)	97.82	84.73	17.87
Quartile deviation (IQR)	20.30	13.95	15.63
Mean	85.94	74.19	11.41
Sample standard deviation (SD)	19.20	21.59	15.81
Standard error of the mean (SEM)	2.89	3.29	2.44
Lower limit of 95% confidence interval for the mean	80.10	67.54	6.48
Upper limit of 95% confidence interval for the mean	91.78	80.83	16.34

Difference = ddPCR – cdPCR; IQR = $Q_3 - Q_1$.

TABLE 5 | Numbers of values lower than or greater than the theoretical quantity (100%).

Criterion	ddPCR	cdPCR
<100%	36 (81.82%)	41 (95.35%)
>100%	8 (18.18%)	2 (4.65%)

by different methods may not obtain the same copy numbers (Kuypers and Jerome, 2017). Furthermore, the use of different quantification standards, which may be plasmid DNA constructs, synthetic RNA or DNA oligonucleotides spanning the complete PCR amplicon, cDNA cloned into plasmid, RNA or DNA from specific biological samples, international biological standards, etc. (Bustin et al., 2009), may also hinder the consistency of the results among various laboratories even when testing the same samples. Absolute quantification of qPCR quantification standards using dPCR that is not dependent on a calibration curve or highly efficient amplification (Streets and Huang, 2014), can provide improved accuracy and comparability of results when determining amounts of microbial pathogens using qPCR in various laboratories.

First, we examined the influence of conformational structure of the plasmid standard on its quantification. Since ddPCR did not allow accurate quantification of circular plasmid forms due to the so-called rain effect and thus creating an impossibility for setting the threshold correctly, we used qPCR for this purpose, where the linearized plasmids quantified by ddPCR were utilized for the calibration curve. Several studies already reported a difference in qPCR amplification depending on the conformation structure of the plasmid (Chen et al., 2007; Hou et al., 2010). In our study, the concentration estimation of the circular plasmid form reached in a range of about 20–30% of concentration of the linear plasmid form. These results indicate that the target sequence in the circular plasmid may not be sufficiently accessible for primers, probe and polymerase annealing probably due to the superhelical nature of the plasmid, which prevents full denaturation of the dsDNA and therefore a large portion of the template target sequence is not amplified. In order to accurately quantify the pathogens using plasmid standards, a linearized form is needed, since the circular form may hamper amplification of the target sequence.

The concentration estimates of the final quantification plasmid standards from dPCR analysis differed in the majority of cases from the concentrations expected based on spectrophotometric measurement of absorbance of the initial stock solution. The concentrations of the standards determined by dPCR were predominantly lower than the expected concentrations indicating an overestimation in spectrophotometric measurement of plasmid standard stock solutions. The results obtained suggest that these differences were probably associated with insufficient purity of the prepared stock solutions measured resulting in incorrect dilution of them to final quantification plasmid standards. Common contamination affecting spectrophotometric measurement may be chromosomal DNA, RNA, proteins, or salts (Sanders et al., 2011). Another explanation could be an inhomogeneity of the plasmid stock solution measured or the presence of plasmids without the target sequence insert, which contribute to higher apparent absorbance, however, this DNA does not carry any DNA of interest. Similar mismatches between expected concentrations of plasmid standards based on spectrophotometric estimates of stock solution and dPCR estimates were found in a recent study quantifying plasmid DNA standards used in qPCR for the detection of *Enterococcus* spp. (Sivaganesan et al., 2018). However, this deviation was attributed to manipulation of plasmid stock solution including restriction digestion, in addition to freezing and thawing of the materials for analysis. Another study also described disagreement between concentration estimates derived by spectrophotometry and dPCR (Sanders et al., 2011). In addition, differences in the concentration estimates between two batches of linear plasmid standards prepared by the same procedure were also observed.

Given the results suggesting an overestimation in spectrophotometric measurement of plasmid stock solution, we investigated various commercially available kits for plasmid DNA isolation to determine whether they are able to remove potential contamination. However, the results showed that the removal of contamination and thus attaining unaffected spectrophotometric measurement of standard stock solutions was not achieved after utilization of any tested isolation kits. Differences in concentration estimations of the individual standards were completely random considering using different isolation kits, as well as between concentration estimations of two standards tested using the same isolation kit. These data suggest that plasmid isolation kits from various manufacturers are generally not able to isolate solely plasmid DNA, but it is likely that some contaminating chromosomal DNA is also purified. Considering the molecular weight of plasmid and chromosomal DNA, even residual presence of chromosomal DNA can affect the final concentration estimation of a plasmid solution by absorbance measurement, which is then reflected in diluting the solution to the final quantification standards. Based on these findings, we recommend that absorbance measurement

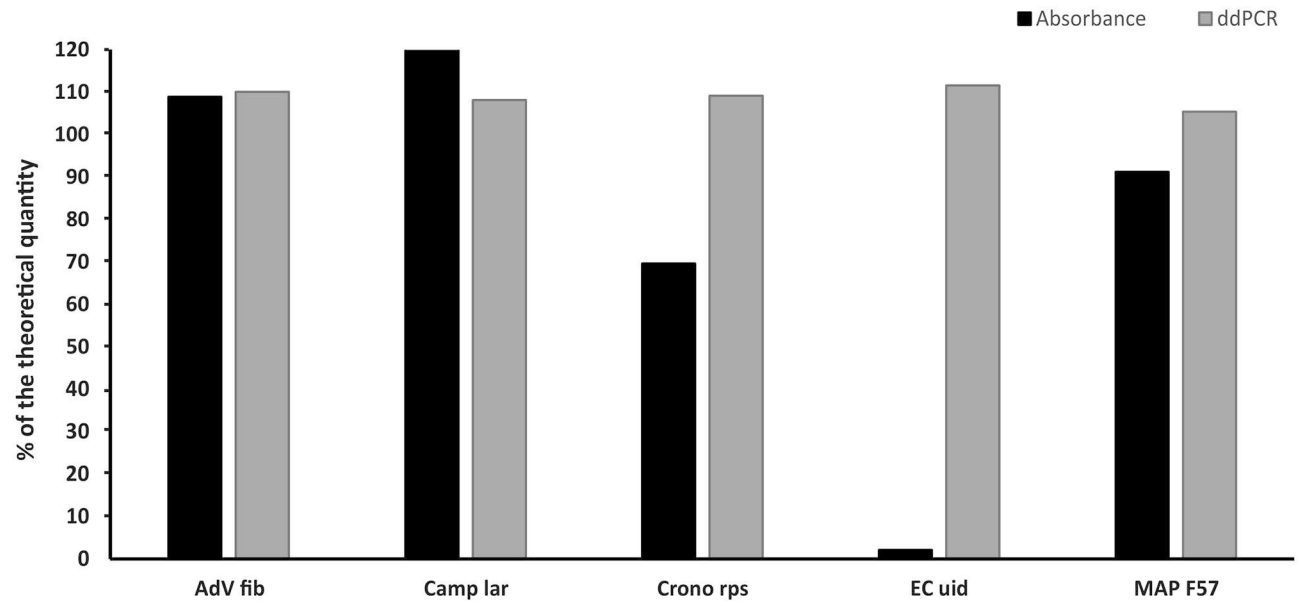

FIGURE 4 | Comparison of the concentrations of selected standards diluted based on spectrophotometric estimation (Absorbance) and re-diluted based on value (MLE) determined by ddPCR (ddPCR). The bars represent the calculated MLE of plasmid standard concentration compared to the theoretical concentration (the values are given in percent).

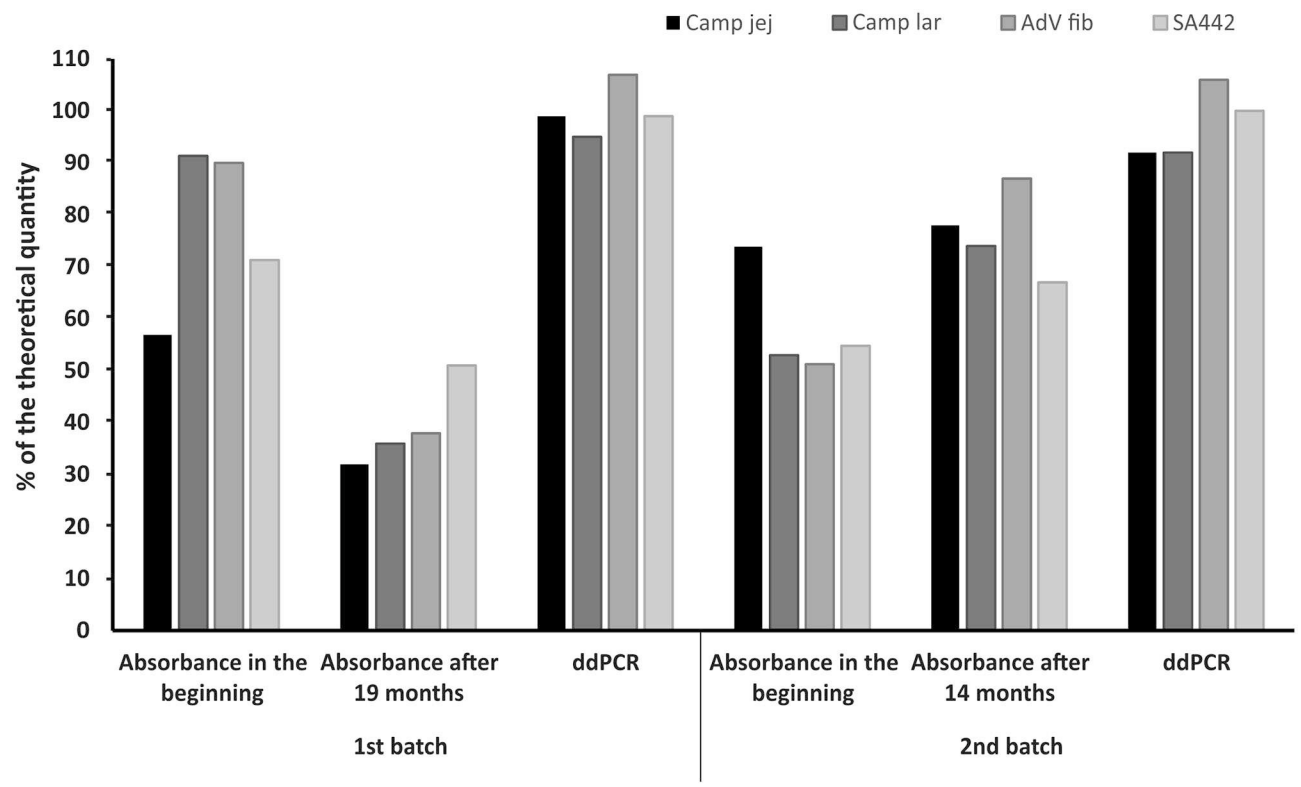

FIGURE 5 | Comparison of the concentrations of linear plasmid standards diluted based on spectrophotometric estimation in the beginning (Absorbance in the beginning), 19 or 14 months thereafter (Absorbance after 19 or 14 months) and re-diluted based on value (MLE) determined by ddPCR (ddPCR). The bars represent the calculated MLE of plasmid standard concentration compared to the theoretical concentration (the values are given in percent).

of standard stock solutions should be used only for a rough concentration estimate. Then, according to this estimate, the standard could be diluted to an order within the dynamic range of dPCR, which subsequently allows absolute quantification of the standard and its more accurate dilution.

Furthermore, we analyzed the linear plasmid standards of 45 qPCR assays using ddPCR and cdPCR platforms. The concentration estimations of the individual plasmid standards obtained by these two dPCR platforms differed significantly. The dynamic range of dPCR depends largely on the amount of the reaction partitions analyzed, and this amount is approximately 20,000 in both dPCR platforms (Pinheiro et al., 2012) utilized in this study, therefore, these two dPCR platforms should have approximately the same dynamic range. However, this was contradicted by the fact that in some cases in cdPCR software analysis of the two edge dilutions of the standard—expected concentration 10^4 and 10^2 copies/μl based on spectrophotometric measurement—the partition results were not clearly separated into negative and positive clusters indicating that these dilutions were already out of the dynamic range and resulting in deterioration of precision (Huggett et al., 2013). This led to a significant loss of linearity at these concentrations in cdPCR and therefore the calculation of MLE of standard concentrations from the three estimations may not give accurate results compared to ddPCR assay, whose response was linear over all three concentrations measured, and therefore probably provided more accurate results. As mentioned above, the absorbance measurements were overestimated in the vast majority of plasmid standard stock solutions, however, one value indicating the opposite was also recorded. In this experiment, the highest concentration estimation measured was recorded for a Camp lar standard with a value corresponding to 120% of the theoretical value when measured by ddPCR. The reason could be either an inhomogeneity of the plasmid stock solution in spectrophotometric measurement of absorbance or a slight underestimation in this measurement, which may also be caused by certain contaminants (Li et al., 2014). Nevertheless, according to this experiment, the approach of transformation of the optimized qPCR assays to dPCR platform seems to be robust and reliable. Only a few quantification standards failed to be analyzed using dPCR analysis—one standard using ddPCR platform and two standards using cdPCR platform out of 45 standards tested. Although, the PCR conditions optimized for qPCR (concentration of primers and probe) were set, it is possible that these conditions were not suitable for the dPCR assays. dPCR assays for which the conditions optimized for qPCR are not suitable, need to be further optimized, which involves, in addition to changes in primer and probe concentrations, changes of annealing temperature or ramp rate during amplification.

Since we concluded the calculation of MLE of concentration was more accurate in ddPCR than cdPCR, we used the values from ddPCR assays for re-dilution of plasmid standards, thus, to evaluate whether ddPCR is suitable for calibration of standards to the required quantity. Five standards were selected to include even those with extreme values, and after re-dilution, their concentration estimations again determined by ddPCR were approximately equal to the required concentration with a deviation of about 10%.

Freezing at −20°C is considered sufficient for storage of DNA for several months, for even longer storage period, freezing at −80°C is applied. To evaluate whether there are any changes in quantity of the linear plasmid standards during long-term storage at −20°C, stock solutions of the standards were again diluted based on the previously determined spectrophotometric estimations after 19 months for the first batch of the standards and after 14 months for the second batch. After 19 months of storage, there was a noticeable decrease in the concentration estimations compared to the standards measured in the beginning. After 14 months, the concentration estimate for one standard was not significantly changed, for the others there was even a slight increase in concentration estimations compared to the standards initially measured. Another study investigating the stability of DNA standards stored for 100 days at 4, 0, and −20°C using real-time PCR observed that freezing at −20°C provided the best storage conditions as it caused the least shift in the resulting *Ct* values (Roder et al., 2010). However, even such a shift would result in a significant deviation of the final sample concentrations if this DNA standard were used for a calibration curve in qPCR. In our study, after evaluating changes in standards concentration after long-term freezing, the standards were recalibrated (re-diluted) based on the values measured by the second ddPCR assay, and by a further additional ddPCR assay it was found that the concentration estimations of re-diluted standards corresponded to required concentrations with a deviation below 10%. Here we suggest that 10% variation (corresponding to 90–110% of the required quantity) in ddPCR results could be the criterion for the verified standard dilution. Long-term storage of standard is costs saving, but as can be seen, the quantity value of the standards may change over time when stored frozen. Therefore, the standards need to be checked by ddPCR at about 6 months whether they maintain the required quantity and recalibrate if necessary.

Results of this study showed that isolation of various plasmid DNAs, their quantification and storage in time represents a complex process, which can be biased on different levels even when maintaining standard operation procedures and standardized isolation kits. Variation may occur in different propagations of cells carrying plasmids, changes in stability of plasmids over time and proneness of the whole process to other factors, of which many of them are difficult to be anticipated. The biases in the concentration estimation of final quantification plasmid standards lead to heterogeneity of quantitative results within a single laboratory over time, which is reflected in determining the amount of a target sequence of microbial pathogens in samples derived from these qPCR standards. Therefore, an independent tool for the routine batch-to-batch control and verification of DNA standard quantity over time is needed. Here we suggest that ddPCR represents a suitable tool for such control. ddPCR is not affected by variation in the plasmid preparation process and can be universally used in any laboratory. It utilizes the same primers and probes as qPCR and amplifies only target DNA, without any interference from other DNA present in the sample. Therefore, standards of

any qPCR method, in-house or commercial, can be verified by ddPCR without any laborious and time-consuming optimization. The accurate recalibration of quantification standards according to the ddPCR would result in obtaining more homogeneous, reproducible and comparable quantitative data by qPCR in various commercial and research laboratories over time. ddPCR could be even proposed as a reference method for the unification of DNA standards across different laboratories detecting identical pathogens via different methods. Quantities of DNA determined by these methods in different laboratories could therefore be more comparable and interpretation criteria would be unified. In other words, the interpretation of quantitative data from different laboratories would be presented in the "same language".

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MB performed the experiments, analysis, and interpretation of data and wrote the manuscript. VB performed the statistical and mathematical analysis. BB provided cdPCR equipment and helped to analyze cdPCR data. MBB provided part of ddPCR equipment and enabling work in her laboratory. PK designed the study was involved in data interpretation, provided biological material, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2020.00155/full#supplementary-material

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Development of a reference standard for the detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* by quantitative PCR

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Quantitative PCR (qPCR) has become a frequently employed direct method for the detection and quantification of *Mycobacterium avium* subsp. *paratuberculosis* (MAP). The quantity of MAP determined by qPCR, however, may be affected by the type of qPCR quantification standard used (PCR product, plasmid, genomic DNA) and the way in which standard DNA quantity is determined (absorbance, fluorescence). In practice, this can be reflected in the inability to properly compare quantitative data from the same qPCR assays in different laboratories. Thus, the aim of this study was to prepare a prototype of an international MAP reference standard, which could be used to calibrate routinely used qPCR quantification standards in various laboratories to promote clinical data comparability. Considering stability, storage and shipment issues, a lyophilised fecal suspension artificially contaminated with a MAP reference strain was chosen as the most suitable form of the standard. The effect of five types of lyophilisation matrices on standard stability was monitored on 2-weeks interval basis for 4 months by F57 qPCR. The lyophilisation matrix with 10% skimmed milk provided the best recovery and stability in time and was thus selected for subsequent comparative testing of the standard involving six diagnostic and research laboratories, where DNA isolation and qPCR assay procedures were performed with the parallel use of the identical supplied genomic DNA solution. Furthermore, the effect of storage conditions on the standard stability was tested for at least 6 months. The storage at room temperature in the dark and under light, at + 4 °C, − 20 °C and − 80 °C showed no significant changes in the stability, and also no substantial changes in MAP viability were found using phage amplification assay. The prepared MAP quantification standard provided homogeneous and reproducible results demonstrating its suitability for utilisation as an international reference qPCR standard.

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of a chronic and infectious disease called paratuberculosis or Johne's disease[1]. The disease occurs throughout the world, and the herd prevalence rates in European countries ranges from 7 to 55%[2], although it is likely to be underestimated in many cases because of limitations of current methodologies used for detection of MAP. Paratuberculosis manifests after a long latent period lasting for years in domestic and wild ruminants and other mammals (e.g. cattle, sheep, goats,

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deer, wild rabbits and foxes) by a diarrhoea, wasting, emaciation, reduced milk production and ultimately ends with the death of the animal due to exhaustion. The faeces and milk of infected animals represent the major source of infection for other healthy individuals[3]. Infected milk also raises human health concerns in connection with Crohn's disease even though a direct link between MAP and disease development has not yet been established[4]. Although the pasteurization treatment of milk is commonly employed, there is an evidence demonstrating the presence of MAP in retail milk and dairy products in many parts of the world[5–7].

The detection of MAP is traditionally based on culture on solid media, which is still widely considered as the "gold standard". However, low sensitivity, long incubation times and sample decontamination requirements led to the development of alternative diagnostic tools, including quantitative polymerase chain reaction (qPCR)[8]. The qPCR detection and quantification are most commonly based on amplification of MAP specific targets IS900 and F57[9–11]. The IS900 sequence is present in 14–20 copies in the MAP genome and therefore is a preferable target for the sensitive detection of MAP in comparison with the single copy element F57, which in turn is preferred for MAP quantification[10].

Using qPCR, the absolute or relative quantification of the target nucleic acid sequence in a sample may be achieved. The major advantages of absolute quantification by qPCR are, compared to relative quantification, the provision of absolute copy number of a particular target and higher reliability for comparisons. However, for the accurate determination of target quantity, properly characterized qPCR quantification standards, used for the construction of a calibration curve, are necessary[12]. The absolute quantification of targets by qPCR assay plays an important role in clinical diagnostics, food safety as well as in monitoring the transmission of diseases. Commonly used standards can take various forms, including PCR products[12], recombinant plasmids[13,14] or genomic DNA[15]. Variety of particular forms of the standard, methods used for its quantification as well as characteristics of the qPCR assay used in the laboratory may be reflected in demonstrable variability in qPCR results. Consequently, different protocols cannot be compared against each other[16] resulting in differences in interpretation in disease diagnosis among laboratories, clinicians and researchers.

The determination of MAP quantity by qPCR, particularly in faeces, is essential for the correct diagnosis and classification of the infection status of animals. The reason for such precision is the phenomenon of "passive shedding" described initially by culture in heavily infected cattle herds in the USA[17]. This concept is based on findings that a high percentage of MAP shedders in infected herds shed low numbers of MAP in their faeces and after the removal of heavy shedders from the herd the overall percentage of MAP shedding animals decreases. These findings were later confirmed by qPCR analysis of the cattle faeces, which, however raised a new issue due to its higher sensitivity compared to culture[18]. Generally speaking, not all qPCR positive animals can be automatically considered to be infected and a threshold of $> 10^4$ MAP cells/g of faeces has been suggested as a selection criterion between truly infected and probably "contaminated" animals[18]. Moreover, this threshold cannot be applied automatically; other factors like the size of the herd, prevalence of paratuberculosis or housing conditions must also be considered.

The passive shedding phenomenon plays therefore an important role in qPCR data interpretations and subsequent control measures adopted. Due to the differences in qPCR assays performed in different laboratories and the variety of quantification standards used, it is difficult to directly compare quantitative data from different laboratories and to find a common consensus on the determination of the threshold between infected and contaminated animals. Therefore, the aim of the study was to develop an international reference standard for quantification of MAP by qPCR, which should facilitate comparison of resulting data from different qPCR assays and from different laboratories. To achieve this goal, a suitable lyophilisation matrix was tested and chosen in the first instance. The selected matrix was then used to produce the prototype lot of the standard, which subsequently underwent interlaboratory comparison by routine DNA isolation and qPCR procedures in six diagnostic and research laboratories. Moreover, the effect of storage under various conditions on the standard stability was tested over time. This part of the study was also related to the determination of MAP cells viability in the standard within the testing period using phage amplification assay.

Methods

Preparation of MAP culture. The field isolate of MAP (strain 7072 from a white deer, RFLP type C1) was used throughout the whole study. The isolate was grown on Herrold's egg yolk medium (HEYM) supplemented with 2 mg/ml Mycobactin J (Allied Monitor, USA) at 37 °C for 3 weeks until visible growth was observed. Afterwards, colonies were harvested and resuspended in 1.2 ml Tris–EDTA (TE) buffer (Amresco, USA) supplemented with Carrier DNA (salmon sperm DNA, final concentration 50 ng/µl; Serva, Germany). The MAP suspension was homogenized by vortexing for 30 s following the addition of 350 mg 1 mm zirconia/silica beads (Biospec, USA). In order to remove large MAP clumps, the suspension was centrifuged at 100 g for 30 s and the upper cell fraction was resuspended in TE buffer with Carrier DNA and diluted to an optical density at 600 nm (OD_{600}) of 0.1, which corresponds to approximately 10^8 MAP cells/ml of suspension[19]. A schematic overview of the entire experimental procedure is shown in Fig. 1.

Lyophilisation matrices—preparation of lyophilisation mixture. In total five different lyophilisation matrices were tested (Table 1) and their effect on the standard stability was monitored at 2-weeks intervals for 4 months. The lyophilisation matrices B, C and D were prepared by dissolving an appropriate amount of skimmed milk powder, sucrose (Amresco, USA) and bovine serum albumin (BSA; Sigma-Aldrich, USA) in sterile distilled water. The matrix E recipe was provided by The Collection of Animal Pathogenic Microorganisms (CAPM; Veterinary Research Institute, Czech Republic).

The lyophilisation mixture was composed of 40% supernatant of fecal suspension, 10% sterile water and 50% tested lyophilisation matrix. The fecal suspension was prepared from 5 g of MAP-negative bovine faeces

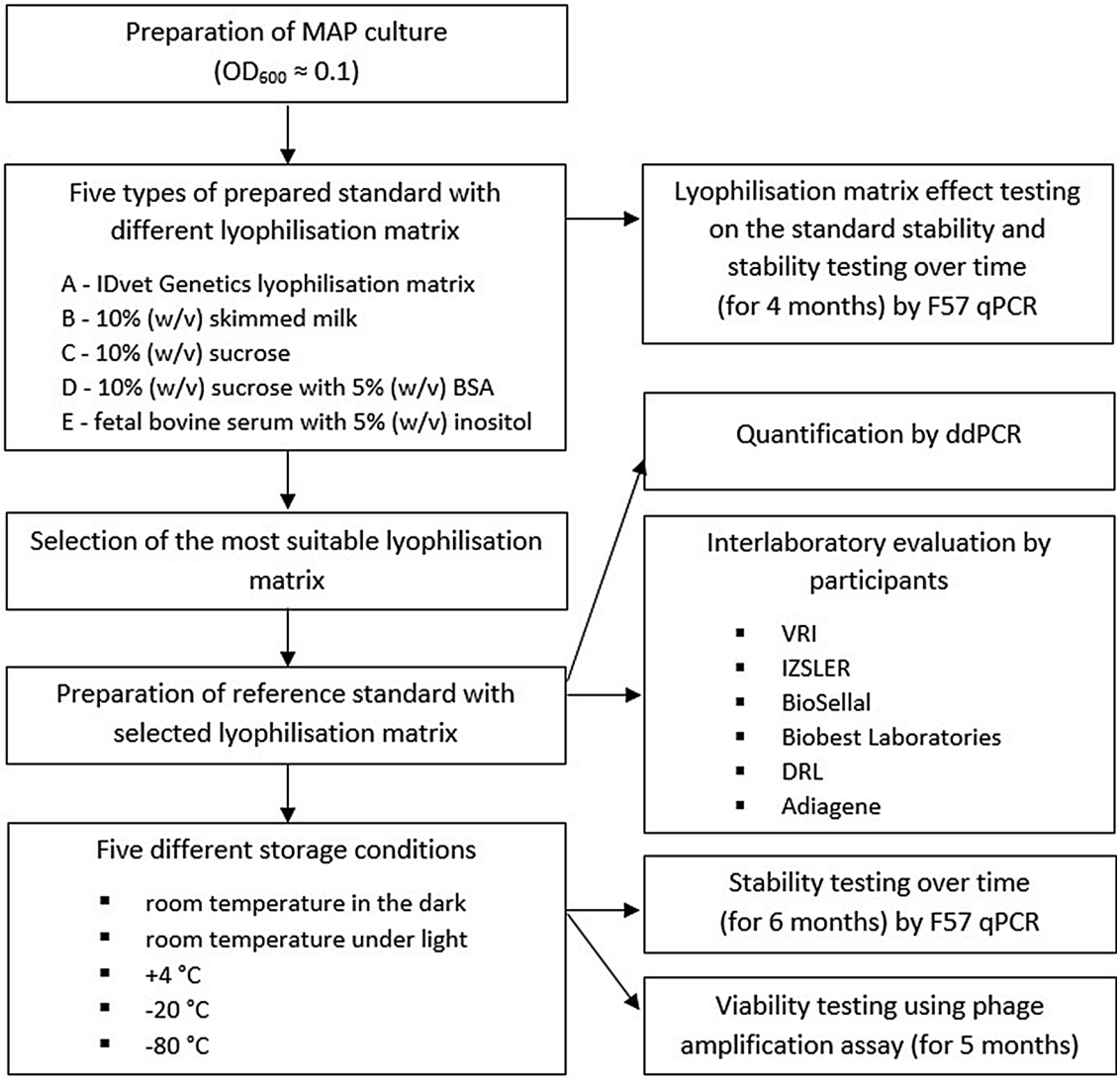

Figure 1. Schematic overview of the entire experimental procedure.

A	IDvet Genetics lyophilisation matrix[a]
B	10% (w/v) skimmed milk
C	10% (w/v) sucrose
D	10% (w/v) sucrose with 5% (w/v) bovine serum albumin
E	Fetal bovine serum with 5% (w/v) inositol

Table 1. The lyophilisation matrices tested. [a]Kindly provided by the Dr. Grewis and Dr. Laffont (IDvet Genetics, Montpellier, France).

(determined by qPCR), which were homogenized in 30 ml of sterile distilled water on a vortex and subsequently centrifuged at 100 *g* for 30 s. After mixing all three components, the lyophilisation mixture was artificially contaminated with the MAP suspension to reach an approximate amount of 10^6 MAP cells per a vial. The lyophilisation mixture was filled into the two-ml serum glass vials (Wheaton, USA) and the lyophilisation was performed on the freeze-dryer (FreeZone Triad, Labconco, USA). The lyophilisation started with precooling to − 20 °C and continued in three steps as follows: − 20 °C for 1 h (ramp rate 5 °C/min), − 10 °C for 2 h (ramp rate 1 °C/min) and 8 °C for 5 h (ramp rate 1 °C/min). The lyophilisates were stored at room temperature in the dark.

Determination of MAP quantity in the standard by droplet digital PCR. A total of 3 batches of lyophilised MAP standard were prepared, in which the exact number of MAP cells was independently determined using droplet digital PCR (ddPCR) targeting MAP-specific element F57. The DNA isolation was based

on a slightly modified protocol from the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research, USA; described in more details in chapter "DNA isolation and qPCR procedures"—*VRI*) and performed on 10 replicates. The ddPCR was carried out using QX200 droplet digital PCR system (Bio-Rad, USA) according to the manufacturer's instructions. The assay was run in duplicate for all analysed samples in a total reaction volume of 22 µl. The reaction mixture contained 11 µl of ddPCR Supermix for Probes (Bio-Rad, USA; No UTP), 5 µl of isolated DNA and F57 probe and primers in concentrations 50 nM and 500 nM, respectively[10]. The reaction mixture was loaded with 70 µl of droplet generation oil into wells of the DG8 cartridge and placed into the QX200 droplet generator. Forty µl of generated droplets was transferred into 96-well PCR plate, which was subsequently sealed using foil heat seal and PX1 PCR plate sealer and placed in a T100 Thermal Cycler for PCR. The PCR included the following conditions: enzyme activation at 95 °C for 10 min followed by 40 cycles of a two steps thermal profile at 94 °C for 30 s and 60 °C for 60 s and a final enzyme deactivation at 98 °C for 10 min while maintaining ramp rate 2 °C/s. Following the PCR the 96-well plate was placed in the QX200 Droplet Reader, where droplets were read and analysed using QuantaSoft Software (Bio-Rad; version 1.7.4.0917). After ddPCR analysis the MAP quantity determined was recalculated per a vial according to the appropriate DNA isolation procedure parameters and this value was considered as a reference.

Stability of MAP reference standard over time and effect of lyophilisation matrices on standard stability. The DNA isolation from a lyophilised MAP reference standard, stored at room temperature in the dark, was based on a slightly modified QIAamp DNA Stool Mini Kit protocol (Qiagen, Germany)[19] including mechanical homogenisation in a bead beating instrument (MagNa Lyser) and elution to 100 µl of TE buffer. The isolated DNA was used for the quantification of MAP cells by a qPCR assay amplifying the single copy fragment F57[10] on a LightCycler 480 instrument (Roche Molecular Diagnostic, Germany). Each sample was analysed in technical duplicates. The absolute numbers of MAP cells were determined using 'Fit-point analysis' in LightCycler 480 software (version 1.5.1.62) according to a calibration curve derived from the serial dilution of circular plasmid standard containing the F57 insert.

The lyophilisation matrix effect testing and initial determination of repeatability and stability in time of the MAP reference standard were performed. After the lyophilisation (day 1), eight vials from each type of the lyophilisation matrix underwent to the DNA isolation and F57 qPCR to assess the quantity of MAP cells and repeatability. To test the stability, two vials of each type of the lyophilisation matrix were analysed at fortnightly intervals for 4 months.

Interlaboratory testing of MAP reference standard. In order to evaluate the MAP reference standard performance in different laboratories, the prototype lot of the standard was prepared and 10 vials of lyophilised standard (with 10% skimmed milk solution as a lyophilisation matrix, which has shown the best performance in previous experiment) were sent out to six laboratories: Veterinary Research Institute (VRI, Czech Republic), Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER, Italy), BioSellal (France), Biobest Laboratories (UK), Disease Research Limited (DRL, New Zealand) and Adiagene (France). Each participating laboratory performed its own routine DNA isolation using one or more DNA isolation kit and one or more PCR assays as described below. To compare resulting data from different laboratories, the lyophilised MAP genomic DNA (gDNA) originating from the MAP strain 7072 was included in testing together with standards. The laboratories were instructed to dilute the gDNA and construct an external independent calibration curve for quantification of the MAP DNA in each qPCR run.

Preparation of MAP gDNA standard. MAP gDNA standard was isolated by a modified protocol from DNeasy Blood and Tissue Kit (Qiagen). Briefly, colonies of MAP were harvested and resuspended in 360 µl of enzymatic lysis buffer, prepared according to the manufacturer's instructions, followed by incubation at 37 °C with shaking at 1400 rpm for 30 min. Subsequent purification steps followed the manufacturer's instructions. The concentration of isolated DNA was measured by NanoDrop 2000c (Thermo Scientific, USA). The DNA (100 ng) was pipetted into screw cap tubes and dried on a thermoblock (Major Science, USA) for about 3 h.

The dried MAP gDNA was reconstituted in 100 µl of water (LightCycler 480 Probes Master H_2O, PCR grade, Roche Molecular Diagnostics) to a concentration of 1 ng/µl, which was determined by ddPCR to correspond to 2×10^5 MAP cells/µl. The reconstituted gDNA was diluted tenfold in a range from 2×10^5 to 2×10^{-1} cells/µl (concentration range 1 ng/µl–1 fg/µl) and amplified in technical duplicate in parallel with samples and standards in all qPCR assays performed.

DNA isolation and qPCR procedures.

I. VRI

DNA from lyophilised MAP reference standards was isolated by a slightly modified protocol from the Quick-DNA Fecal/Soil Microbe Microprep Kit (Zymo Research). The lyophilisates were dissolved in 750 µl of BashingBead Buffer and transferred to a BashingBead Lysis Tube. Then the samples were subjected to mechanical homogenisation in the MagNA Lyser at 6400 rpm for 30 s, left until they have cooled down and the homogenization step was repeated. Following centrifugation at 10,000*g* for 1 min, 400 µl of supernatant was processed using an isolation kit according to the manufacturer's instructions. The DNA was eluted into 25 µl of DNA Elution Buffer and 5 µl was used as a template for the F57 qPCR assay as described above[10].

II. IZSLER

The lyophilisates were dissolved in 500 µl of DEPC (diethyl pyrocarbonate) water and submitted to mechanical homogenisation in Tissue Lyser II (Qiagen) at 30 Hz for 10 min with 10 mg of acid washed glass beads (Sigma-Aldrich, Milan, Italy). Two-hundred µl of the supernatant were processed using DNeasy Blood & Tissue Kit (Qiagen) by adding 200 µl of buffer AL, 20 µl of Proteinase K and incubating for 10 min at 70 °C. After addition of 200 µl of ethanol, the mixture was centrifuged through columns provided by the kit. After washing according to the manufacturer's instructions, the DNA was eluted into 200 µl of DNA Elution Buffer and 5 µl were used in IS900 qPCR assay already used[20] and carried out on a StepOne qPCR system (Thermo Fisher, software version 2.3).

III. BioSellal

The lyophilisates were resuspended in 600 µl of ATL buffer (Qiagen) and transferred to a grinding tube (BioPrep MAP-2). The tubes were grinded in a FastPrep® grinder (MP BioMedicals) 4 times for 45 s at 6.5 m/s. After centrifugation at 10,000*g* for 1 min, 400 µl of supernatant was used for the DNA extraction using BioExtract® Superball® and BioExtract® Column (BioSellal) according to the manufacturer's instructions. Five lyophilisates were extracted per extraction methods. The elution volume was 100 µl for both extraction kits. The detection and quantification of the MAP IS900 sequence was performed, according to the manufacturer's instructions, using 5 µl of extracted DNA and Bio-T kit® Mycobacterium avium paratuberculosis (BioSellal) on AriaMx thermal cycler (Agilent Technologies).

IV. Biobest Laboratories

In the extraction protocol 1, DNA from lyophilised MAP standard material was extracted by a slightly modified protocol from the MagMAX Total Nucleic Acid kit (Applied Biosystems, USA). The lyophilised material was reconstituted with 300 µl MagMAX lysis/binding solution, this material was then transferred to a bead beating tube and processed as per the manufacturer's guidelines. In the extraction protocol 2, DNA from lyophilised MAP standard material was extracted by a slightly modified protocol from the MagMAX CORE kit with mechanical lysis module (Applied Biosystems). The lyophilised material was reconstituted with 400 µl MagMAX CORE clarifying solution, this material was then transferred to a bead beating tube and processed as per the manufacturer's guidelines. Five vials of lyophilised MAP standard material were processed by each extraction method.

DNA from each extract was tested in duplicate by two PCR tests. PCR 1 used the VetMAX-Gold MAP PCR kit (Applied Biosystems) with an additional 1.3 µl of water, 1.5 µl of IS900 primers and 1.2 µl of IS900 probe (primers and probes are described in study by Ravva and Stanker[21]). PCR 2 also used the VetMAX-Gold MAP PCR kit (Applied Biosystems) with an additional 4 µl of water. In both PCR reactions, 4 µl of DNA was added to a total mastermix of 21 µl. PCR tests were run on the Rotor-Gene 6000 (previously Corbett Life Science, now sold by Qiagen as the Rotor-Gene Q).

V. DRL

DNA from the lyophilised MAP reference standards was recovered using either of two different methods representing protocols employed routinely by the DRL laboratory for low-throughput ($n \leq 5$) or high-throughput sample numbers. For low-throughput extractions, individual spin columns (Quick-DNA Fecal/Soil Microbe Microprep Kit; Zymo Research) were utilised with minor modifications to the supplied protocol. Briefly, the reference standards were resuspended in 1 ml of the kit lysis buffer and transferred to a 2 ml skirted, screw top microcentrifuge tube containing 0.5 ml of a 50:50 mix of 0.5 and 0.1 mm zirconia beads (BioSpec, Corp., Bartesville, OK, USA). Samples were mixed briefly before the tube was placed in a vigorously boiling water bath for 5 min. Following boiling lysis, samples were immediately further disrupted by bead beating for 5 min at 1750 bpm in a GenoGrinder instrument (Spex SamplePrep, NJ, USA). Following bead beating, the samples were centrifuged at 18,000*g* for 5 min to pellet undissolved solids before 370 µl of cleared lysate was removed and processed for DNA extraction according to the manufacturer's protocol. Nucleic acids were eluted in 110 µl of DNA elution buffer and 3 µl was used for PCR amplification.

For high-throughput extractions, a 96 well plate extraction format was utilised. Samples were resuspended in 1100 µl of lysis buffer and subjected to thermal, chemical and mechanical lysis. Eight hundred microliters of clarified lysate were extracted on a semiautomated DNA extraction platform resulting in 150 µl of eluted DNA. Three microliters of eluate were used for PCR amplification.

For qPCR quantitation, samples were assayed for IS900 using an in-house, hydrolysis probe based real time PCR assay using an Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA).

VI. Adiagene

Two DNA isolation procedures were applied to the lyophilised MAP reference standards. In the isolation procedure 1, five lyophilisates were dissolved in 500 µl of Nuclease free water and transferred to a tube containing 300 mg glass beads (ADIAPURE™ aliquoted Glass beads, Bio-X Diagnostics, Belgium). Samples were grinded in the Mixer Mill at 30 Hz for 10 min and centrifuged at 15,000*g* for 5 min. 350 µl of supernatant was used for DNA extraction using QIAamp DNA Mini kit (Qiagen).

In the isolation procedure 2, five lyophilisates vials were dissolved in 400 µl of ATL buffer and proteinase K from QIAamp DNA Mini kit. Samples were incubated for 1 h at 56 °C and then transferred to a tube containing 300 mg glass beads (ADIAPURE™ aliquoted Glass beads). Samples were grinded in the Mixer Mill at 30 Hz for 10 min and centrifuged at 15,000*g* for 5 min. Subsequently, 220 µl of supernatant was used for DNA extraction using QIAamp DNA Mini kit.

All DNA samples were eluted with 200 µl of AE buffer and 5 µl was used as a template for the qPCR assay using ADIAVET™ PARATB REAL TIME according to the manufacturer's instructions (Bio-X Diagnostics).

The effect of storage conditions on the standard stability over time and determination of MAP cells viability using phage amplification assay. To evaluate the MAP reference standard stability under different storage conditions, the vials with lyophilised standards were stored under five conditions: room temperature in the dark, room temperature under light, + 4 °C, − 20 °C, − 80 °C and all were analysed in 2-weeks intervals for at least 6 months. DNA from the lyophilised standards was isolated using Quick-DNA Fecal/Soil Microbe Microprep Kit as described above with analysis by F57 qPCR assay[10]. DNA isolation was carried out in duplicate from each condition tested and the gDNA standard gradient (described above) was included in each qPCR run performed and used for quantification.

In parallel with the DNA isolation and F57 qPCR, the slightly modified phage amplification assay previously described by Stanley et al.[22] was performed. The goal was to assess stability of the MAP cells in the standard in a complex manner. Specifically, two vials of lyophilised standard from each storage condition were analysed in 2-weeks intervals. The vials were dissolved in 1 ml of Middlebrook 7H9 broth (Difco Laboratories, USA) supplemented with 10% Middlebrook OADC enrichment (Becton Dickinson, USA), 2 mM $CaCl_2$ (Penta, Czech Republic) and 1.25% PANTA (Becton Dickinson). The antibiotic mixture PANTA was prepared according to the manufacturer's instructions and was used to suppress natural microflora present in faeces. After overnight incubation at 37 °C with shaking at 100 rpm, 100 µl of bacteriophage D29 (kindly provided by Dr. Botsaris, Cyprus University of Technology, Cyprus and Dr. Rees, University of Nottingham, UK; stock concentration 10^9 PFU/ml) was added to each sample and incubated at 37 °C for 2 h with shaking at 100 rpm. Then the bacteriophages that have not infected mycobacterial cells were eliminated by the addition of 100 µl of 100 mM ferrous ammonium sulphate (FAS; Lach-Ner, Czech Republic). After incubation of samples at room temperature for 5 min, 5 ml of enriched medium was added for FAS neutralization followed by the preparation of tenfold dilutions of the samples. Then 1 ml of *Mycobacterium smegmatis* mc^2155, grown for 48 h at 37 °C with shaking at 100 rpm in 50 ml of Middlebrook 7H9 broth with 10% OADC (10^8 CFU/ml), was added to each sample dilution. All samples were transferred into a sterile Petri dish and 6 ml of molten 1.6% Middlebrook 7H10 agar (Difco Laboratories) cooled to 55 °C was added to each plate. The content of the plate was carefully mixed and left at room temperature until the agar set. The number of plaques formed was counted after overnight incubation of plates at 37 °C. Positive and negative controls were included in each analysis.

Mathematical and statistical analysis. *Stability of MAP reference standard over time and effect of lyophilisation matrices on standard stability.* The repeatability and stability over time of standards with a different lyophilisation matrix (A–E) was tested using one-way analysis of variance (ANOVA). The repeatability and stability were estimated from the model as "mean square error of sample (within-subject variance)" and "mean square error of Week (within-subject variance)", respectively. In addition, R^2, mean, sample standard deviation and coefficient of variation were calculated.

Interlaboratory testing of MAP reference standard. The MAP DNA copy number detected by a particular isolation procedure and qPCR assay was calculated from the raw crossing point (Cq) values according to the respective regression equation of gDNA gradient from the actual run. This value was then converted to the amount of MAP cells in the initial sample (lyophilised vial) according to the appropriate isolation procedure parameters using this formula:

$$y = copy\ number \times \frac{elution\ volume}{volume\ of\ DNA\ in\ qPCR\ reaction} \times \frac{volume\ to\ dissolve\ the\ lyophilisate}{volume\ of\ lysate\ collected\ for\ subsequent\ isolation\ steps}$$

Particular recalculation formulas for individual DNA isolation and PCR procedures used by participating laboratories are given in Table 2. The mean with standard deviation for the particular DNA isolation and qPCR assay performed were calculated. For statistical evaluation of mean MAP quantity estimations in the standard obtained by each method in the participating laboratory, one-way ANOVA and one-sample t-test were used.

The effect of storage conditions on standard stability over time and determination of MAP cells viability using phage amplification assay. The stability and cell viability of MAP standard stored under five conditions over time were analysed using two-way ANOVA.

Results

Preparation of MAP reference standard. With regard to stability, storage and shipment issues, the lyophilised form of the MAP reference standard appeared to be the most suitable. The optimised protocol for a large scale (approximately 200 vials) lyophilisation provided the successful freeze drying of all five standard types with a different lyophilisation matrix. A highly porous and easy to reconstitute cake was obtained for all types of lyophilised standard.

In the 1st batch of lyophilised standard, intended for the interlaboratory testing, the exact MAP quantity was determined to be 1.0×10^6 cells. In the 2nd and 3rd batch, which were used for the stability study under five storage conditions, the MAP quantities were defined to be 9.3×10^5 and 9.8×10^5 cells, respectively. Only for the initial stability study evaluating various lyophilisation matrices, the exact MAP quantity determination in lyophilised vials was not performed.

Stability of MAP reference standard over time and effect of lyophilisation matrices on standard stability. The determination of MAP cells quantity in eight biological replicates of the standard by F57 qPCR assay analysed immediately after the lyophilisation demonstrated a high repeatability of four types (B–E)

Laboratory	Method	Recalculation[c]	Mean of detected cells	SD
VRI[a]		GE number[d] × (25/5) × (750/400)	1.82×10^6	3.63×10^5
IZSLER[a]		GE number × (200/5) × (500/200)	5.16×10^5	2.07×10^5
BioSellal	BioExtract® Column[b]	GE number × (100/5) × (600/400)	9.75×10^5	1.33×10^5
	BioExtract® Superball®[b]	GE number × (100/5) × (600/400)	4.56×10^5	8.40×10^4
Biobest Laboratories	**Isolation method 1**			
	PCR 1[b]	GE number × (50/4) × (300/200)	3.20×10^5	5.28×10^4
	PCR 2[b]	GE number × (50/4) × (300/200)	3.08×10^5	5.04×10^4
	Isolation method 2			
	PCR 1[b]	GE number × (90/4) × (400/300)	6.40×10^5	2.35×10^5
	PCR 2[b]	GE number × (90/4) × (400/300)	6.23×10^5	2.28×10^5
DRL	Low throughput extractions[b]	GE number × (110/3) × (1000/370)	2.84×10^5	1.68×10^4
	High throughput extractions[b]	GE number × (150/3) × (1100/800)	1.42×10^6	1.23×10^5
Adiagene	Isolation method 1[b]	GE number × (200/5) × (500/350)	1.02×10^6	4.62×10^5
	Isolation method 2[b]	GE number × (200/5) × (400/220)	7.34×10^5	3.10×10^5

Table 2. The number of MAP cells in the lyophilised standard detected by various DNA isolation procedures and PCR assays in different laboratories. *GE* genome equivalent, *SD* standard deviation. [a]Data originated from 10 repeats. [b]Data originated from 5 repeats. [c]Recalculation of MAP quantities per a vial. [d]Number of MAP genome equivalents detected by qPCR.

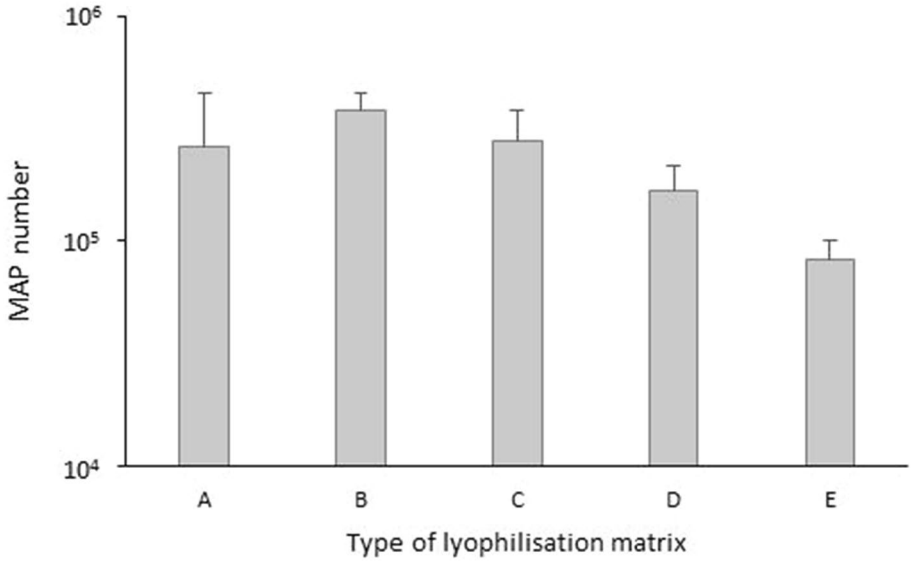

Figure 2. The mean amount of MAP cells recovered using F57 qPCR in day 1 from five types of standard with different lyophilisation matrix. Error bars represent standard deviations obtained from eight biological replicates.

	A	B	C	D	E
Repeatability	0.346	0.012	0.058	0.044	0.015
R^2 of model	0.998	0.885	0.936	0.982	0.933
Mean	5.271	5.576	5.418	5.205	4.914
SD	0.402	0.081	0.171	0.145	0.087
Coeff. of variation (%)	7.63	1.45	3.15	2.79	1.77

Table 3. Descriptive statistic regarding repeatability testing of five types of standard with different lyophilisation matrix.

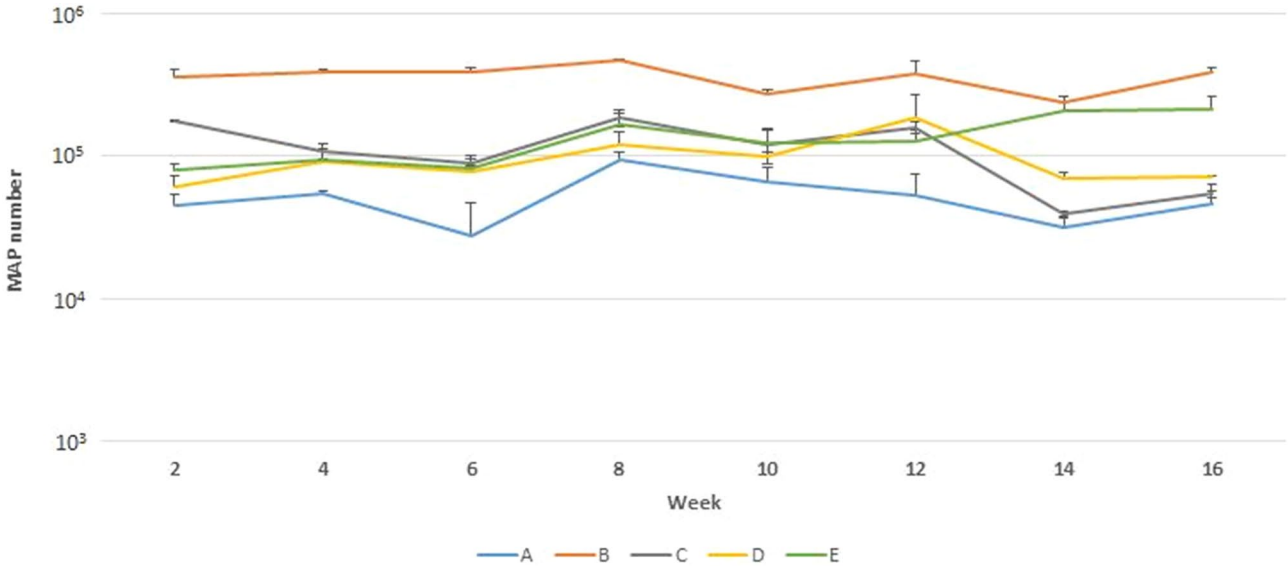

Figure 3. The stability of MAP reference standard with five different lyophilisation matrices in time determined by F57 qPCR assay. Error bars represent standard deviations for the means of two biological replicates.

	A	B	C	D	E
Variance	0.153	0.036	0.183	0.079	0.117
R^2 of model	0.556	0.763	0.929	0.641	0.724
Mean	4.662	5.542	5.036	4.942	5.094
SD	0.249	0.104	0.218	0.170	0.191
Coeff. of variation (%)	5.35	1.87	4.32	3.43	3.75

Table 4. Descriptive statistic regarding stability testing of five types of standard with different lyophilisation matrix.

of standard tested (Fig. 2; Table 3). The best uniformity of results and the highest yield were recorded for the standard with lyophilisation matrix B (10% skimmed milk solution) during the 16 weeks of the initial stability determination (Fig. 3; Table 4). Based on these parameters, the 10% skimmed milk solution was selected as the most suitable lyophilisation matrix for the preparation of the lyophilised reference standard. The selected lyophilisation matrix was used in further testing.

Interlaboratory testing of MAP reference standard. The performance of the lyophilised MAP reference standard was evaluated in six diagnostic and research laboratories by various isolation and qPCR methodologies routinely employed by these laboratories. The number of MAP cells detected by qPCR were recalculated per a vial according to the parameters of the particular DNA isolation (Table 2). Comparability of the resulting data from different laboratories was ensured by the utilization of MAP gDNA, the exact concentration of which was determined using ddPCR and which was used as a calibrator for quantification of the analysed standards by each laboratory. The statistically significant variability in MAP quantity estimates obtained by different laboratories and methods used was recorded ($P < 0.01$; ANOVA factor effect F-test) with estimations ranging from 2.84×10^5 to 1.82×10^6.

The effect of storage conditions on standard stability over time. No statistically significant differences were found between the individual storage conditions ($P > 0.05$; ANOVA factor effect F-test; Fig. 4) during 6-month testing period, therefore, all five storage conditions can be considered equivalent in terms of the effect on standard stability. Also, differences in MAP quantity estimates in lyophilised standard obtained during the testing period are not statistically significant ($P > 0.05$; ANOVA factor effect F-test).

Determination of MAP cells viability using phage amplification assay. The difference in viable MAP quantities determined for the standard stored at room temperature in the dark, under the light and at − 20 °C and for the standard stored at − 80 °C and + 4 °C was recorded (Fig. 5). This can be explained by separate preparations of these standards due to the high demand for a number of lyophilised vials for the stability study. However, no substantial difference in trends during 5-months testing period was observed between the two batches. The MAP viability has been relatively constant over time under all five storage conditions. The main

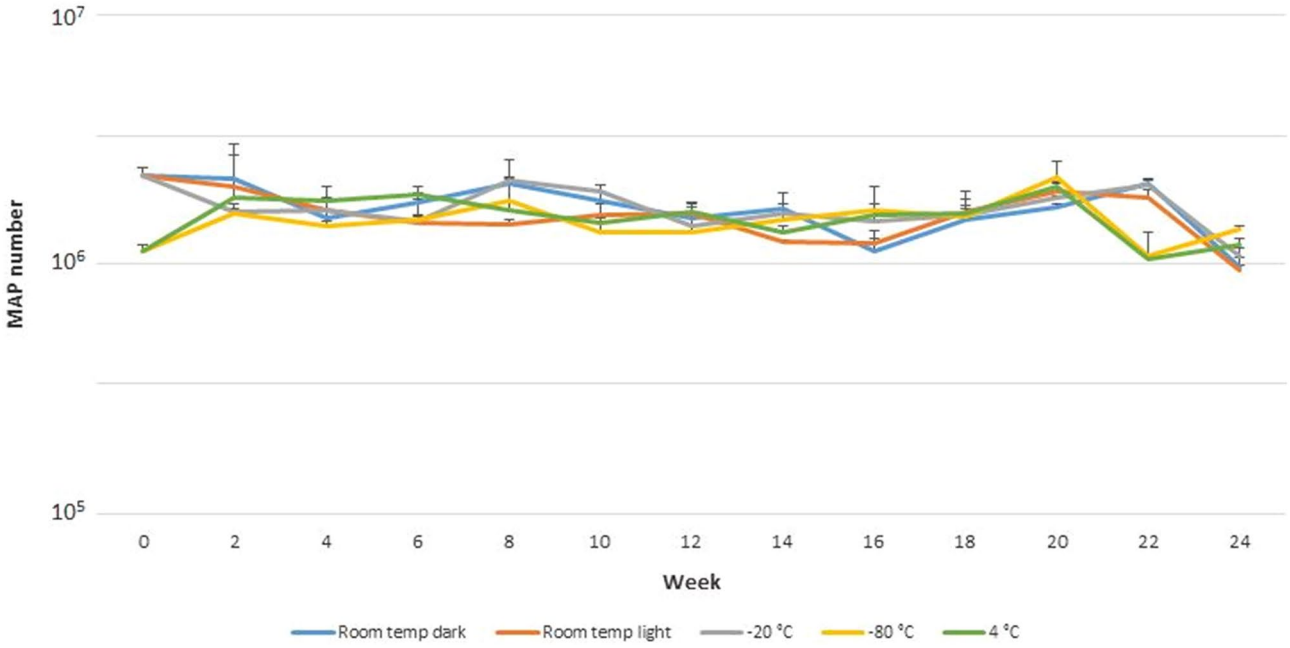

Figure 4. The stability of MAP reference standard stored in five different storage conditions over time determined by F57 qPCR assay. Error bars represent standard deviations for the means of two biological replicates.

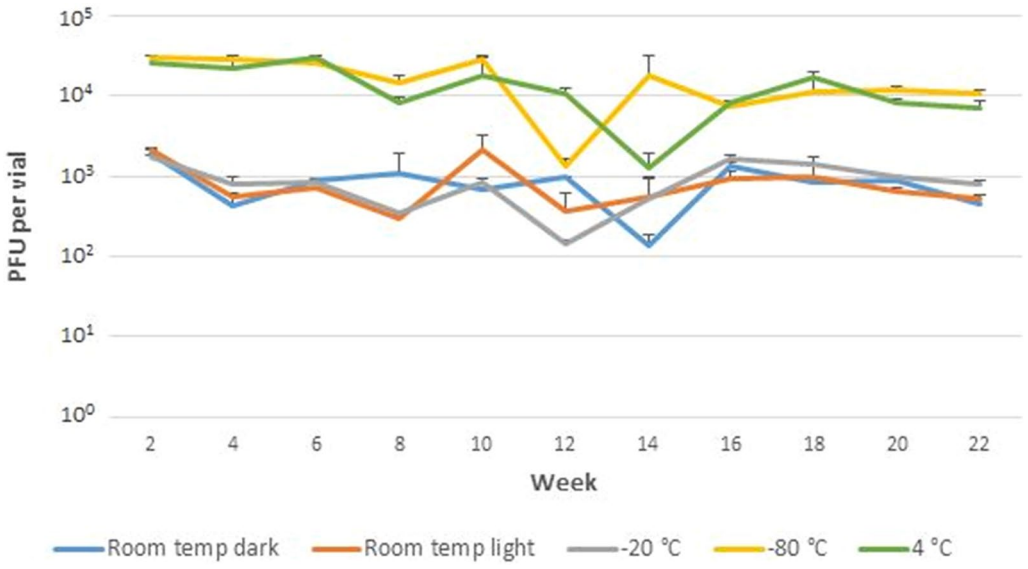

Figure 5. The viability of MAP cells in the reference standard stored in five storage conditions over time determined by phage amplification assay. Error bars represent standard deviations for the means of two biological replicates.

deviation was the decrease in the values of viable MAP cells of around one order of magnitude in the 12th and 14th week.

Discussion

The aim of the present study was to develop an international reference standard for accurate MAP quantification by qPCR. Nowadays, no reference standard for MAP quantity determination is available; various diagnostic and research laboratories use different DNA extraction and subsequent amplification procedures, whether commercial or in-house. Utilization of variety of calibration materials (recombinant plasmid, genomic DNA, PCR product, etc.) and methods for DNA quantity assessment of these materials significantly influences the results of particular qPCR and subsequent data interpretation[12,14,15]. Besides, the degradation of calibration materials maintained in a frozen state over time can significantly affect PCR efficiency[12]. These combined sources of variability

result in the inability to compare quantitative data from various qPCR assays from different laboratories[23]. Through the establishment of a reference standard, it is possible to address this issue and promote unification of DNA quantification standards (independently on the DNA extraction and qPCR) in qPCR detection and quantification of pathogens. Universally available reference materials for nucleic acid quantity testing have been established, for example, for hepatitis viruses, human immunodeficiency virus and human parvovirus B19[24–28], which play an important role in blood screening and diagnostic fields. The consensus in quantitative DNA results between clinical laboratories and between serial samples from a particular patient within one laboratory is essential for definition of generally accepted clinical threshold values for viral infection and for monitoring disease initiation and progression[16].

In diagnosis of paratuberculosis, qualitative interpretation of qPCR data is not sufficient for the determination of infectious status of animals due to the effect of passive shedding phenomenon. The passive shedding where MAP is present in animal faeces as a result of the oral ingestion of MAP organisms from environment massively contaminated by MAP but unrelated to the current host infection with paratuberculosis, was first demonstrated by fecal culture and later confirmed by qPCR testing of fecal samples[18,29]. As molecular methods such as qPCR are capable of detecting very low levels of DNA, establishment of cut-off value is relevant in order to correctly conclude whether the presence of MAP in faeces is the result of real ongoing infection of animals or only reflects a high environmental infectious pressure. Thus, the quantity of 10^4 MAP cells/g of faeces has been suggested as the threshold to discriminate between truly infected and probably "contaminated" animals[18]. The accurate determination of MAP load in faeces and correct interpretation of qPCR data are crucial for the reliable animal infection status assessment and subsequent reasonable paratuberculosis control measures adopted. The variability in the efficiency of DNA extraction from fecal sample and amplification by the particular qPCR may lead to the underestimation of MAP load in the clinical sample, which can affect subsequent decision regarding control or eradication interventions and affect the prevalence of MAP-infected individuals in the herd.

The lyophilised form of the MAP reference standard, where residual moisture and oxygen are minimized, was decided to be the most suitable as this form can be dispatched at ambient temperature and is stable over time[30]. The composition of the standard is based on a fecal suspension, which is also of advantage as it closely simulates a real sample. In total, five types of lyophilisation matrix were analysed, and after freeze-drying there were no visible significant differences among lyophilisation matrices used. Also, all five types were easily reconstituted in a lysis buffer. The lyophilised standard with 10% skimmed milk as a lyophilisation matrix (type B), however, provided the best recovery and stability over time and was thus subjected to further testing.

To ensure accurate and independent MAP quantity value determination in the MAP reference standard, the ddPCR was utilized and shown to be the most appropriate approach. The ddPCR, a single-molecule amplification technique based on limiting dilution, is a highly accurate and precise method for absolute DNA quantification, which does not require the creation of the calibration curve and exhibits increased tolerance to inhibitors compared to qPCR. Currently, the ddPCR is considered as a higher-order reference measurement method for nucleic acid testing of infectious disease[31,32]. In present study, three batches of lyophilised MAP standard were separately prepared and independently quantified using ddPCR with all results oscillating around the theoretical value of 10^6 MAP cells per a vial (derived from the rough OD determination), which demonstrates ddPCR to be a robust and reliable technique for exact reference standard quantification. These values corresponded to the amount of 10^6 MAP cells from the subsequent qPCR analyses. In some cases, however, minimal deviations were recorded between qPCR results and the MAP quantity in the standard determined by ddPCR, which can be attributed to manipulation with samples including dilutions and pipetting errors. The ddPCR procedure used here have proven its suitability in an earlier study when allowed to achieve a defined quantity of quantification plasmid standards for various viral and bacterial pathogens[33]. Besides, the ddPCR technology was reported to produce more precise, reproducible and statistically significant results required for publication quality data for samples with low levels of nucleic acids and/or variable amounts of chemical and protein contaminants[34].

No significant effect on MAP standard stability was found over 6 months under five different storage conditions using F57 qPCR, keeping the number of MAP cells recovered from the standard at a roughly constant level during testing period. These findings were supported by results of a phage amplification assay, the method allowing a rapid enumeration of viable MAP cells in various kinds of matrices including milk and faeces using a lytic mycobacteriophage D29[35]. No substantial changes in number of viable MAP cells in the standard were found during 5 months of testing period. The main deviation recorded was the decrease in the values of viable MAP cells of around one order of magnitude in the 12th and 14th week, however, this decrease occurred in all storage conditions and may therefore be due to some circumstances other than properties of the samples. It is likely that this was due to a combination of various factors, including pipetting, sample manipulations, and the possible presence of MAP cells clumps in the sample may also play a role. Two batches of lyophilised standard were prepared for stability study under five storage conditions, which was reflected in the number of viable MAP cells determined. In one batch (room temperature in the dark and under light, and − 20 °C) the value of viable MAP cells was of the order of 10^3, while for the next batch (− 80 °C and + 4 °C) this value was around 10^4 cells. Nevertheless, the determined viability did not affect the qPCR results. The viability determination showed that the standard in its current form is rather not suitable for the culture standardization.

Although the values of viable MAP cells in the standard determined by phage amplification assay were up to about three orders of magnitude lower than qPCR values, this is in concordance with results of cultivation experiments of Kralik et al.[19] when cultivation provided lower MAP CFU counts by approximately two $\log_{10}$, compared to F57 qPCR. This was explained by the tendency of MAP cells to form clumps and the ability of cultivation to detect only viable cells. The presence of fecal suspension in a standard, that has been found to contain components inhibiting phage infection[35], may also contribute to a further reduction in number of cells detected by the phage amplification assay. Moreover, for phage amplification assay, the antibiotic mixture PANTA was necessary to be added to the media to suppress the growth of natural microflora present in the fecal suspension.

The microflora not only competed with the growth of "background" formed by *M. smegmatis* cells, but also hampered the counting of plaques on an agar. Overall, the results of the stability study show that a short time at elevated or decreased temperatures during shipment should not affect MAP quantity or viability of the standard.

To demonstrate the routine application of the standard and to evaluate the MAP reference standard performance in various laboratories from different countries, the interlaboratory study was performed. Certain differences ($<1\ \log_{10}$) were found among MAP quantity estimations, which reflects various methodologies (particularly efficiencies of the DNA isolation procedures) that were employed by participants. However, these differences in quantitative data can be considered as minor in comparison with previous studies performed to establish other international reference standards for hepatitis viruses and human parvovirus B19[26,28,36] that reached 1.5–2.5 $\log_{10}$ difference. In addition, with the exception of four results, all fell within acceptable standards of variation, defined as the expected result $\pm 0.50\ \log_{10}$[37]. On the other hand, the achieved results variance underlines the importance of introducing a reference standard for accurate and uniform MAP quantity value determination by qPCR assays.

The intended concept of using MAP reference standard considers calibration of the quantification standard of a particular qPCR according to the MAP reference standard. If the uniform procedure would be used for extraction and quantification of DNA from both the reference standard and fecal samples, the DNA losses during the DNA extraction and amplification process of samples would be eliminated enabling measurement precision of a MAP load in the sample. In addition to that, quantities of DNA in samples determined in different laboratories based on this reference standard could therefore be more comparable and interpretation criteria could be unified.

This study was performed with the aim of establishing an international reference standard for the exact MAP quantification using qPCR and to facilitate comparability of quantitative data among various laboratories. Obtained results demonstrated striking data homogeneity of prepared MAP reference standard. The applicability of the reference standard for routine MAP quantity assessment by qPCR was proven through interlaboratory testing involving six diagnostic and research laboratories employing different PCR assays and through stability study over time in various storage conditions. The introduction of the first international reference standard for MAP qPCR assay calibration would be an initial important step in quality improvement and standardization of this laboratory tool. The next step will be an applying for approval from the OIE Biological Standards Commission to include qPCR standard among OIE-Approved Reference Standards.

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Author contributions

Mo.B. performed the experiments, analysis and interpretation of data, and wrote the manuscript. Ma.B. performed all analyses regarding ddPCR. S.M., E.S., M.R., R.O. and B.B. participated in the interlaboratory evaluation of the reference standard. I.S. revised the manuscript. V.B. performed the statistical analysis. P.K. designed the study, was involved in data interpretation and revised the manuscript. All authors approved the submitted version.

Competing interests

The authors declare no competing interests.

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Mycobacterium avium subsp. *paratuberculosis* viability determination using *F57* quantitative PCR in combination with propidium monoazide treatment

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (*MAP*) is known to be a very slow-growing organism. The fact that cells typically need several weeks to form visible colonies severely compromises the suitability of plate counting for assessment of viable cell counts. This problem might be overcome by the application of fast molecular methods containing a viability component. We have evaluated a promising technology combining sample treatment with propidium monoazide (PMA) prior to DNA extraction for selective detection of cells with intact cell membranes with detection of sequence element *F57* by quantitative PCR (*F57* qPCR). Element *F57* is unique for *MAP* and is not known to exist in any other bacterial species. Conditions of PMA treatment were optimised for *MAP* isolate 7082 using live and heat-killed cells and comparing different DNA extraction procedures. The subsequent successful application of the optimised protocol to four other *MAP* isolates of different origins suggested that the optimised protocol might be broadly applicable to different *MAP* strains. Furthermore, different equations were compared to use the data resulting from this technology to optimally predict the percentage of live *MAP* cells in mixtures containing both live and dead cells. The presented protocol holds promise to be used routinely for detecting MAP with intact cell membranes in research applications.

1. Introduction

Mycobacterium avium subspecies *paratuberculosis* (*MAP*) causes chronic intestinal inflammation in animals eventually leading to systemic infection referred to as paratuberculosis or Johne's disease. Paratuberculosis primarily affects domestic and wild ruminants and is clinically manifested by intermittent diarrhoea, weight loss, decreased milk production, and ultimately death of the infected animal (Ayele et al., 2001; Chacon et al., 2004). For humans, there is strong evidence that *MAP* might be linked with the development of Crohn's disease (Behr and Kapur, 2008; Uzoigwe et al., 2007).

Direct detection of viable *MAP* is conducted by culture on solid and liquid media which is considered to be the "gold standard" (Slana et al., 2008b). However, *MAP* cultivation requires long incubation periods (at least 12 weeks). For certain *MAP* strains isolated from sheep, atypical hosts or Crohn's disease patients, cultivation times of more than 9 months have been reported (Pavlik et al., 1999). Detection by cultivation is further complicated by the facts that the sensitivity of detection can be compromised by decontamination steps often performed before plating (Chiodini et al., 1984) and that strains have been described that could not be successfully grown *in vitro* (Pavlik et al., 1999). Counting itself is challenged by the tendency of *MAP* cells to form clumps which can result in underestimation of cell numbers (Pickup et al., 2005).

Molecular techniques based on end-point PCR and quantitative real time PCR (qPCR), on the other hand, have the ability to detect and quantify *MAP* within one day with great sensitivity (Slana et al., 2008a,b). The main disadvantage of PCR-based methods, however, is their inability to discriminate between live and dead cells. This problem nullifies the advantage of speed and makes the application of these methods pointless in studies that assess *MAP* survival under different conditions of interest (Grant et al., 1996).

Two molecular approaches have been described that can add a viability component to PCR. The first is based on the amplification of RNA. Detection of unstable mRNA species was suggested to be a good choice for the determination of bacterial viability (Birmingham et al., 2008). While this is true, work with RNA is nevertheless cumbersome due to the very same instability that is the basis for viability determination. Great care has to be taken to avoid contamination with RNases (resulting in false negative results) and with DNA (resulting in false positive results). Furthermore, degradation of RNA depends on environmental conditions, the cause of cell death, the nature and stability of the particular RNA transcript, and the region that is targeted for amplification (Birch et al., 2001). Most importantly, however, isolation of mRNA from complex environmental samples containing only a few target cells in a background of many

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non-target species still poses a substantial technical challenge in terms of detection sensitivity.

Alternatively, samples can be subjected to treatment with DNA-modifying dyes prior to molecular analysis. Two different dyes have been described, which selectively enter cells with compromised membranes, but not cells with intact cell membranes. Once inside the cell, the corresponding dye intercalates into DNA. Exposure of samples to bright light results in photoactivation of the dye, which in turn leads to irreversible modification of the DNA and strongly interferes with its subsequent PCR amplification. Excess dye, which has not penetrated membrane-compromised cells, is at the same time inactivated by the light exposure making sure that DNA from previously intact cells does not become modified during the DNA extraction procedure. Comparing the qPCR data obtained from dye-treated and non-dye-treated sample portions can in turn provide information about the ratio of dead and live cells encountered in this sample.

The first fluorescent dye described for determining the viability of bacterial pathogens was ethidium monoazide (EMA; Nogva et al., 2003). Whereas EMA efficiently suppresses the PCR amplification of DNA from membrane-compromised cells, a problem was found in that that EMA can also penetrate the membranes of live cells (Cawthorn and Witthuhn, 2008; Flekna et al., 2007; Kobayashi et al., 2009; Lee and Levin, 2009; Nocker et al., 2006; Pan and Breidt, 2007; Rueckert et al., 2005). The extent of dye entry into intact cells is species-dependent. Propidium monoazide (PMA), on the other hand, seems to be highly selective for membrane-damaged cells, no entry into live cells has been described so far. The higher selectivity of PMA for dead cells can most probably be explained by the higher positive charge compared with that of EMA (Nocker and Camper, 2009).

Since its first description, PMA has been successfully applied to a large spectrum of bacterial species including important foodborne pathogens like *Listeria monocytogenes* (Nocker et al., 2006; Pan and Breidt, 2007; Pan et al., 2009), *Staphylococcus aureus* and *Staphylococcus epidermidis* (Kobayashi et al., 2009; Nocker et al., 2006), *Enterobacter sakazakii* (Cawthorn and Witthuhn, 2008), *E. coli* O157:H7 (Nocker et al., 2006), and others. In addition to vegetative bacterial cells, PMA has been applied to detect live *Bacillus subtilis* spores (Rawsthorne et al., 2009) and fungal species (Vesper et al., 2008). PMA treatment was further found to be applicable for studies of survival of faecal bacteria in wastewater (Bae and Wuertz, 2009a,b; Varma et al., 2009), for monitoring the effect of disinfectants on various bacterial pathogens (Nocker et al., 2007; Rieder et al., 2008) and *Nitrosomonas europaea* (Wahman et al., 2009), and for determining the viability of probiotic bacteria both in culture (Kramer et al., 2009) and in milk products (Garcia-Cayuela et al., 2009). In addition to its application in combination with qPCR, PMA can confer viability information to microarray results in the case that PCR amplicons are used for hybridization. (Nocker et al., 2009).

The principal proof that PMA can be applied to mycobacteria was provided in 2006 using the example of *Mycobacterium avium* complex (Nocker et al., 2006). A follow-up study validated the suitability of PMA to monitor the effect of different disinfection procedures on different bacterial species including *M. avium*, which was subjected to heat (Nocker et al., 2007). It was shown that temperatures of up to 60 °C (for 15 min each) did not result in any substantial PMA-induced signal reduction. Increasing the temperature from 60 °C to 70 °C, however, resulted in a significant difference of around 6 cycles between crossing points (CP) values of PMA-treated and non-PMA-treated culture portions. This difference corresponds to approx. two orders of magnitude in cell numbers (Nocker et al., 2007). Culturability was completely lost at 70 °C.

The aims of this study were (i) to assess the suitability of PMA treatment for *MAP*, (ii) to identify a suitable DNA preparation procedure for the purification of *MAP* genomic DNA from PMA-treated cultures, (iii) to optimise the PMA-*F57* qPCR method for the application to *MAP* as an alternative to viability determination by cultivation, and (iv) to validate the approach using defined mixtures of different ratios of live and dead cells.

2. Materials and methods

2.1. MAP isolates and culture conditions

Experiments for optimising PMA treatment conditions and for studying the effect of different DNA extraction procedures were performed with *MAP* isolate 7082. Optimised PMA treatment conditions were applied to an additional four *MAP* isolates listed in Table 1. All isolates were cultured on Herrold's egg yolk medium (HEYM) containing Mycobactin J (Allied Monitor, Fayette, MO, USA) and incubated at 37 °C for 12 to 15 weeks. A single colony from each isolate was inoculated into liquid Middlebrook 7H9 broth (Difco, Livonia, MI, USA), supplemented with Middlebrook AODC enrichment (Difco) and Mycobactin J (Allied Monitor) and cultured for a maximum of 5 weeks to avoid excessive cell clumping.

2.2. Killing conditions

MAP cell portions with volumes of 500 µl were exposed to 80 °C for 15 min using a standard laboratory heat block. This temperature was sufficient to kill cells, but cells appeared intact under the microscope after the Ziehl–Neelsen staining procedure. Killing efficiency was checked by seeding heat-treated *MAP* cells on HEYM with Mycobactin J and incubating at 37 °C for 12 to 15 weeks.

2.3. Optimisation of PMA concentrations and treatment conditions

The basic procedure of PMA treatment was adopted from a previous study by Nocker et al. (2006). One milligram of PMA (Biotium Inc., Hayward, CA, USA) was dissolved in 1.96 ml of 20% DMSO to obtain a stock solution of 1 mM. This solution was aliquoted into light-impermeable tubes and stored at −20 °C. All subsequent steps employing active PMA were performed under minimal light conditions. PMA was added to 500 µl portions of heat-killed and live *MAP* cells or mixtures thereof in M7H9 broth and kept in light-transparent 2 ml screw cap tubes to reach final dye concentrations of 5, 10, 25, 50 and 100 µM. As controls, identical volumes of 20% DMSO (without PMA) were added to portions of heat-killed and live cells. PMA-treated and non-treated cells were incubated for 5 min in the dark on a vortex with mild continuous shaking (20 Hz) followed by brief spinning. Light exposure tubes were placed on ice in a diagonal position to allow maximal light penetration. Light exposure was performed for 2 min using a halogen lamp with a 650 W bulb (B & H PhotoVideo, New York, NY, USA) placed at a distance of approx. 20 cm from the tubes. Cells were subsequently harvested by centrifugation at 8000×*g* for 5 min. The supernatant was discarded and the cell pellet was subjected to DNA isolation.

In order to maximise the difference in CP values between live and dead *MAP* cells, prolonged PMA incubation times (20 and 50 min) and

Table 1
Mycobacterium avium subsp. *paratuberculosis* isolates used in this study.

Isolate	Source
MAP 7082	White-tail deer, intestinal tissue, CZ[a]
CAPM 6381	Collection of Animal Pathogenic Microorganisms[b]
MAP 8819	Cow, faeces, CZ[a]
MAP 8672	Cow, faeces, CZ[a]
MAP 12146	Cow, faeces, CZ[a]

[a] Czech Republic.
[b] Veterinary Research Institute, Brno, Czech Republic.

repeated PMA treatment steps were applied as described previously for other organisms (Pan and Breidt, 2007; Rawsthorne et al., 2009; Rogers et al., 2008). Based on the findings from PMA optimisation experiments, a final PMA concentration of 25 µM was chosen for subsequent experiments.

2.4. DNA isolation and crude lysate preparation

To determine the effects of different DNA extraction procedures on DNA recovery and removal of excess PMA, two commercial DNA isolation kits DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and the QuickGene DNA Tissue Kit (Fujifilm, Tokyo, Japan) were used. The latter was used in combination with the QuickGene Mini80 instrument. For both kits the respective protocols for gram-positive bacteria (provided by the manufacturer) were followed. The kits were compared with a method to prepare crude lysates, which consisted of resuspending pellets of PMA-treated and untreated cells in 200 µl of sterile Tris–EDTA (TE) buffer (Amresco, Solon, OH, USA), followed by cell lysis at 100 °C for 20 min. Tubes were subsequently centrifuged at 18,000×*g* for 5 min to remove cell debris. The supernatant served as the template for PMA-*F57* qPCR amplification. DNA isolation and crude lysate preparation were performed in triplicate.

2.5. F57 qPCR and analysis of data

For the quantitative determination of genome copies, the previously developed competitive duplex *F57* qPCR with internal amplification control was used (Slana et al., 2008a). All *F57* qPCR reactions were carried out on the LightCycler 480 (Roche Molecular Diagnostic, Mannheim, Germany). Determination of CP values and absolute cell numbers was performed using the "Fit point analysis" option of the LightCycler 480 software (version 1.2.0.0625).

Absolute numbers of *MAP* cells were calculated based on a calibration curve for *F57* targets. Additionally we compared differences in raw CP values of heat-killed and live *MAP* cells treated with PMA and their respective untreated controls according to the following formulas: (1) $\Delta CP_{\text{dead with PMA–live with PMA}}$, (2) $\Delta CP_{\text{dead with PMA–dead without PMA}}$, and (3) $\Delta CP_{\text{live with PMA–live without PMA}}$. All experiments were performed in triplicate to obtain mean values and standard deviations.

2.6. Validation of optimised PMA treatment with different MAP isolates

Optimised PMA treatment conditions were applied to five different *MAP* isolates to determine potential strain-specific differences. The optimised procedure comprised two consecutive PMA treatments (with 25 µM PMA each), incubation in the dark for 5 min on a vortex (20 Hz), and light exposure for 2 min. DNA was isolated using the QuickGene DNA Tissue Kit (Fujifilm). *F57* qPCR and data analysis were carried out as described above.

2.7. Mixture of heat-killed and live MAP cells and calculation of viable MAP cells percentage

Suspensions of heat-killed and live *MAP* cells of isolate 7082 in M7H9 broth were mixed in defined ratios of 100:0, 99:1, 90:10, 75:25, 50:50, 25:75, 10:90, 1:99 and 0:100 to give final volumes of 500 µl each. Mixtures were subjected to PMA treatment following the previously optimised protocol. These mixtures were compared to samples with identical cell numbers containing only non-heat exposed cells. To determine the percentage of viable cells in the mixtures we used two basic approaches. The first approach was based on the absolute numbers of *MAP* cells obtained from a *F57* qPCR standard curve. The standard curve was used to determine cell numbers for mixtures subjected to PMA treatment (cells in mixtures + PMA) and for live cells subjected to PMA treatment (Live cells + PMA) or not (Live cells–PMA). The percentage of live *MAP* cells in mixtures was calculated according to the equation:

$$\text{Live } MAP\,(\%) = \text{Cells in mixture}_{\text{PMA}} / \text{Live cells}_{+\text{PMA}} \times 100 \quad (1)$$

or according to a similar equation employing non PMA-treated live cells (Live cells–PMA) instead of 'Live cells + PMA':

$$\text{Live } MAP\,(\%) = \text{Cells in mixture}_{\text{PMA}} / \text{Live cells}_{-\text{PMA}} \times 100 \quad (2)$$

The second equation was used to demonstrate differences between Eqs. (1) and (2) caused by the impact of PMA on non-heat-treated portions.

Another approach for the calculation of the percentage of live cells in mixtures of live and dead cells was based on the raw CP values (instead of using a standard curve to derive absolute cell numbers). CP values served for calculating absolute genome copy numbers using the theoretical equation describing PCR amplification. The basic equation was modified as follows:

$$\text{Live } MAP\,(\%) = 2^{-[CP(\text{Mixture}+\text{PMA})-CP(\text{Live } cells+\text{PMA})]} \times 100 \quad (3)$$

where "2" corresponds to the optimal qPCR amplification efficiency. As before, CP (Live cells + PMA) can be replaced by CP (Live cells–PMA).

$$\text{Live } MAP\,(\%) = 2^{-[CP(\text{Mixture}+\text{PMA})-CP(\text{Live cells}-\text{PMA})]} \times 100 \quad (4)$$

2.8. Statistical analysis

The significance of differences between CP values (ΔCP) and of the percentage of viable *MAP* cells in mixtures of dead and live cells were calculated by an unpaired Student's *t*-test. *P* values lower than 0.05 were considered to be statistically significant.

3. Results

3.1. Optimisation of PMA concentration

In a first step, live and dead cells were exposed to different PMA concentrations (Fig. 1). The effect of PMA on heat-killed cells was shown to increase with higher PMA concentrations, the greatest difference in CP values between PMA-treated and untreated dead cells ($\Delta CP_{\text{dead with PMA–dead without PMA}}$) was observed with a PMA concentration of 100 µM (Fig. 1A). Higher PMA concentrations, however, also resulted in higher CP values for live cells compared with untreated live controls. Whereas PMA concentrations of 25 µM and lower had a very moderate effect on the $\Delta CP_{\text{live with PMA–live without PMA}}$ (less than 1 cycle), concentrations of 50 µM and higher resulted in a substantial difference (almost 2 cycles with 50 µM and 3 cycles with 100 µM). This effect on live cells was an important feature to be considered in the analysis of all subsequent optimisation steps.

Combining the two trends for dead and live cells, subtraction of CP values of PMA-treated live cells from the ones of PMA-treated dead cells ($\Delta CP_{\text{dead with PMA–live with PMA}}$) showed an increasing difference between live and dead cell signals for PMA concentrations up to 25 µM. No further improvement was achieved with PMA concentrations of 50 µM and 100 µM. These trends were also visible when plotting absolute numbers of *MAP* cells (as determined with a *F57* qPCR standard curve) for different PMA concentrations (Fig. 1B). For live cells, treatment with 100 µM PMA resulted in a difference of almost one order of magnitude (Fig. 1B). Due to the fact that the $\Delta CP_{\text{dead with PMA–live with PMA}}$ was not significantly different for PMA concentrations of 25 µM, 50 µM and 100 µM, a dye concentration of 25 µM was chosen for subsequent experiments.

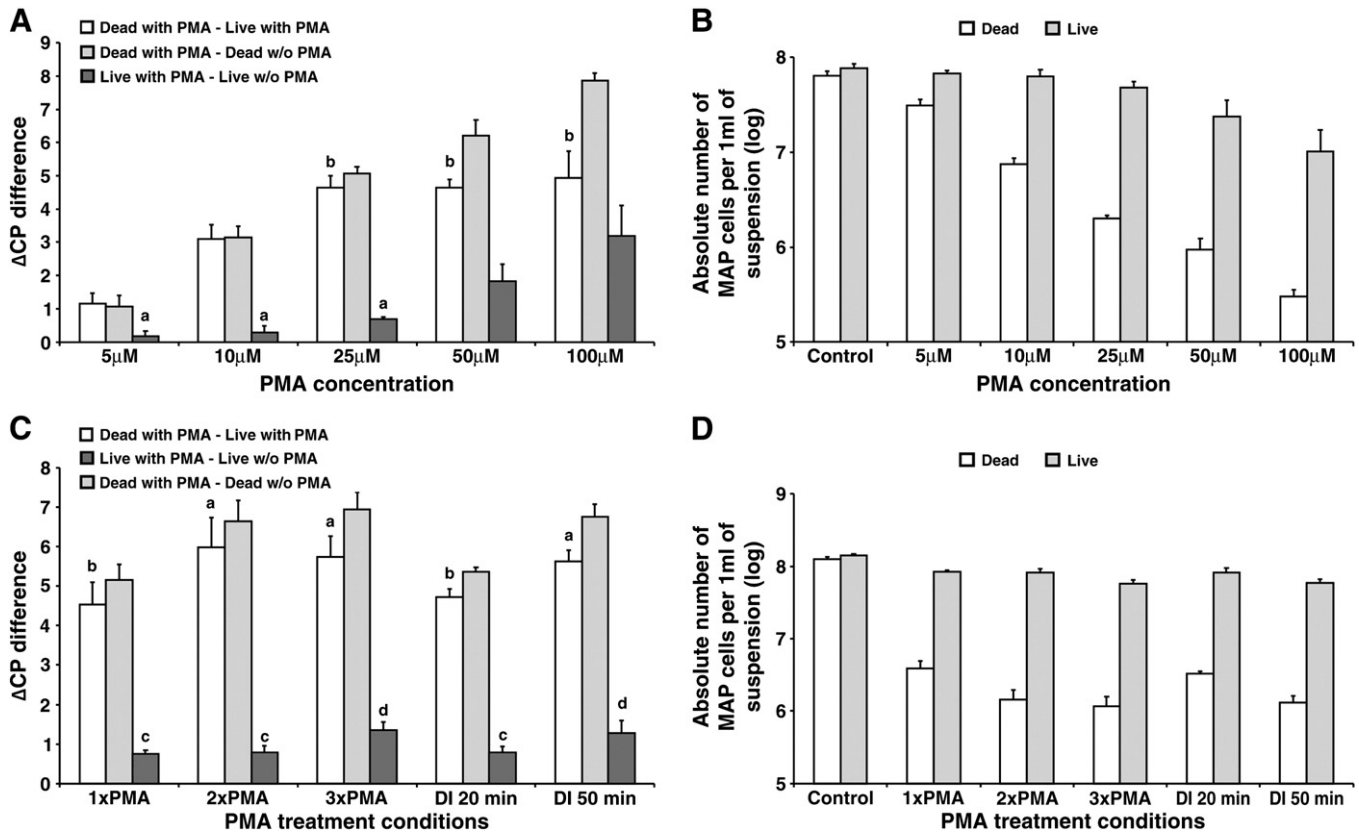

Fig. 1. Effect of different PMA concentrations (A and B), multiple PMA treatments, and different PMA exposure times (C and D) on CP values obtained from live and heat-killed *MAP* cells. Results are presented as ΔCP differences (A and C) or as absolute cell numbers calculated from *F57* qPCR results with the help of a standard curve (B and D). DI stands for dye incubation time. Statistically insignificant differences ($P>0.05$) are indicated by subscript letters, different letters indicate statistically significant differences ($P<0.05$). Error bars represent standard deviations obtained from three independent physical replicates (DNA isolations).

3.2. Optimisation of PMA treatment

In a next step we addressed the effect of multiple PMA treatments and longer dye exposure times (Figs. 1C and D). When treating culture portions with 25 μM PMA once, twice, or three times (with intermediate harvesting of cells by centrifugation), double treatment resulted in a $\Delta CP_{\text{dead with PMA–live with PMA}}$ that was approx. 1.5 cycles higher compared to a single PMA treatment. A third PMA exposure further increased the CP values obtained from dead cells, but also equally affected the CP value obtained from live cells and therefore did not add further value compared to the double PMA treatment.

In comparison to a 5 min dye incubation (DI) time, a prolonged incubation of 20 min resulted in no significant increase in $\Delta CP_{\text{dead with PMA–live with PMA}}$ compared to a single PMA treatment, whereas a PMA exposure time of 50 min had a more pronounced effect. Like a triple PMA treatment, however, the 50 min PMA exposure resulted not only in an increase in CP values for dead cells, but also for live cells. This effect minimised the $\Delta CP_{\text{dead with PMA–live with PMA}}$ thus the 50 min DI was not found to be beneficial.

Comparing all PMA treatment conditions, the greatest CP difference between PMA-treated dead and live cells ($\Delta CP_{\text{dead with PMA–live with PMA}}$) was obtained with a double PMA treatment with 25 μM PMA for 5 min. This condition was considered optimal and applied in the following experiments.

3.3. Effect of different genomic DNA isolation procedures on PMA-F57 qPCR results

Different DNA isolation procedures (crude lysate preparation, Fujifilm QuickGene DNA Tissue Kit, QIAGEN DNeasy Blood & Tissue Kit) were compared in respect to their effects on PMA-*F57* qPCR. Whereas the commercial kits include a DNA purification step, this is not the case for the crude lysate preparation. No statistically significant differences in ΔCP values were obtained for live and dead cells (Fig. 2A). The same held true for absolute numbers of *MAP* cells calculated with the help of a standard curve (Fig. 2B). Comparing calculated live and dead cell numbers, the overall $\Delta CP_{\text{dead with PMA–live with PMA}}$ difference was around two orders of magnitude independent of the DNA isolation procedure. Considering the similarity of results obtained with different DNA isolation procedures, the crude lysate preparation was considered a good alternative for DNA isolation kits. Advantages of the crude lysate preparation are the speed and cost saving compared to the commercial kits. Nevertheless, the QuickGene DNA Tissue Kit (Fujifilm) protocol for the gram-positive bacteria was applied as default method as it allows partial automation using the QuickGene-Mini80 instrument.

3.4. Comparison of different MAP isolates treated with optimised PMA procedure

MAP isolate 7082 was compared with an additional four isolates to address potential strain-specific differences in combination with PMA-*F57* qPCR. All isolates were subjected to the optimised PMA treatment conditions described earlier followed by subsequent DNA isolation using the Fujifilm kit (Figs. 3A and B). No statistically significant differences between isolates were observed for $\Delta CP_{\text{dead with PMA–live with PMA}}$. Absolute differences $\Delta CP_{\text{dead with PMA–live with PMA}}$ were on average around 2 orders of magnitude. Parameter $\Delta CP_{\text{live with PMA–live without PMA}}$ was also not statistically different and did not exceed a difference of one cycle. We concluded that *MAP* isolates of different origin behave similarly when treated with PMA and that optimised PMA treatment conditions can be applied to different *MAP* isolates.

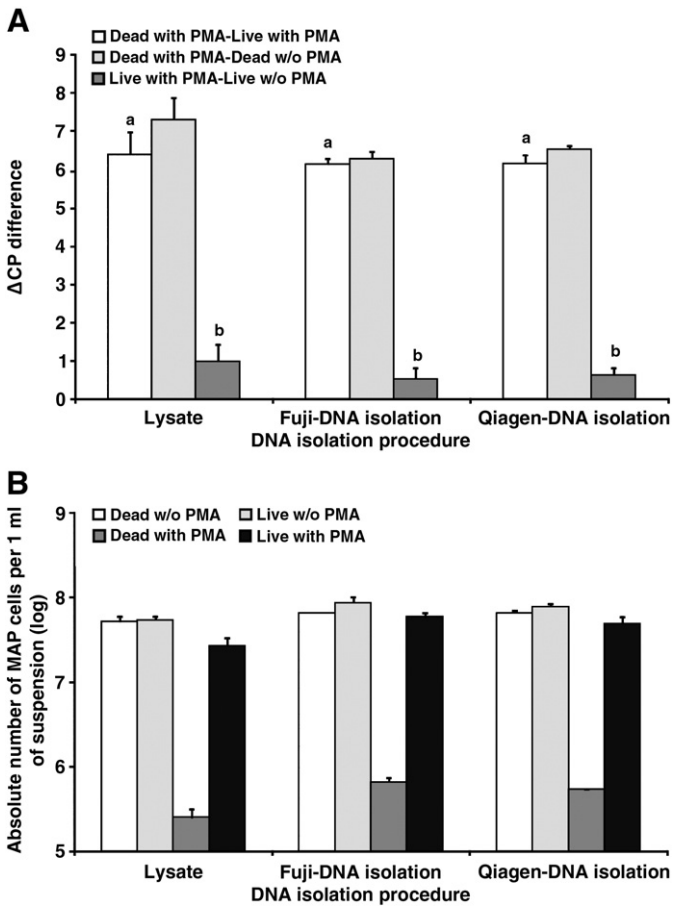

Fig. 2. Effects of different DNA isolation procedures on PMA-*F57* qPCR. Results are presented as ΔCP differences (A) or as absolute cell numbers calculated from *F57* qPCR results with the help of a standard curve (B). Statistically insignificant differences ($P>0.05$) are indicated by subscript letters, different letters indicate statistically significant differences ($P<0.05$). Error bars represent standard deviations obtained from three independent physical replicates (DNA isolations).

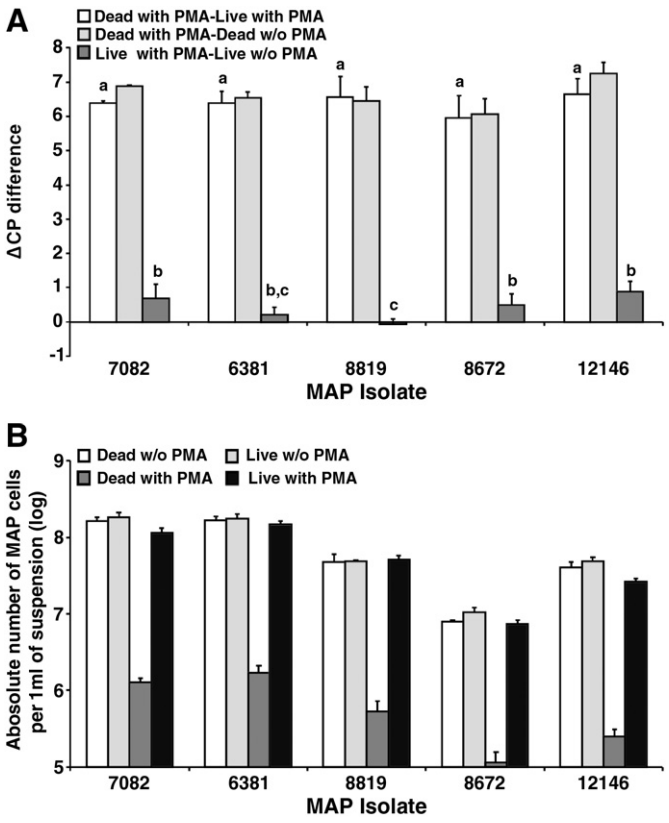

Fig. 3. Comparison of PMA treatment on live and dead cells of five selected *MAP* isolates based on differences in raw CP values (A) and based on absolute cell numbers as determined by *F57* qPCR with the help of a standard curve (B). Statistically insignificant differences ($P>0.05$) are indicated by subscript letters, different letters indicate statistically significant differences ($P<0.05$). Error bars represent standard deviations obtained from three independent physical replicates (DNA isolations).

3.5. Defined mixtures of dead and live MAP cells and calculation of percentages of viable MAP cells

Different ratios of dead and live *MAP* cells were subjected to the optimised PMA treatment protocol. As shown in Fig. 4A, high dead–live ratios resulted in a maximal difference in CP values of approx. 6 cycles between heat-treated and live portions with PMA, whereas increasing percentages of non-heated cells resulted in an increasing similarity of CP values obtained from these portions.

In a next step we addressed the possibility to optimise the calculation of the percentage of viable *MAP* cells in these mixtures. Using Eqs. (1) and (3), which consider the PMA effect on non-heat-treated portions, calculated dead–live ratios reflected the known ratios of the corresponding mixtures very well, especially in the range from 90:10 to 25:75 (Fig. 4B). Eqs. (2) and (4), on the other hand, lead to the underestimation of the percentage of viable MAP cells as they do not consider the effect of PMA treatment.

4. Discussion

This study addressed the optimisation of PMA-*F57* qPCR for preferential detection of *MAP* cells with intact cell membranes. The application of this cultivation-independent diagnostic method with the ability to provide viability information gains special relevance for bacterial species like *MAP* where the application of cultivation as a gold standard for viability assessment is greatly compromised by very long growth times.

The study presented here showed that PMA-*F57* qPCR can be used to determine the viability of *MAP* and confirmed the usefulness of the technique for mycobacterial species as demonstrated earlier for *M. avium* complex (Nocker et al., 2007). Results can be obtained within one day. However, results for *M. avium* complex were not as optimal as for other bacterial species used. PMA-induced signal reductions between live and heat-killed cells were only around 7 cycles and therefore significantly lower than signal reductions observed for other bacterial species using the identical PMA treatment conditions (Nocker et al., 2007). Optimisation of *MAP* could be expected to be even more important than for *M. avium* complex due to the more fastidious character of this species.

PMA treatment of *MAP* isolate 7082 revealed that increasing dye concentrations resulted not only in an increasing signal reduction with heat-killed cells, but also in a signal reduction with non-heat-treated cells, although to a lesser extent. Similar findings, where PMA treatment resulted in higher threshold cycles for live cells, have been reported for fungi (Vesper et al., 2008) and for *S. aureus* and *S. epidermidis* (Kobayashi et al., 2009). In the first case, the authors concluded that fungal suspensions already contained a certain percentage of dead cells, in the second case authors did not discuss this finding. The observed signal reductions with untreated cultures, expressed in our study as $\Delta CP_{\text{live with PMA–live without PMA}}$, could either be due to the presence of membrane-compromised cells in these culture portions, the carryover of PMA into qPCR reactions (PMA at higher concentrations was found to inhibit PCR amplification; Nocker et al., 2006), and the possibility that PMA might also enter live cells to a certain extent, or a combination thereof. Whereas the presence of a certain proportion of membrane-compromised cells in untreated cultures seems probable (especially considering the growth of *MAP* over several

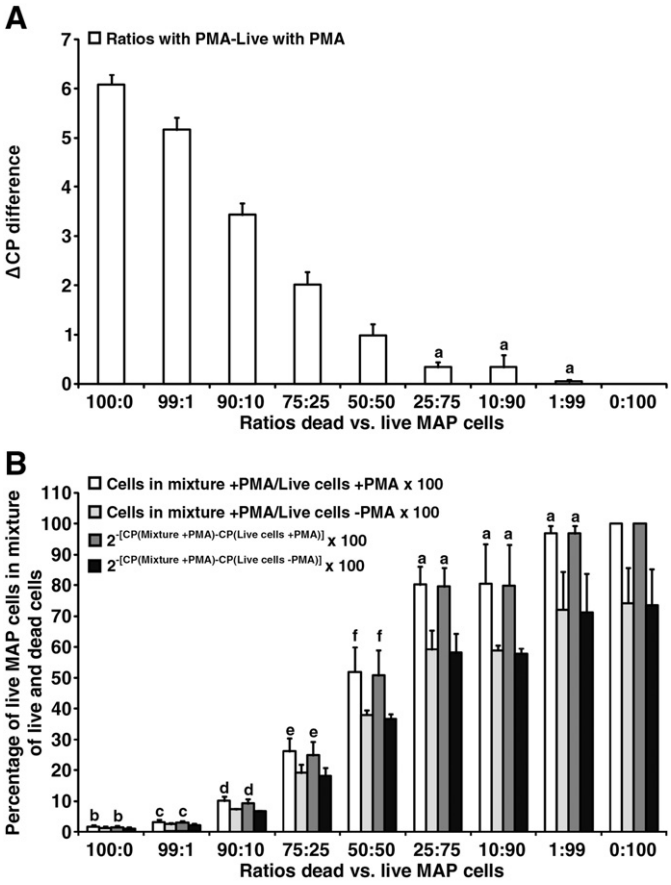

Fig. 4. Differences in raw CP values (ΔCP) for defined mixtures of live and dead *MAP* cells (A) and the effect of different calculation procedures for determining percentages of live cells from these mixtures. Statistically insignificant differences ($P>0.05$) are indicated by subscript letters, different letters indicate statistically significant differences ($P<0.05$). Error bars represent standard deviations obtained from three independent physical replicates (DNA isolations).

weeks), the steady increase in $\Delta CP_{\text{live with PMA–live without PMA}}$ with increasing PMA concentrations up to 100 μM indicates an important role of the other two factors.

The further improved resolution for live–dead distinction by consecutive PMA treatments is in concordance with previously published data where double PMA treatment of heat-killed *Listeria* increased the average CP by 2.8 cycles compared to identical portions treated only once with PMA (Pan and Breidt, 2007). In our study, it should be noted that a double PMA treatment with 25 μM of PMA did not lead to higher CP values of live cells, whereas a significant signal reduction (meaning increase in CP values) of approx. 1.5 cycles was obtained with heat-killed cells. This fact made a double PMA treatment with 25 μM PMA appear more beneficial than a single PMA treatment with 50 μM PMA as the latter resulted in an equally great increase in CP values for dead and live cells. The same phenomenon was observed with a triple PMA treatment, which was therefore not found to be beneficial. We conclude that *MAP* is relatively sensitive to PMA, especially when exposed to higher PMA concentrations. Two consecutive treatments with lower PMA concentrations were found to be more beneficial than applying a higher PMA dose. Prolonged PMA incubation, on the other hand, as applied in studies with bacterial species found in cystic fibrosis sputum samples (Rogers et al., 2008), *B. subtilis* spores (Rawsthorne et al., 2009) or clinical wastewater samples (Rieder et al., 2008) resulted in increased signal reduction of dead cells in our study when choosing a 50 min incubation period, whereas a 20 min incubation period was directly comparable with the default 5 min incubation. As the 50 min PMA exposure, however, also had a negative impact on the signal from untreated live *MAP*, we continued with the 5 min incubation time.

Apart from the susceptibility of live cells to higher concentrations of PMA, however, one also has to consider that *MAP* cells tend to clump when cultured in broth *in vitro*. Although this empirical observation is known, no detailed information is available about this phenomenon. As clumps tend to form primarily in later stages of liquid cultures, the cultivation time in this study was limited to 5 weeks, which does not mean, however, that cultures were free of clumps. On the other hand, it can be speculated that clumps are likely to contain dead cells in the centre. This in turn would explain a certain proportion of dead cells in cultures which overall can be considered to be in log-phase. Clumping phenomena might also have affected results in other studies where differences in absolute numbers of live (untreated) cells were observed depending on whether samples were PMA-treated or not (Kobayashi et al., 2009; Varma et al., 2009; Vesper et al., 2008). Different extents of clumping might also have contributed to differences when applying optimised PMA treatment conditions on five different *MAP* isolates of different origins. The mean $\Delta CP_{\text{live with PMA–live without PMA}}$ ranged between slightly negative (isolate 8819) and nearly 1 cycle (isolate 12146). Further studies addressing the extent of dead cells in broth cultures will be necessary to gain more insight into PMA-induced differences in CP values.

The role of carryover PMA and potential inhibition of amplification by residual dye in qPCR reactions as a third possible explanation for obtaining higher CP values for PMA-treated live cells has not become clear. Such an explanation was most likely for qPCR reactions that used portions of crude lysates as a template for amplification. We assumed that the DNA purification step should efficiently remove PMA and therefore there should be no effect of PMA on the increase of CP values from PMA-treated live cells. However, no significant differences between the different extraction procedures were observed.

Considering the susceptibility of non-heated cells to PMA treatment, different equations were compared for their abilities to optimally express the known dead–live ratios in mixtures of dead and live cells. Equations were fed either with absolute cell numbers (determined by PMA-*F57* qPCR using a standard curve) or with raw CP values from PMA-*F57* qPCR. Good correlations between theoretical and experimental values were obtained with Eqs. (1) and (3) (Fig. 4B). These equations take into consideration the susceptibility of non-heat-treated cells to PMA (caused either by the presence of dead cells or by factors discussed above). The correlations were suboptimal, on the other hand, when taking Eqs. (2) and (4), which do not take into account this susceptibility. The deviations between theoretical and experimental values were more pronounced with higher proportions of live cells in the mixtures.

It should be noted that calculations of percentages of viable *MAP* cells from raw CP data using Eq. (3) yielded similar results (without statistically significant difference) compared to calculations from absolute *MAP* numbers using Eq. (1). Very modest differences were caused mainly by different experimental PMA-*F57* qPCR amplification efficiencies (which in Eq. (3) was set to "2" which reflects the optimal PCR efficiency). If the experimental qPCR amplification efficiency differs from the optimum, the calculation of the percentage of live cells is negatively affected. Eq. (3) should be very useful, however, when absolute cell numbers are unknown. It has to be pointed out that CP determination has to be performed with high precision as differences in CP values will be amplified when calculating percentage values in mixtures due to the exponential nature of PCR amplification.

In summary, we present evidence that sample treatment with PMA in combination with *F57* qPCR can be useful for assessing the number of *MAP* cells with intact cell membranes. Optimal PMA treatment conditions consisted of a double exposure of cells to 25 μM of dye for 5 min each followed by 2 min light exposure. This procedure in combination with *F57* qPCR quantification represents a

fast, reliable, and relatively cheap alternative to the conventional culture on solid or liquid media. It has to be acknowledged, however, that in contrast to other bacterial species, where PMA treatment of membrane-compromised cells resulted in a signal decrease of up to 4 log units, the overall difference for *MAP* was only about 2 log units. A further development of the PMA technology will be necessary to overcome this difficulty. Nevertheless, the exclusion of 99% of dead *MAP* cells already represents a significant advantage over regular qPCR and raises hopes that this fast technology can be applied on a broader basis, which in turn could significantly increase our knowledge about *MAP* survival in food matrices and provides new insights on the pathogenesis of paratuberculosis or Crohn's disease. Due to the fact that PMA treatment was already applied directly to bacteria in different matrices like wastewater, biofilms, or foods like milk or fish fillets (Lee and Levin, 2009; Pan et al., 2009; Rieder et al., 2008; Varma et al., 2009; Vesper et al., 2008), it can be expected that *MAP* viability will be soon determined directly in such matrices.

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Effect of short- and long-term antibiotic exposure on the viability of *Mycobacterium avium* subsp. *paratuberculosis* as measured by propidium monoazide F57 real time quantitative PCR and culture

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP), the causative agent of paratuberculosis in ruminants, has a lipid-rich cell wall which facilitates its survival and persistence in the environment. This property of the organism is exploited when it is cultured as decontaminating agents and antibiotics are used to suppress the growth of contaminating microflora, but such treatments can also negatively affect the isolation of MAP itself. The objective of this study was to assess the effect of the 'VAN' antibiotics (vancomycin, amphotericin B and nalidixic acid) on the viability of MAP using a propidium monoazide real time quantitative PCR (PMA qPCR) and culture.

Long-term (5 week) treatment with VAN antibiotics resulted in a larger decrease in bacterial numbers compared to short-term (3 day) exposure. The PMA qPCR assay indicated that 50 μg/mL of vancomycin, 50 μg/mL of nalidixic acid, and 200 μg/mL of amphotericin B were 'threshold' concentrations, respectively, above which the decline in the viability of MAP was statistically significant. Using culture, these threshold concentrations were 100 μg/mL of vancomycin, 50–100 μg/mL of nalidixic acid, and 100 μg/mL of amphotericin B, respectively. Given that the two methods were found to be comparable, the PMA qPCR is a potentially more convenient and effective alternative to culture in detecting MAP.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the aetiological cause of paratuberculosis (Johne's disease) in ruminants, where the faeces of infected animals contaminate the environment and provide a ready source of infection. The environmental sustainability of MAP is directly related to the lipid-rich cell wall of the organism (Sung and Collins, 1998; Whan et al., 2001; Beckler et al., 2008), and this property is exploited during their *in vitro* culture when samples are decontaminated using quaternary ammonium compounds such as hexadecylpyridinium chloride (HPC), and are subsequently grown on media containing antibiotics that suppress the growth of faster-growing contaminant microflora.

Different individual and combinations of antibiotics have been tested to determine the concentration with maximal effect on contaminating microorganisms yet with minimal influence on the mycobacteria. Antibiotic combinations include the commercially available PANTA PLUS (Whittington et al., 1998; Gumber and Whittington, 2007), a mixture of vancomycin (VCM), amphotericin B (AMP-B) and nalidixic acid (NAL), referred to as 'VAN' (Reddacliff et al., 2003; Whittington, 2009), or the less frequently used combination of penicillin, chloramphenicol and AMP-B (Jorgensen, 1982). Apart from their inhibitory effect on contaminating bacteria and moulds, these antibiotics can also exert a negative influence on the growth of the mycobacteria, which is dependent on the mycobacterial strain and antibiotic concentration (Gumber and Whittington, 2007).

Most of the studies which have assessed the viability of mycobacteria to antibiotic exposure have used culture media (Gumber and Whittington, 2007; Radomski et al., 2010). However, an alternative, culture-free detection method using the selective dye propidium monoazide (PMA) is now available (Nocker et al., 2006). Various stressors such as heat, antibiotics, and disinfectants, increase the permeability of the cell wall of killed bacteria to PMA. Once inside the bacterium, the PMA covalently binds to the organism's DNA after exposure to bright light and forms an insoluble complex, which is lost in the subsequent DNA isolation procedure and therefore is not amplified during any subsequent real time quantitative PCR (qPCR). As the PMA cannot penetrate the cell wall of viable bacteria, this assay can be used to distinguish live from killed organisms (Nocker et al., 2006).

Nocker et al. (2006, 2007) used this PMA qPCR assay to distinguish viable from killed *M. avium* in monitoring the effectiveness

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of heat and disinfection treatments, and a modification of this method, amplifying the MAP unique F57 sequence, was recently developed by Kralik et al. (2010). The PMA qPCR assay has been employed in distinguishing viable bacteria in human faeces (Fujimoto et al., 2011), fermented milk (Garcia-Cayuela et al., 2009), chicken carcasses (Josefsen et al., 2010), and in waste water (Varma et al., 2009).

The aim of the present study was to assess the effect of VAN antibiotics on the viability of MAP isolates during culture. Different concentrations of this antibiotic combination were added to cultures of MAP which were then cultivated short- (3 days) or long- (5 weeks) term. The experiment was carried out using two field and one laboratory strain of MAP, and bacterial survival was assessed using the modified PMA qPCR method and conventional culture.

Materials and methods

Bacterial isolates used and culture conditions

The MAP field isolates 283/08 and 12146, and a laboratory reference strain CAPM 6381 (The Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Czech Republic) were used. The mycobacteria were cultured in liquid M7H9 Middlebrook media (Difco) with added 10% Middlebrook AODC enrichment (Difco) and Mycobactin J (Allied Monitor) at 37 °C for 5 weeks to obtain cells in logarithmic growth phase (OD_{600}, 0.3–0.4), and to circumvent excessive clumping of the organisms.

Antibiotic treatments

For short-term treatments, appropriate amounts of VCM, AMP-B and NAL (Sigma) were added to 1500 μL of each isolate prepared as indicated above. The mycobacteria were cultured with the antibiotics in a rotator at 37 °C for 3 days and then processed according to the PMA qPCR and culture protocols (Pavlik et al., 2000; Kralik et al., 2010). For the long-term exposure, 300 μL of bacterial cultures in logarithmic phase were added to sterile bottles with appropriate amounts of VAN and 10 mL of M7H9 broth with added AODC and Mycobactin J. Mycobacterial suspensions containing the antibiotic mixtures were cultivated with agitation at 37 °C for 5 weeks. Various combinations of all three antibiotics were added to each short- or long-term sample (Table 1). Concentrations of antibiotics commonly used in culturing mycobacteria (100 μg/mL of VCM, 100 μg/mL of NAL, and 50 μg/mL of AMP-B) were chosen as 'standard' concentrations (Merkal and Richards, 1972; Reddacliff et al., 2003; Whittington, 2009). Two antibiotic combinations with a lower, and two with a higher concentration than the standard were also tested. Control samples with no added antibiotics were also used. Following incubation with VAN, 250 μL of the test suspensions were washed twice with M7H9 broth, reconstituted in 500 μL of M7H9, and used for the PMA treatment.

Table 1
Concentrations of vancomycin (VCM), amphotericin B (AMP-B) and nalidixic acid (NAL) used in the short and long-term exposure of *Mycobacterium avium* subsp. *paratuberculosis* in culture.

Changing concentration of one of the antibiotics	Final concentrations of all antibiotics		
	Vancomycin (μg/mL)	Amphotericin B (μg/mL)	Nalidixic acid (μg/mL)
VCM 25	25	50	100
VCM 50	50	50	100
VCM 100	100	50	100
VCM 200	200	50	100
VCM 400	400	50	100
NAL 25	100	50	25
NAL 50	100	50	50
NAL 100	100	50	100
NAL 200	100	50	200
NAL 400	100	50	400
AMP-B 12.5	100	12.5	100
AMP-B 25	100	25	100
AMP-B 50	100	50	100
AMP-B 100	100	100	100
AMP-B 200	100	200	100
Control	–	–	–

Propidium monoazide treatment

The modified PMA qPCR method used was adopted from the study by Kralik et al. (2010). In brief, 12.5 μL of 1 mM PMA stock solution (Biotium) were added to 500 μL of mycobacterial suspension to a final concentration of 25 μM, and was incubated in the dark for 5 min with mixing at 20 Hz. The test vials were then placed on ice and exposed to intense light from a 650 W halogen bulb (B & H Photo-Video) for 2 min. The entire process of 'dark' incubation–'light' exposure was repeated after the addition of 12.5 μL PMA to a final concentration of 50 μM. The suspension was centrifuged at 7000 *g* for 5 min and the supernatant replaced with 500 μL of Tris–EDTA buffer supplemented with 50 ng/μL of fish sperm DNA (Serva Electrophoresis). After lysis at 100 °C for 20 min the cells were centrifuged (18,000 *g* for 5 min), and used as a template in the qPCR. The PMA treatment on each VAN combination and control was performed in triplicate and analysed in a single qPCR run.

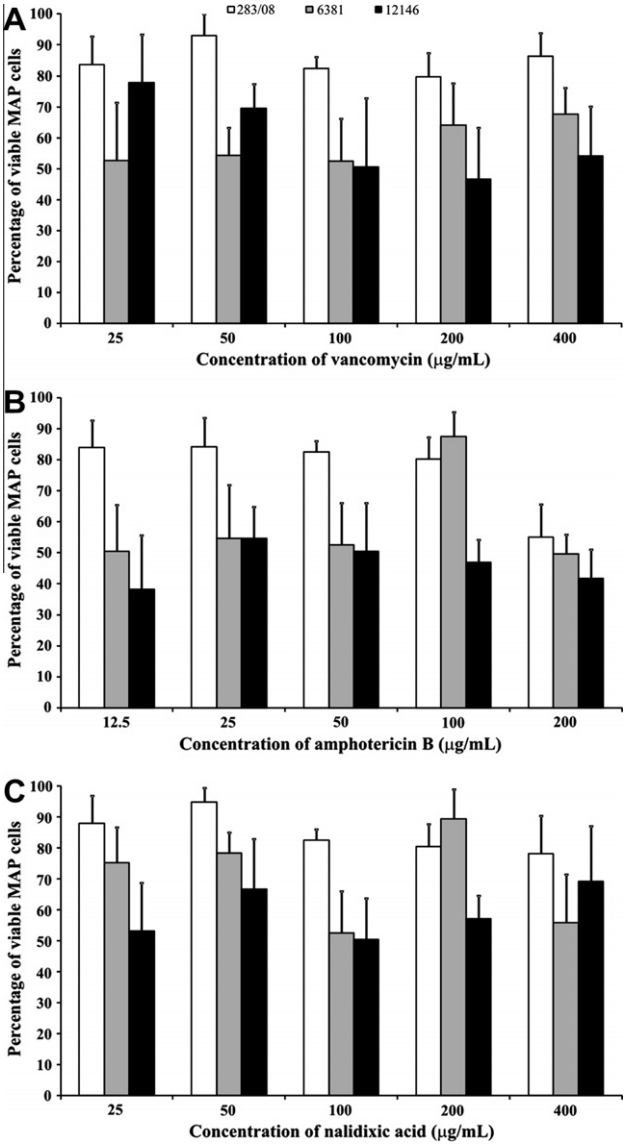

Fig. 1. Short-term treatment of three *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates (283/08, 12146 and 6381) with VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics as measured by propidium monoazide real time PCR: (A) concentration of vancomycin (VCM), 25–400 μg/mL. Concentration of nalidixic acid (NAL) and amphotericin B (AMP-B), 100 and 50 μg/mL, respectively; (B) concentration of VCM and NAL, 100 μg/mL in each case. Concentration of AMP-B, 12.5–200 μg/mL; and (C) concentration of NAL, 25 –400 μg/mL. Concentration of VCM and AMP-B, 100 and 50 μg/mL in each case, respectively.

Determination of mycobacterial viability by qPCR

The F57 qPCR procedure used to detect and quantify the MAP bacteria has been described previously (Slana et al., 2008). Quantification was carried out according to a standard curve derived from plasmid standards. Amplification was performed using a LightCycler 480 instrument (Roche Molecular Diagnostic) and 'fit-point analysis' software (v. 1.2.0.0625) was applied to determine the absolute numbers of bacterial cells present. Organism viability was determined as the quotient of absolute numbers of PMA-exposed, VAN-treated cells and PMA-exposed control (VAN-untreated) cells (Kralik et al., 2010).

Determination of mycobacterial viability by culture on solid media

Herrold's egg yolk medium (HEYM) with added Mycobactin J and without antibiotics was used to culture the mycobacteria. Two hundred microlitres of each 100 and 10,000 dilutions (in liquid M7H9) short- and long-term experimental samples were cultivated on three HEYM at 37 °C for 3 months. The viability of VAN-treated cells was determined as the quotient of CFU (colony forming units) in the treated sample, control, and untreated sample (100%) for each isolate. The culture of each VAN combination and control was performed in triplicate to facilitate direct comparison of the results of culture and the PMA qPCR assay.

Statistical analysis

Sources of statistically significant variability were identified based on two factors: (1) the MAP isolate without regard to changes in antibiotic concentration and (2) the increasing concentration of one antibiotic in concurrence with the other two antibiotics in the VAN combination disregarding differences among specific isolates. Statistical analysis of the variability at the level of isolates and antibiotic concentrations was performed using a two-way ANOVA (Statistica 9, StatSoft Inc.). When only a single parameter (isolate or concentration) had statistically significant variability, a one-way ANOVA (Statistica 9) was used to confirm the result of the previous test. Detailed information about groups with statistically significant variability was determined using Tukey's HSD post hoc test (Statistica 9). P values <0.05 and <0.01 were considered statistically significant and highly significant, respectively.

Results

Viability of MAP isolates following short-term VAN treatment

Neither the MAP isolates, nor the various concentrations of VAN were significant sources of variability when culture was used (data not shown). The MAP isolate was a significant source of variability when the PMA qPCR was used, as manifested by the greater viability (resistance to VAN) of isolate 283/08 compared to 6381 and 12146, respectively (Fig. 1; Table 2). With increasing concentrations of VCM and AMP-B, the number of surviving mycobacteria was significantly lower ($P < 0.01$) for the 12146 and 6381 isolates compared to 283/08 (Table 2). Although a similar trend was observed for different concentrations of NAL, the decreased viability of the 6381 isolate was not statistically significant due to the influence of a 6381 'outlier' (Fig. 1C; Table 2). A combination of the 'standard'/'commonly used' concentrations of VCM and NAL (100 μg/mL in each case) had the greatest impact on isolates 12146 and 6381 (Fig. 1B). Higher concentrations of AMP-B further decreased the viability of these isolates and also negatively impacted on the survival of the 'resistant' 283/08 isolate (Fig. 1B). Changes in antibiotic concentration was a highly significant source of variability ($P < 0.01$) only in the case of AMP-B (Table 3).

Viability of MAP isolates following long-term VAN treatment

In contrast to short-term treatment, substantial changes in *MAP* viability were found following longer-term antibiotic exposure (Figs. 2 and 3). The laboratory strain 6381 was most sensitive, while the 12146 field isolate was most resistant (Figs. 2 and 3). The 5 week treatment also had a negative impact on the viability of isolate 283/08, the most resistant isolate to the short-term treatments (Figs. 1–3). When measured by PMA qPCR, isolate 283/08 only withstood combinations of VAN when VCM or NAL concentrations were at their lowest (Fig. 2). A similar trend was found using culture (Fig. 3). Using both techniques, MAP isolates were a significant ($P < 0.05$) or highly significant ($P < 0.01$), source of variability (Table 4).

Both methods revealed that the concentration of antibiotics and their interactions were a significant source of variability (Table 5). The threshold concentration, above which the decrease in bacterial numbers was highly significant, was determined as 50 and 100 μg/mL of VNC as detected by PMA qPCR and culture, respectively (Table 5). Using culture, 50–100 μg/mL of NAL and 100 μg/mL of AMP-B were considered significant in terms of decreased viability of MAP (Table 5). Such a trend was not found in the case of NAL and AMP-B when the PMA qPCR detection method was employed (Fig. 2). Although the existence of 'outlier' data complicated the interpretation of the results, 50 μg/mL of NAL and 200 μg/mL of AMP-B were found to significantly decrease MAP viability as detected by PMA qPCR (Fig. 2; Table 5).

Discussion

The detection of viable MAP in biological samples is hindered by the necessary decontamination steps, as well as the addition of antibiotics required to prevent the overgrowth of faster-growing microorganisms (Bull et al., 2009). These processes can negatively impact on the recovery of MAP, so samples containing low concentrations of bacteria can appear as 'false negatives'. An alternative detection method, based on the penetration of PMA dye through bacterial cell walls, was thus established to distinguish viable from dead bacteria independent of culture procedures (Nocker et al., 2006; Kralik et al., 2010). While Pholwat et al. (2011) reported the effects of antibiotic treatment on the culture of *Mycobacterium tuberculosis*, this is the first study to investigate such effects on MAP using PMA qPCR.

The short-term treatment we adopted was previously used for the treatment of MAP-positive faecal samples following decontamination using HPC (Reddacliff et al., 2003; Whittington, 2009). Culture of MAP bacteria for 5 weeks was chosen as such a timeframe results in an exponential growth phase of organisms that do not

Table 2
Statistical significance of the differences in viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates following short-term treatments with VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics measured using the propidium monoazide real time PCR method. Each isolate was treated with a combination of three VAN antibiotics: while the concentrations of two of the antibiotics remained the same, the concentration of the third varied.

MAP isolate	Vancomycin[a]			Amphotericin B[b]			Nalidixic acid[c]		
	283/08	6381	12146	283/08	6381	12146	283/08	6381	12146
283/08	–			–			–		
6381	$P < 0.01$	–		$P < 0.01$	–		ns	–	
12146	$P < 0.01$	ns	–	$P < 0.01$	$P < 0.01$	–	$P < 0.01$	ns	–

$P < 0.01$, highly significant difference (Tukey's HSD test); ns, not significant.
[a] 25–400 μg/mL of vancomycin (VNC), 50 μg/mL of amphotericin B (AMP-B), and 100 μg/mL of nalidixic acid (NAL).
[b] 100 μg/mL of VNC, 12.5–200 μg/mL of AMP-B, and 100 μg/mL of NAL.
[c] 100 μg/mL of VNC, 50 μg/mL of AMP-B, and 25–400 μg/mL of NAL.

Table 3
Statistical significance of the differences in viability of all *Mycobacterium avium* subsp. *paratuberculosis* isolates as measured by propidium monoazide real time PCR following short-term exposure to different concentrations of amphotericin B.

Concentrations of amphotericin B (μg/mL)[a]	12.5	25	50	100	200
12.5	–				
25	ns	–			
50	ns	ns	–		
100	$P < 0.01$	ns	ns	–	
200	ns	$P < 0.01$	ns	$P < 0.01$	–

$P < 0.01$, highly significant difference (Tukey's HSD test); ns, not significant.
[a] Concentration of amphotericin B changed as stated: the concentrations of both vancomycin and nalidixic acid were 100 μg/mL in all cases, respectively.

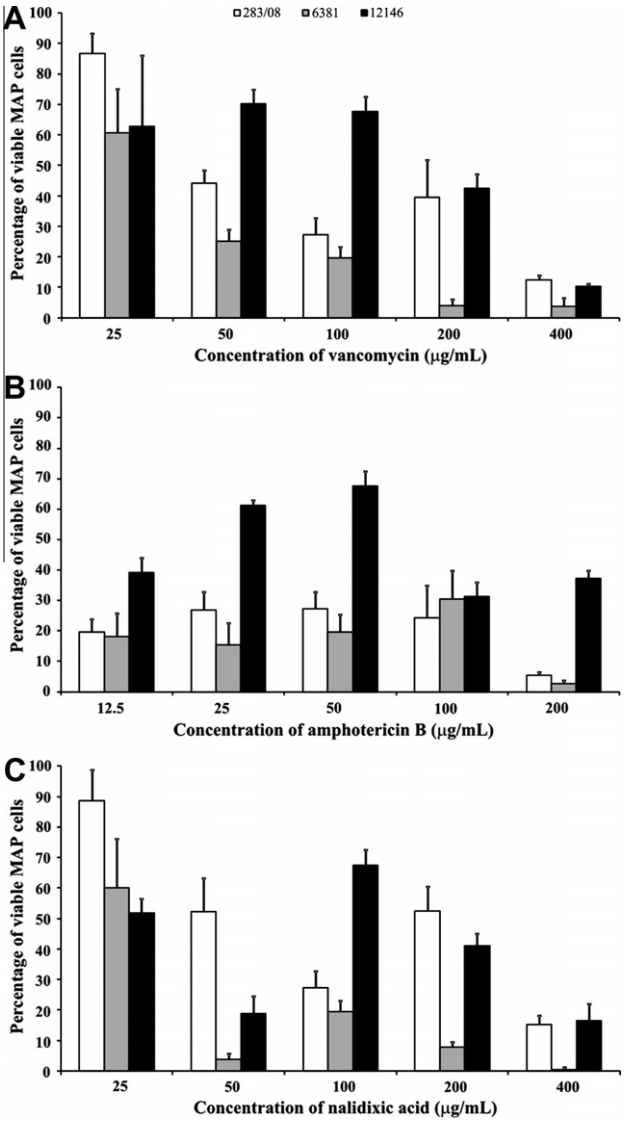

Fig. 2. Long-term treatment of three *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates (283/08, 12146 and 6381) with VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics as measured by propidium monoazide real time PCR: (A) concentration of vancomycin (VCM), 25–400 μg/mL. Concentration of nalidixic acid (NAL) and amphotericin B (AMP-B), 100 and 50 μg/mL, respectively; (B) concentration of VCM and NAL, 100 μg/mL in each case. Concentration of AMP-B, 12.5–200 μg/mL; and (C) concentration of NAL, 25 –400 μg/mL. Concentration of VCM and AMP-B, 100 and 50 μg/mL in each case, respectively.

tend to clump, in contrast to mycobacteria cultured for longer periods (Kralik et al., 2010). In general, no major differences in the

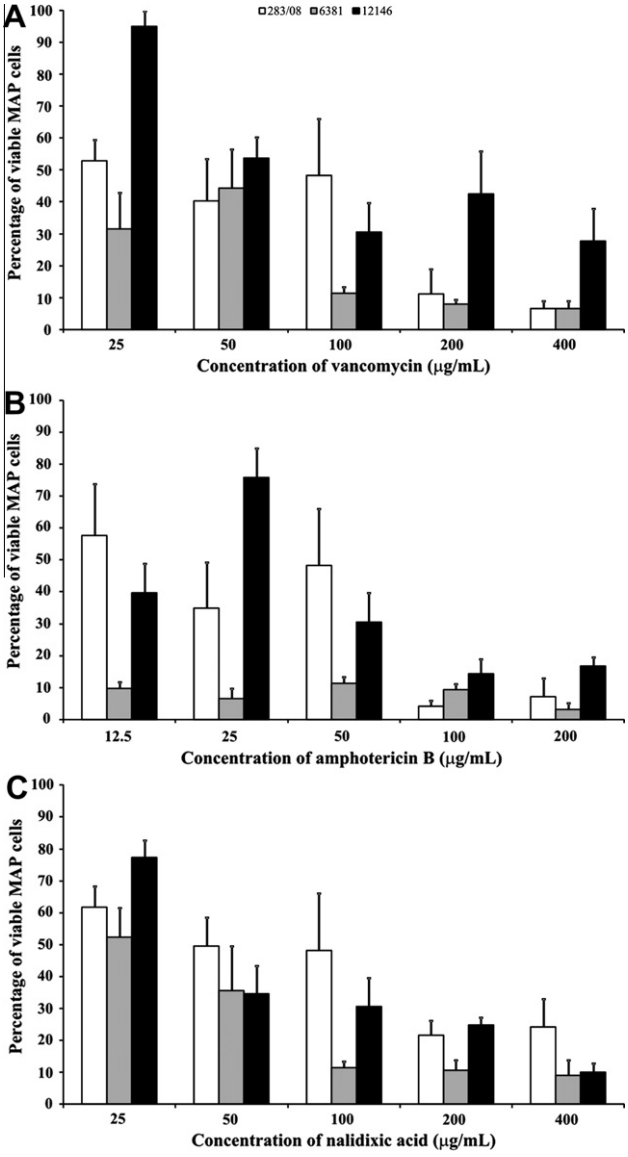

Fig. 3. Long-term treatment of three *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates (283/08, 12146 and 6381) with VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics as measured by culture: (A) concentration of vancomycin (VCM), 25–400 μg/mL. Concentration of nalidixic acid (NAL) and amphotericin B (AMP-B), 100 and 50 μg/mL, respectively; (B) concentration of VCM and NAL, 100 μg/mL in each case. Concentration of AMP-B, 12.5–200 μg/mL; and (C) concentration of NAL, 25–400 μg/mL. Concentration of VCM and AMP-B, 100 and 50 μg/mL in each case, respectively.

viability of individual isolates and in individual VAN concentrations were identified with the short-term treatment. In contrast, and as expected, the effect of VAN was more detrimental when applied longer-term, leading us to speculate that such an approach to culturing MAP could have a detrimental impact on subsequent assessment of mycobacterial viability and response to stress factors such as pH, temperature and disinfection procedures.

We therefore recommend that MAP is cultured in the absence of antibiotics in all experiments assessing the viability of the organism using PMA qPCR. Absolute viability of the culture prepared for experiments with PMA can be determined in control, non-stressed cells exposed to PMA, compared to control non-stressed cells that are not exposed to PMA. However, the comparison could be misleading due to the significant differences in the C_q between PMA-exposed and PMA non-exposed organisms (Vesper et al., 2008; Kobayashi et al., 2009; Kralik et al., 2010). Such an approach

Table 4
Statistical significance of the differences in viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates as measured by propidium monoazide real time PCR and culture following long-term exposure to VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics. Each isolate was treated with a combination of three VAN antibiotics: while the concentrations of two of the antibiotics remained the same, the concentration of the third varied.

Antibiotic	MAP isolate		MAP isolate 283/08	MAP isolate 6381	MAP isolate 12146	
Vancomycin [a]	283/08	PMA qPCR	-	*P*<0.01	*P*<0.01	Culture
	6381		*P*<0.01	-	*P*<0.01	
	12146		*P*<0.01	*P*<0.01	-	
Amphotericin B [b]	283/08	PMA qPCR	-	*P*<0.01	ns	Culture
	6381		ns	-	*P*<0.01	
	12146		*P*<0.01	*P*<0.01	-	
Nalidixic acid [c]	283/08	PMA qPCR	-	*P*<0.01	ns	Culture
	6381		*P*<0.01	-	*P*<0.05	
	12146		*P*<0.01	*P*<0.01	-	

P < 0.01, highly significant difference (Tukey's HSD test); *P* < 0.05, significant difference (Tukey's HSD test); ns, no significant difference.
[a] 25–400 μg/mL of vancomycin (VNC), 50 μg/mL of amphotericin B (AMP-B), and 100 μg/mL of nalidixic acid (NAL).
[b] 100 μg/mL of VNC, 12.5–200 μg/mL of AMP-B, and 100 μg/mL of NAL.
[c] 100 μg/mL of VNC, 50 μg/mL of AMP-B, and 25–400 μg/mL of NAL.

Table 5
Statistical significance of the differences in viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP) isolates as measured by propidium monoazide real time PCR and culture following long-term exposure to different concentrations of VAN (vancomycin, amphotericin B and nalidixic acid) antibiotics.

Antibiotic of variable concentration	Concentration of antibiotic (μg/mL)		Concentration of antibiotic (μg/mL) [d] 25 (12.5)	50 (25)	100 (50)	200 (100)	400 (200)	
Vancomycin (μg/mL) [a]	25	PMA qPCR	-	ns	*P*<0.01	*P*<0.01	*P*<0.01	Culture
	50		*P*<0.01	-	*P*<0.01	*P*<0.01	*P*<0.01	
	100		*P*<0.01	ns	-	ns	*P*<0.01	
	200		*P*<0.01	*P*<0.01	ns	-	ns	
	400		*P*<0.01	*P*<0.01	*P*<0.01	*P*<0.01	-	
Amphotericin B (μg/mL) [b]	12.5	PMA qPCR	-	ns	ns	*P*<0.01	*P*<0.01	Culture
	25		*P*<0.01	-	ns	*P*<0.01	*P*<0.01	
	50		*P*<0.01	ns	-	*P*<0.01	*P*<0.01	
	100		*P*<0.01	*P*<0.01	ns	-	ns	
	200		*P*<0.01	*P*<0.01	*P*<0.01	*P*<0.01	-	
Nalidixic acid (μg/mL) [c]	25	PMA qPCR	-	*P*<0.05	*P*<0.01	*P*<0.01	*P*<0.01	Culture
	50		*P*<0.01	-	ns	*P*<0.01	*P*<0.01	
	100		*P*<0.01	*P*<0.01	-	ns	ns	
	200		*P*<0.01	*P*<0.05	ns	-	ns	
	400		*P*<0.01	*P*<0.01	*P*<0.01	*P*<0.01	-	

P < 0.01, highly significant difference (Tukey's HSD test); *P* < 0.05, significant difference (Tukey's HSD test); ns, no significant difference.
[a] Vancomycin (VNC) 25–400 μg/mL, nalidixic acid (NAL) and amphotericin B (AMP-B) 100 μg/mL and 50 μg/mL, respectively.
[b] AMP-B 12.5–200 μg/mL, VNC and NAL 100 μg/mL, respectively.
[c] NAL 25–400 μg/mL, VNC and AMP-B 100 μg/mL and 50 μg/mL, respectively.
[d] Concentrations valid for VNC and NAL (in parenthesis for AMP-B).

can identify healthy ($\Delta C_q \leqslant 2$), partially damaged ($\Delta C_q = 2$–4) and severely damaged/dead ($\Delta C_q > 4$) bacteria, in the sample (Kobayashi et al., 2010).

Although substantial differences in viability were revealed using relative comparisons, the differences between the lowest viability in stressed cells and non-stressed controls were similar (data not shown). When culture is used as the detection method, VAN antibiotics are found to have a negative effect on the isolation of MAP, although this effect is insignificant compared to their effect on the growth of other bacteria (Merkal and Richards, 1972; Whittington, 2009). As little as 5 μg/mL of VCM for 10–20 min suppresses the growth of *Streptococcus pneumoniae* (Haas et al., 2005), and 10 μg/mL of NAL represents a lethal concentration for *Escherichia coli* (Goss et al., 1965).

With both the short and long treatments, a combination of 100 μg/mL of both VCM and NAL (with any concentration of

AMP-B) generally resulted in the largest decrease in the viability of all MAP isolates. In addition to NAL and AMP-B, VCM is included in commercial liquid MGIT medium while it is not present in BACTEC medium. Gumber and Whittington (2007) speculated that the presence of VCM was the main factor delaying the growth of an ovine strain of MAP. Staphylococcal bacteria treated with gentamicin, which acts mainly through targeting protein synthesis, were more viable than those treated with VCM (Kobayashi et al., 2010). NAL also acts indirectly by inhibiting topoisomerase II, an enzyme that facilitates DNA replication. In the current study we did not observe the same effect for NAL as for gentamicin, possibly due to differences in the bacteria used or the co-effects of the other antibiotics used.

Differential reactions to stress were noted in the different isolates tested. The 6381 laboratory strain was most sensitive and had a lower viability compared to the other isolates, a feature previously documented for yeasts (Redon et al., 2011). Kvitek et al. (2008) demonstrated that a laboratory strain of yeast was phenotypically distinct from other strains in terms of sensitivity to stress, gene expression, and mitochondrial content. The 283/08 field isolate of MAP was little influenced by short-term antibiotic treatment: its viability decreased only when exposed for the longer time period. Surprisingly, the viability of the 12146 isolate did not decrease with increasing exposure to VAN when measured using the PMA qPCR. Nevertheless, the 'abnormal' feature of increased viability with increasing concentration of antibiotics observed with isolate 12146 requires further investigation.

Although culture and PMA qPCR produced numerically distinct data, which were more pronounced following long-term exposure, the interpretation of the results using both methods was ultimately similar. However, since each of these methods measures bacterial viability in fundamentally different ways, they cannot, strictly speaking, produce numerically identical results. The relative viability of the bacteria (treated/control bacterial cells × 100%) determined during the optimisation of the PMA qPCR procedure (Kralik et al., 2010), was the most suitable for this study, and to facilitate better comparison of culture and PMA qPCR, an identical approach was applied for culture. It must be highlighted that the experimentally determined difference between completely live and dead MAP organisms following PMA treatment is approximately $2 \log_{10}$ (Kralik et al., 2010). In culture, a decrease in CFUs of this amount does not represent the death of all the bacteria in the sample. The trends of viability determination using both methods were comparable, which suggests that PMA can be used for the determination of MAP viability independently of culture.

Conclusions

Given the responses of MAP isolates to different concentrations of antibiotics over short- and long-term treatments were similar between culture and PMA qPCR detection methods, we propose that PMA qPCR could replace culture in experiments assessing the effects of different treatments on mycobacterial viability. Overall, 50 μg/mL of VNC, 50 μg/mL of NAL and 200 μg/mL of AMP-B, respectively, were considered to be threshold concentrations for decreasing the viability of MAP organisms. Using culture, these threshold concentrations were 100 μg/mL, 50–100 μg/mL and 100 μg/mL, respectively. Given that exposure to VAN antibiotics could negatively affect the results of studies using PMA qPCR to assess the response and viability of MAP to various stressors, we recommend that the viability of control cells be verified by comparing PMA-treated and non PMA-treated organisms. The PMA qPCR method can identify differences in the viability of organisms both treated and not treated with NAL, an antibiotic which does not primarily affect cell wall synthesis.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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Repeated cycles of chemical and physical disinfection and their influence on *Mycobacterium avium* subsp. *paratuberculosis* viability measured by propidium monoazide *F57* quantitative real time PCR

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ABSTRACT

Mycobacterium avium subsp. *paratuberculosis* (MAP) has a high degree of resistance to chemical and physical procedures frequently used for the elimination of other bacteria. Recently, a method for the determination of viability by exposure of MAP to propidium monoazide (PMA) and subsequent real time quantitative PCR (qPCR) was established and found to be comparable with culture. The aim of this study was to apply the PMA qPCR method to determine the impact of increasing concentration or time and repeated cycles of the application of selected disinfectants on MAP viability. Different MAP isolates responded to the same type of stress in different ways. The laboratory strain CAPM 6381 had the highest tolerance, while the 8819 low-passage field isolate was the most sensitive. Ultraviolet exposure caused only a partial reduction in MAP viability; all MAP isolates were relatively resistant to chlorine. Only the application of peracetic acid led to the total elimination of MAP. Repeated application of the treatments resulted in more significant decreases in MAP viability compared to single increases in the concentration or time of exposure to the disinfectant.

Introduction

Mycobacterium avium subspecies *paratuberculosis* (MAP) causes Johne's disease (paratuberculosis) in ruminants and other mammals (Beard et al., 2001; Stevenson et al., 2009). MAP and other mycobacteria have a lipid-rich cell wall, which is less permeable to biocides compared to other bacteria (Russell, 1998) and confers acid-fastness, hydrophobicity and increased tolerance to physical and chemical stresses, such as increased temperature (Sung and Collins, 1998), disinfection agents (Le Dantec et al., 2002) and antibiotics (Pribylova et al., 2012). These features enable MAP to survive in different environments for a relatively long time (Whittington et al., 2004, 2005). Removal of MAP from contaminated stables and farms is a long and complicated process, which contributes to the high levels of infection in cattle worldwide (Nielsen and Toft, 2009).

Since mycobacteria have increased tolerance to external stressors, they are often included in studies comparing the effects of disinfectants (Griffiths et al., 1999; Taylor et al., 2000; Wang et al., 2005). In most of these studies, culture methods have been used to quantify MAP cells after exposure to the stress. Culture is recognised as the gold standard, but has disadvantages in that it requires a long incubation time, has low sensitivity (Hernandez et al., 2003), MAP tend to form clumps (Sung and Collins, 1998) and some strains of MAP cannot be cultured (Kopecna et al., 2008). Moreover, negative culture results do not necessarily mean the absence of viable bacteria, but could be caused by the inability of injured bacteria to grow after being exposed to disinfectants (Sung and Collins, 1998; Gumber and Whittington, 2007; Pribylova et al., 2012).

A molecular method using the selective dye propidium monoazide (PMA) in combination with qPCR has been used as an alternative to culture to examine the effects of heat and alcohol on the viability of MAP (Nocker et al., 2006). The method has also been used to determine the effects of disinfectants and heat on the viability of *Mycobacterium avium* complex subsp. *avium* (Nocker et al., 2007). Using this method, the viability of MAP is determined by the ability of PMA to penetrate the cell wall of damaged or dead cells but not viable cells with intact cell walls. Once absorbed, PMA binds covalently to DNA and prevents its amplification by real-time quantitative PCR (qPCR). Since qPCR is not inhibited in viable and undamaged MAP, the method enables discrimination between live and dead bacteria (Nocker et al., 2006).

The PMA qPCR method has been used to distinguish between live and dead cells of a range of bacterial species in various environments (Varma et al., 2009; Josefsen et al., 2010; Kaushik and Balasubramanian, 2013). Since PMA is able to bind and inactivate exogenous DNA, this method has also been applied in the health care industry as a rapid method for quantification of environmental contamination (Schnetzinger et al., 2013). The combination of

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PMA with amplification of a single *F57* sequence (PMA *F57* qPCR) has been developed to determine the specific viability of MAP (Kralik et al., 2010). Pribylova et al. (2012) showed that the results of culture and PMA *F57* qPCR are comparable.

Due to the high resistance of MAP to biocides compared to other bacteria and due to problems with culture of MAP, the use of PMA qPCR for determining MAP viability would be useful. The aims of this study were: (1) to investigate the effect of a single exposure to ultraviolet (UV) light, chlorine and peracetic acid (PAA) on the viability of three MAP isolates; and (2) to determine the effect of repeated cycles of UV, and repeated application of chlorine and PAA on the viability of the three MAP isolates.

Materials and methods

MAP isolates and preparation of MAP suspensions

Three MAP isolates originating from different sources were used. Low passage field isolate 8819 and high passage field isolate 12146, which had undergone several passages, were used. The laboratory reference strain CAPM 6381 (Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Czech Republic) was also included. All isolates originated from cattle and were of restriction fragment length polymorphism type C1.

The isolates were grown on solid Herrold's egg yolk medium (HEYM) with antibiotics (penicillin G, chloramphenicol and amphotericin B; Becton Dickinson), supplemented with 2 μg/mL mycobactin J (Allied Monitor) at 37 °C for 3 months. Using a loop, colonies of all isolates were scraped off from HEYM and resuspended in 1.2 mL Middlebrook 7H9 (M7H9) broth (Difco) supplemented with 10% (V/V) Middlebrook OADC enrichment (Difco). After addition of 350 mg sterilised 1 mm zirconia/silica beads (Biospec), the MAP suspension was homogenised by vortexing for 30 s. The suspension was centrifuged at 100 *g* for 30 s to remove large clumps and the upper cell fraction was resuspended in fresh M7H9 broth. The suspension was diluted to an optical density at 600 nm (OD_{600}) of 0.15–0.20, which corresponds to ~10^8 MAP cells/mL of suspension (Kralik et al., 2011).

Experimental design

To determine the efficacy of the disinfection procedures, each treatment with UV, free chlorine and PAA was repeated three times. On the first day, the MAP suspension of each isolate was divided into 18 tubes (8 mL per tube; five treatments and control in biological triplicates). After exposure to the disinfection treatment (for details see paragraphs below), MAP cells were centrifuged at 7000 *g* for 3 min, then the supernatant was discarded and an equal volume of fresh M7H9 broth was added. At this point, samples for the PMA treatment (technical triplicates of 500 μL from each tube) were collected (one-time exposure). The remaining suspensions were transferred to an incubator at 37 °C for overnight recovery. The same procedure as described above was repeated on the second and third days (repeated exposure; 'treatments 2 and 3'). The volumes of chlorine and PAA solutions were kept equal to the decreasing volumes of the MAP suspensions.

Ultraviolet disinfection

In a biohazard box, 8 mL MAP cell suspensions were poured onto 90 mm Petri dishes without lids and exposed to a UV lamp (supplied with the box) for 10, 20, 30, 45 and 60 min. The dishes were at a distance of ~7 cm from the UV tube. The declared parameters of the UV tube were 44 mWs/cm^2. A new lamp was employed to minimise the variation of strength of UV bulb during the experiment.

Chlorine disinfection

The chlorine stock solution (100 parts per million, ppm) was prepared by dissolving the commercially available chlorine powder disinfectant Chloramin T (active chlorine concentration 28%; Bochemie) in water. The concentration of free chlorine in the stock solution was determined colorimetrically according to the standardised *N,N*-diethyl-*p*-phenyldiamine (DPD) method (CSN ISO 7393-2). The chlorine stock solution was diluted to 10, 25, 50, 75 and 100 ppm. The MAP cell suspensions were centrifuged at 7000 *g* for 3 min and the M7H9 broth was replaced with an equal volume of chlorine solution (water for controls). After 30 min exposure, 2 mL sodium thiosulphate solution (0.05% in phosphate buffered saline, PBS; Sigma) was added to neutralise the remaining free chlorine. Due to the instability of chlorine solutions, fresh dilutions were prepared for each day of testing.

Peracetic acid disinfection

The stock solution of PAA (Sigma) was diluted to 0.01, 0.05, 0.1, 0.5 and 1% solutions in PBS. The samples were processed identically as for chlorine disinfection, i.e., the MAP suspensions were centrifuged and the M7H9 medium was replaced with an equal volume of PAA. The samples were exposed to PAA for a contact time of 10 min, after which PAA was neutralised using M7H9 broth with 1.135% β-cyclodextrine, 0.5% sodium thiosulphate, 0.1% L-histidine and 0.2% glycerol (Sigma; Steinhauer et al., 2010).

Propidium monoazide treatment

The PMA method was adopted from the study of Kralik et al. (2010), which is a modification of the PMA procedure described by Nocker et al. (2006). A volume of 12.5 μL of 1 mM PMA stock solution dissolved in 20% dimethyl sulphoxide (DMSO; Biotium) was added to 500 μL of each MAP suspension (final concentration 25 μM PMA) and incubated in the dark for 5 min with mixing at 20 Hz. The vials were then placed on ice and exposed to a light from a 650 W halogen bulb (B & H PhotoVideo) for 2 min. The entire process of dark incubation and light exposure was then repeated once after the fresh addition of 12.5 μL PMA (final concentration 50 μM). In control samples, the PMA solution was replaced by an identical volume of 20% DMSO. The suspension was centrifuged at 7000 *g* for 5 min and the supernatant was replaced with 500 μL Tris-ethylene diamine tetraacetic acid (TE) buffer supplemented with 50 ng/μL fish sperm DNA (Serva Electrophoresis). After lysis at 100 °C for 20 min, the cells were centrifuged at 18,000 *g* for 5 min and used as a template in *F57* qPCR. The PMA treatment was performed in technical triplicates and analysed in a single qPCR run.

F57 *qPCR*

For the detection and quantification of MAP, a previously published assay amplifying the single copy fragment *F57* was used (Slana et al., 2008). Quantification was performed according to a standard curve derived from a plasmid standard gradient. Amplification runs were carried out on a LightCycler 480 instrument (Roche Molecular Diagnostic). The 'Fit point analysis' option of the LightCycler 480 software (version 1.5.0.39) was used to determine the absolute numbers of MAP cells. MAP viability was determined as the quotient of absolute numbers of PMA-exposed stress factor-treated cells and the PMA-exposed control (stress factor-untreated) MAP cells (Kralik et al., 2010).

Statistical analysis

All statistical calculations were performed using Statistica 9.0 software (StatSoft). Exploratory data analysis was used to find the most suitable statistical model for the analyses of experiments with UV and chlorine. On the basis of this analysis, multiple regression analysis was employed to determine the differences among MAP isolates examined. The model used was $VC = \beta_0 + \beta_1 T + \beta_2 E$, where VC is the percentage of viable cells, T is the number of treatments, E is the exposure time or concentration of disinfectant and β_i represents the parameters of the model (β_0 is a constant term; β_1 and β_2 can be interpreted as a change in the value of VC after one additional treatment and after an additional 1 min of UV exposure or an additional 10 ppm of chlorine, respectively). Since different MAP isolates responded to the different treatments in distinct ways (Figs. 1–3), it was decided to create an individual model for each disinfection treatment and MAP isolate (Table 1), although this was not

Table 1
Multiple regression models[a] for respective *Mycobacterium avium* subsp. *paratuberculosis* isolates and treatment methods.

MAP isolate	Treatment	*F* test[b]	R^2 statistic[c]	Parameters of model[d]		
				β_0	β_1	β_2
6381	UV	53.379	0.718	79.572	–14.978	–0.522
8819	UV	76.108	0.784	87.344	–12.691	–0.755
12146	UV	117.624	0.849	137.513	–36.800	–0.579
6381	Chlorine	19.101	0.476	85.336	–14.060	–0.236
8819	Chlorine	87.945	0.807	116.626	–29.122	–0.373
12146	Chlorine	53.362	0.718	147.682	–28.084	–0.471

[a] The model used was $VC = \beta_0 + \beta_1 T + \beta_2 E$, where VC is the percentage of viable cells, T is the number of treatments, E is the exposure time or concentration of disinfectant and β_i represents the parameters of the model (β_0 is a constant term; β_1 and β_2 can be interpreted as a change in the value of VC after one additional treatment and after an additional 1 min of UV exposure or an additional 10 ppm of chlorine, respectively.

[b] Test of the suitability of the model (all corresponding P values < 0.01).

[c] Proportion of the response variable (VC) variation which is explained by the model.

[d] All parameters were statistically significant (P values for t tests < 0.01).

possible for the PAA treatment. Suitability of the models was confirmed using the *F* test and the significances of parameters were verified by the *t* test. The statistical analysis of the effect of PAA on MAP viability was performed using two-way analysis of variance (ANOVA), with subsequent testing of the statistical significance of partial differences using Tukey's honest significant difference (HSD) test. *P* values < 0.05 were considered to be statistically significant.

Results

Ultraviolet light

Regression models showed that the effect of repeated exposure to UV light had a higher impact on MAP viability than prolonged time of exposure for all three MAP isolates (Table 1). The viability of the reference strain CAPM 6381 was least influenced by the time of exposure. Conversely, in the 8819 low-passage isolate, the effect of time of exposure was more pronounced in terms of viability reduction (Fig. 1). The statistical model showed that the highest reduction of viability can be expected in the 12146 high-passage isolate after repeated treatment with UV (36.8% reduction; Table 2, which is a consequence of the model described in Table 1). When considering the effect of additional exposures (10 min), the reduction of MAP viability was similar for all three MAP isolates (Table 2).

Chlorine

As in the case of UV treatment, the effect of repeated chlorine treatments on MAP viability was more significant than one-time exposure to increasing chlorine concentrations (Fig. 2). Moreover, increases in chlorine concentration generally had a mild effect on decreasing MAP viability. The laboratory strain CAPM 6381 and the high-passage isolate 12146 were tolerant to a one-time increase in chlorine concentration. According to the statistical model, a higher decrease in viability can be expected in 8819 and 12146 isolates compared to the 6381 strain after the additional treatments using chlorine (Table 3, which is a consequence of the model described in Table 1). Also, additional exposures (10 ppm) of chlorine should have a slightly higher impact on MAP 8819 and 12146 than on strain 6381 (Table 3).

Peracetic acid

Due to the significant effect of PAA on the viability of MAP, only concentrations of 0.01, 0.05 and 0.1% were taken into account in the statistical analysis. In all isolates tested, both the number of treatments and increasing concentration of PAA were statistically significant sources of variability (Fig. 3). In all examined isolates, the second and third treatments led to statistically significant decreases in viability ($P < 0.01$) compared to the first treatment. No statistically significant difference ($P > 0.05$) was found between the second and third treatments. The same pattern was observed for different concentrations of PAA. Exposure of MAP to 0.05 and 0.1% PAA led to statistically significant decreases in MAP viability compared to 0.01% PAA ($P < 0.01$). However, no statistically significant difference in MAP viability ($P > 0.05$) was found between 0.05% and 0.1% PAA.

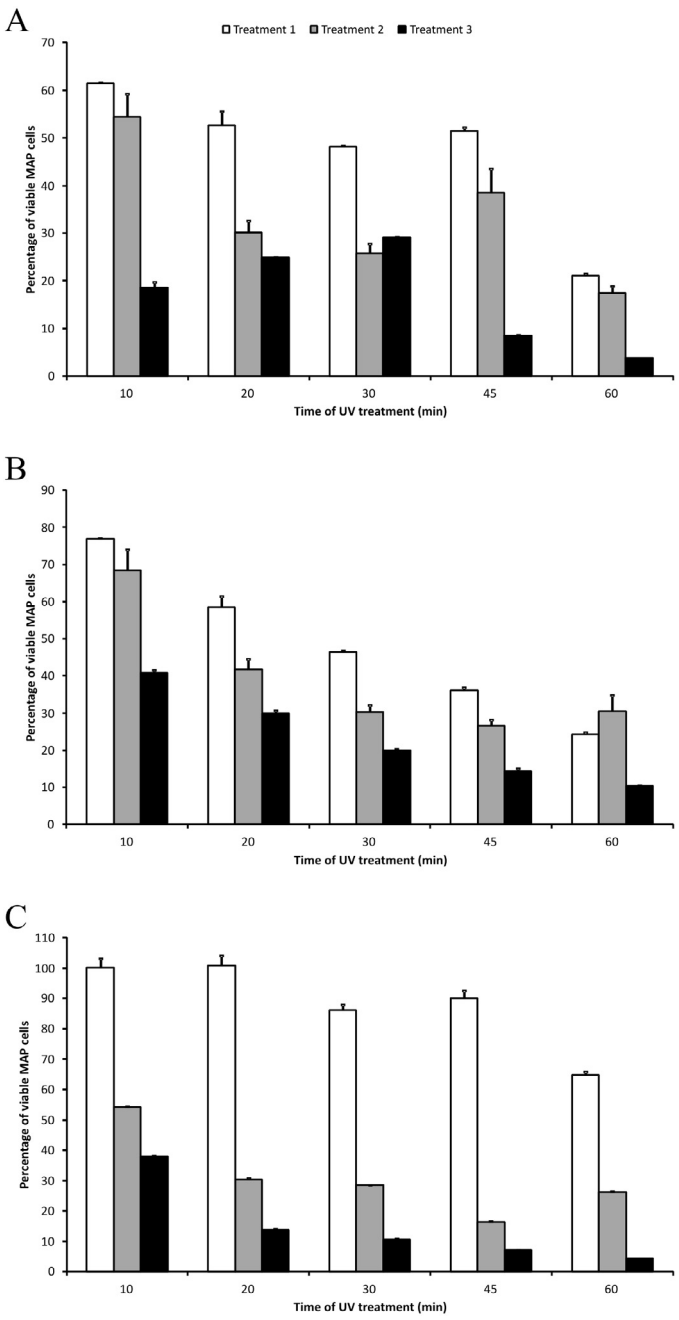

Fig. 1. Effect of ultraviolet (UV) light on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) viability. A, collection strain CAPM 6381; B, low passage isolate 8819; C, high passage isolate 12146. The same procedure ('Treatment 1') was repeated on the second (repeated exposure 'Treatment 2') and third days (repeated exposure 'Treatment 3'). Vertical bars represent sample standard deviations.

Table 2
Effect of repeated ultraviolet treatment and an additional 10 min of exposure on the viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

MAP isolate	% MAP viability reduction after additional treatment	% MAP viability reduction after an additional 10 min of exposure
6381	15.0	5.2
8819	12.7	7.6
12146	36.8	5.8

Table 3
Effect of repeated chlorine treatment and an additional 10 ppm of chlorine on the viability of *Mycobacterium avium* subsp. *paratuberculosis* (MAP).

MAP isolate	% MAP viability reduction after additional treatment	% MAP viability reduction after additional 10 ppm
6381	14.1	2.4
8819	29.1	3.7
12146	28.1	4.7

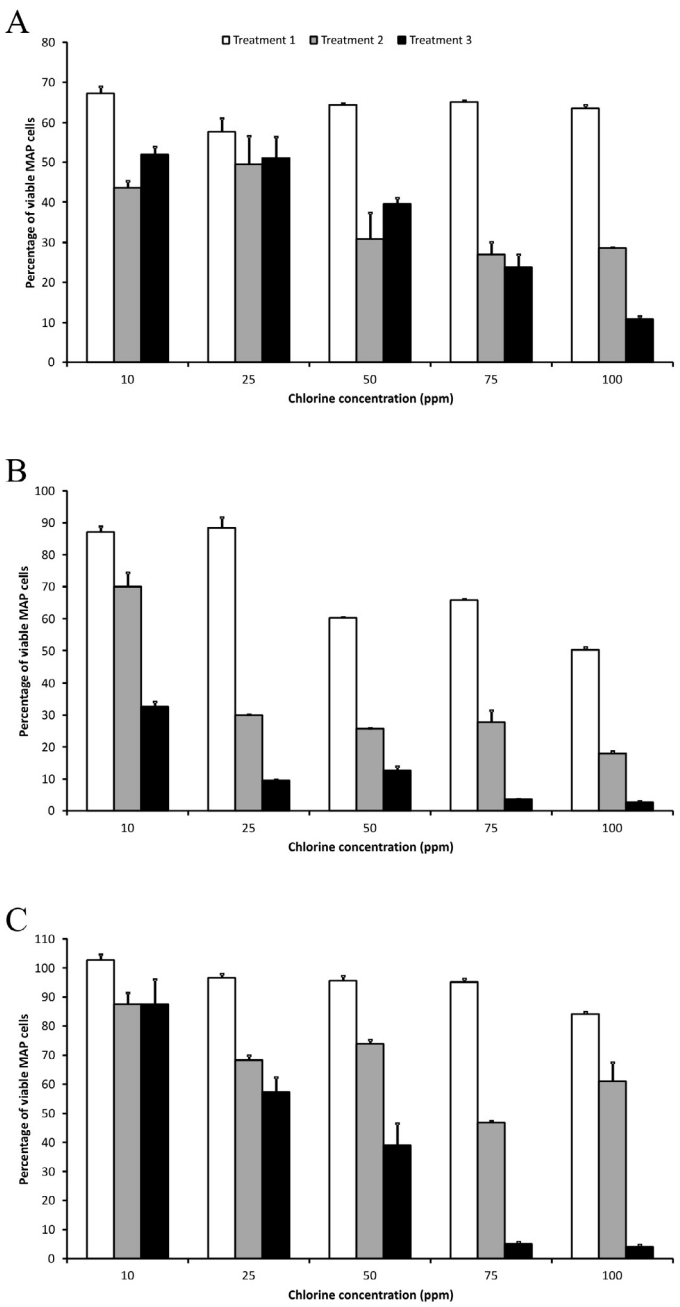

Fig. 2. Effect of chlorine on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) viability. A, collection strain CAPM 6381; B, low passage isolate 8819; C, high passage isolate 12146. The same procedure ('Treatment 1') was repeated on the second (repeated exposure 'Treatment 2') and third days (repeated exposure 'Treatment 3'). Vertical bars represent sample standard deviations.

Discussion

Disinfection processes employ chemical or physical procedures to kill or reduce the number of viable microorganisms on the treated surface. UV as a physical type of disinfection and chloramine and PAA as chemical disinfectants were selected for the purposes of this study as disinfection agents broadly employed in practice and at the same time effective against mycobacteria. UV disturbs the reproduction and protein synthesis of organisms by reacting with DNA molecules. Its application is wide and ranges from the disinfection of surfaces or materials to the disinfection of water or air. For water disinfection, chlorine-releasing agents, which denature the proteins of target organisms, are also used. Apart from water sanitation, they are broadly used in the food, medical and agricultural industries (Fraise, 1999; McDonnell and Russell, 1999; Vlkova et al., 2008). PAA is a strong oxidising agent that increases the permeability of cell walls by denaturing proteins and enzymes. It has strong bactericidal effects on vegetative bacteria, spores and mycobacteria, and is used in the medical sphere, pulp industry, for water disinfection or in preventing biofilm formation (Fraise, 1999; McDonnell and Russell, 1999). Neither the effect of UV, nor chlorine or PAA, has been studied previously in MAP using PMA qPCR. Since disinfectants not only kill but also suppress the growth of bacteria, the use of PMA qPCR, with its capability to detect even non-

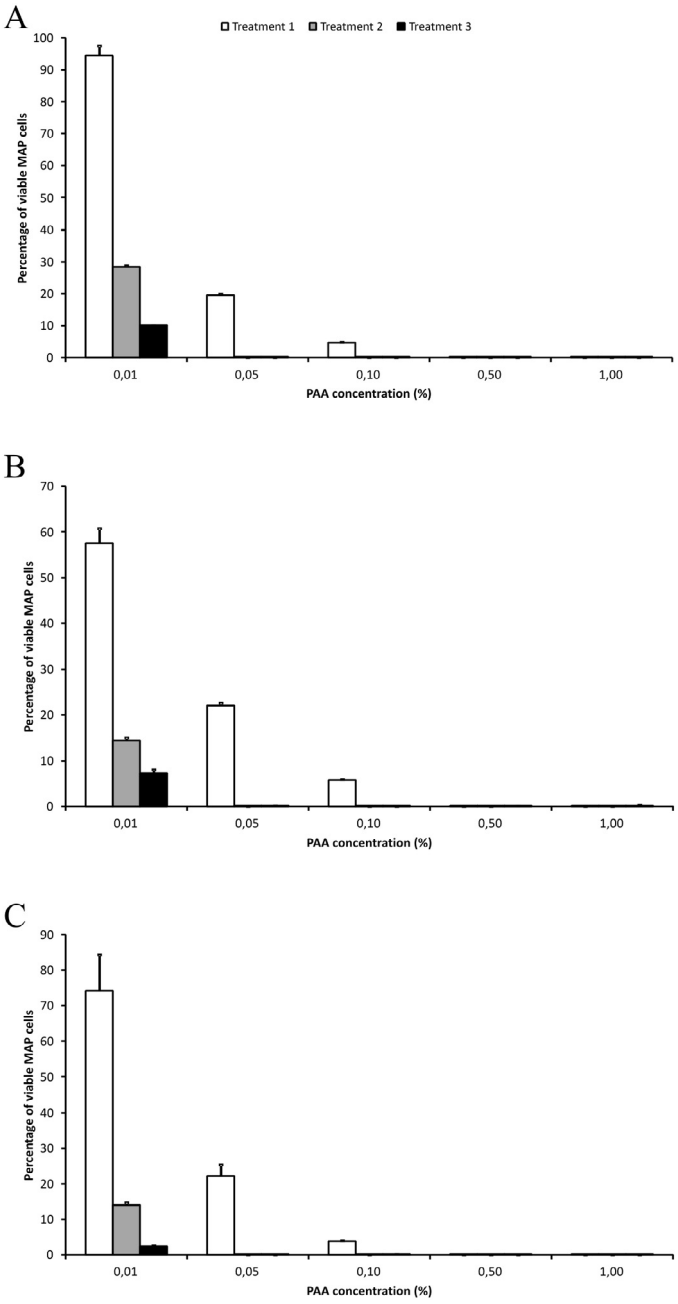

Fig. 3. Effect of peracetic acid (PAA) on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) viability. A, collection strain CAPM 6381; B, low passage isolate 8819; C, high passage isolate 12146. The same procedure ('Treatment 1') was repeated on the second (repeated exposure 'Treatment 2') and third days (repeated exposure 'Treatment 3'). Vertical bars represent sample standard deviations.

growing microorganisms, would be beneficial compared to standard culture techniques. Moreover, the effect of repeating exposure of selected disinfectants on MAP is described here for the first time.

The effect of UV light strongly correlates with the environment in which the mycobacteria are treated. In water, complete killing of MAP (10^5–10^6 cells) was achieved using 15 mWs/cm^2 (Collins, 2003), while >20 mWs/cm^2 was needed to achieve a 4 log reduction of MAP (Shin et al., 2008), or 3 log reduction of MAP in PBS (Lee et al., 2010). In milk, the use of >1000 mWs/cm^2 led to <1 log reduction in MAP (Donaghy et al., 2009). We observed that one-time increasing of time of UV exposure did not have a substantial impact on cells, except in the case of the 8819 isolate. This may be related to the fact that 8819 is a low-passage field isolate. The lower response of MAP isolates to increasing durations of UV radiation could be related to the presence of MAP in rich laboratory liquid medium (Altic et al., 2007). In all MAP isolates, a significant decline was observed after repeated cycles of UV radiation (Table 2).

Differences in response to UV stress were observed for different isolates (Fig. 1). Differing responses of MAP isolates, as well as other mycobacterial species, to UV and other disinfectants have been described previously (Le Dantec et al., 2002; Wang et al., 2005; Donaghy et al., 2009; Lee et al., 2010). Surprisingly, repeating of UV exposure caused a much larger decrease in the viability of the 12146 isolate compared to CAPM 6381 (Fig. 1; Table 2), although both were high-passage isolates. It is not easy to explain this phenomenon, since the same experimental conditions were followed for all the isolates.

Nocker et al. (2007) reported that PMA qPCR is not applicable for determining viability after UV treatment due to the inability of UV of eliciting membrane permeability. In their study using *Escherichia coli*, quantification cycle (Cq) values of UV-treated cells with PMA compared to Cq values of identical but non-UV exposed cells were the same (Nocker et al., 2007). However, in the present study, differences in Cq values were observed. The reason for this is not clear and may be due to the use of different intensities of radiation, distance from the source of radiation, exposure of cells in different cultivation media or the different species employed in our study compared to the study of Nocker et al. (2007). Nalidixic acid is an antibiotic, which, similarly to UV, should not affect the permeability of the cell wall. However, decreases in MAP viability after exposure to nalidixic acid using PMA qPCR have been described (Pribylova et al., 2012).

Due to the problems with mycobacteria in tap water, the majority of disinfection studies focus on the effect of different chlorine-releasing agents on mycobacteria (Falkinham, 2003; Wang et al., 2005; Luh et al., 2008; Lee et al., 2010). Taylor et al. (2000) found that the resistance of mycobacteria to chlorine-releasing agents in water is higher than that of bacteria growing in culture media. Along with growth in different environments or laboratory conditions, the effects of temperature, distinct colony types, cells in different growth phases or the diversity of mycobacteria strains are responsible for the differing responses of mycobacteria to chlorine-releasing agents (Le Dantec et al., 2002; Falkinham, 2003; Lee et al., 2010). As could be predicted, all strains and isolates used in this study showed high resistance to free chlorine. Low susceptibility to chlorine-based chemicals, such as hexadecylpyridinium chloride, confirms this generally known feature of MAP, which is used for the decontamination of samples for culture. The laboratory isolate CAPM 6381 showed the highest resistance to one-time increases in chlorine concentrations, but also to repeated chlorine treatments. It is known that laboratory strains behave differently compared to field isolates if exposed to different stress factors (Falkinham, 2003; Gumber and Whittington, 2007). The higher resistance to chlorine was probably connected with its long-term exposure to in vitro culture conditions (Fig. 2; Table 3).

PAA was shown in this study to be a potent disinfectant. One-time application of 0.1% PAA for 10 min led to a significant reduction of all three MAP isolates (less than 5% viable bacteria). No bacteria could be detected when a concentration of 0.5% was used. Repeated exposure to 0.05% PAA led to a total elimination of all MAP isolates (Fig. 3). Wang et al. (2005) showed that 0.2% PAA for 10 min resulted in 5 log reductions of *Mycobacterium tuberculosis* and *Mycobacterium chelonae* subsp. *abscessus*. PAA concentrations ~0.3% are reliably effective against all mycobacteria; this is exploited in commercial disinfection products, such as Perasafe (0.26%) and Nu-Cidex (0.35%). Using these products, a 5 log reduction in mycobacteria can be achieved within 5 min (Griffiths et al., 1999; Hernandez et al., 2003).

The repeated application of disinfectants is a very important aspect of their mechanisms of action. In all MAP isolates, a significant reduction in the number of viable cells could be observed for all disinfection procedures after repeated exposure to appropriate disinfection procedures (Figs. 1–3; Tables 2 and 3). Riazi and Matthews (2011) subjected pathogenic bacteria to repeated exposure to chlorhexidine digluconate. The second treatment resulted in a more considerable decrease in cell viability compared to the first treatment. The same was observed in this study using PMA qPCR. This phenomenon may be explainable by a decreased ability of previously stressed cells to cope with the same stress again (Riazi and Matthews, 2011). In our conditions, even a third treatment did not result in zero viability, except in the case of PAA. Similarly, repeated treatment with 100 ppm chlorhexidine digluconate also did not lead to a total elimination in *Salmonella* and *Staphylococcus* spp. (Riazi and Matthews, 2011). Likewise, repeated treatment of drinking water with 50 mg/L chlorine consistently for 2.5 years did not lead to the elimination of *Legionella pneumophila*, but led to the discovery of seven persistent *L. pneumophila* strains (Cooper et al., 2008).

Conclusions

This study concerned one-time increasing concentration/time and repeated exposition of UV, free chlorine and PAA on the viability of MAP cells using the PMA qPCR method.

Different MAP isolates responded in different ways. As expected, the laboratory strain CAPM 6381 showed the highest tolerance, while the 8819 low-passage field isolate was the most sensitive. UV light exposure elicited only a partial reduction in MAP viability. As could be expected, all MAP isolates were relatively resistant to chlorine. PAA had a profound effect on MAP viability, indicating that disinfection agents containing PAA will be effective in MAP removal. An improved effect of disinfection procedures was achieved by the repeated administration of individual treatments rather than one-time increasing of concentration or time of exposure.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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RESEARCH LETTER

Evaluation of viable *Mycobacterium avium* subsp. *paratuberculosis* in milk using peptide-mediated separation and Propidium Monoazide qPCR

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Abstract

The causative agent of paratuberculosis in ruminants, *Mycobacterium avium* subsp. *paratuberculosis* (MAP), although still a matter of debate, has been linked with Crohn's and other human diseases. The availability of rapid methods for assessing the viability of MAP cells in food, in particular milk, could be of great use for risk management in food safety. MAP viability is generally assessed using culture techniques that require prolonged incubation periods for the growth of MAP. To differentiate between viable and nonviable MAP cells in milk samples, this study explores the combination of two already described techniques: peptide magnetic bead separation followed by Propidium Monoazide qPCR. Using an Ordinal Multinomial Logistic Regression model to analyze the results obtained after spiking milk samples with mixtures containing different percentages of viable/dead cells, we were able to assess the probability of the viability status of MAP found in milk. This model was applied to contaminated pasteurized milk to ascertain the efficacy of heat treatment in MAP killing. The method reported herein can potentially be used for direct detection of MAP viability in milk.

Introduction

Mycobacterium avium subsp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis, a chronic and infectious disease mainly affecting domestic and wild ruminants. Although no definitive studies have been reported so far, MAP has been linked to some human pathologies, such as Crohn's disease, type I diabetes and others (Hermon-Taylor & Bull, 2002; Scanu *et al.*, 2007; Sechi *et al.*, 2008; Cossu *et al.*, 2011).

The exposure for humans to MAP is generally related to the consumption of contaminated milk, meat and water (Gill *et al.*, 2011); however, it remains uncertain if MAP *per se*, irrespective of its viability status (or of its ability to sustain infection), can be related to the pathogenesis of Crohn's and other human diseases (Momotani *et al.*, 2012). More recently, one study reported the capacity of MAP to form spore-like structures, underlying its potential ability to survive even in adverse conditions (Lamont *et al.*, 2012).

The commonly used techniques for the detection of viable MAP cells in milk and its derivatives are cultural methods, both in liquid and solid media (Okura *et al.*, 2012). Many drawbacks have been reported regarding these methodologies, such as prolonged incubation periods needed for the growth of MAP, overgrowth of other organisms such as molds and possible loss of sensitivity because of the preliminary chemical decontamination steps (Dundee *et al.*, 2001).

In order to overcome these problems, different methodologies have been proposed for evaluation of the viability of MAP in milk samples. One method was based on the retro-amplification of RNA followed by qPCR (Dzieciol *et al.*, 2010), while others used phage-amplification assay with or without prior specific peptide-magnetic

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separation (PMS) (Foddai *et al.*, 2011; Botsaris *et al.*, 2013). Another approach, based on the use of qPCR coupled with propidium monoazide (PMA) treatment, permitted the differentiation between viable and nonviable bacteria (Nocker & Camper, 2009; Fittipaldi *et al.*, 2012). The PMA mechanism of action is due to its ability to selectively enter within cells with compromised membranes and chemically modify the DNA after light exposure, herein defined as inactivated DNA according to (Fittipaldi *et al.*, 2011), finally resulting in inhibition of PCR.

Two recent papers (Kralik *et al.*, 2010; Pribylova *et al.*, 2012) described the use of PMA for the assessment of the viability of MAP. However, this assay has so far been reported only for transparent culture liquid media, while its use on complex matrices, like milk, was not explored.

The aim of our study was thus to combine two already known techniques, PMS and PMA treatment followed by qPCR, in order to develop a tool able to determine MAP viability directly in opaque media, like milk.

Materials and methods

Bacterial strains, bacterial killing and culture conditions

A laboratory reference strain MAP ATCC 19698 and two MAP field isolates, 917/11 and 653/11, (National Reference Centre for Paratuberculosis, Piacenza, IZSLER, IT) were used in the study. The field strains (Type C), were isolated from cattle feces and subcultured at 37 °C for 4 weeks in liquid 7H9 Middlebrook medium (BD, Milan, Italy), supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC) enrichment (BD) and mycobactin J (2 mg L^{-1}; ID Vet, Montpellier, France). After incubation, ATCC19698 and field strains were washed in 7H9 Middlebrook medium and vortexed with 150–212 μm diameter glass beads (Sigma-Aldrich, Milan, Italy) to disrupt clumps. Taking care to use the top fraction, 100 μL of MAP cell suspension was collected in a 1.5-mL tube (Eppendorf, Milan, Italy). The killing of MAP cells was carried out by heating for 15 min at 90 °C to obtain a suspension of completely dead MAP cells (Kralik *et al.*, 2010). Loss of cultivability/viability was checked by spreading 100 μL of both killed and viable cell suspensions in duplicate (*c.* 10^6 CFU mL^{-1}) onto Herrold's egg yolk medium, containing mycobactin J (2 mg L^{-1}). The plates were sealed with PTFE sealing tape (Sigma-Aldrich) and incubated at 37 °C for 4 weeks.

Spiking of milk samples

Bulk milk used as a negative matrix for preparing spiked samples was collected from a Paratuberculosis-free dairy herd in Piacenza, Italy (all animals were negative for the presence of paratuberculosis for the past 10 years by ELISA testing and fecal cultures). With the exception of experiments relative to the evaluation of the method in pasteurized artificially contaminated raw milk, all spiked samples were prepared by adding 900 μL of milk to 7H9 liquid aliquots (100 μL) of live or dead MAP cells. Ten independent experiments for ATCC strain and three independent experiments for field stains (IZSLER 917/11 and 653/11) were conducted.

For the experiments relative to milk samples with mixed populations (containing different percentages of dead and live cells MAP cells), suspensions of heat-killed and live cells of ATCC 19698 in 7H9 broth were mixed in ratios of 100 : 0, 75 : 25, 50 : 50, 25 : 75 and 0 : 100 (killed : live cells) to a final volume of 100 μL. Nine hundred microliters of raw milk was added to each sample and subsequently submitted to PMS and PMA-qPCR. Five independent experiments were conducted.

PMS

Milk samples were then vortexed and centrifuged at 2500 ***g*** for 15 min. The supernatant was discarded, and the pellets were suspended in 1 mL of 0.05% PBS-Tween20.). After addition of 10 μL of magnetic beads (MyOne Tosylactivated Dynabeads, Life Technologies) coated with biotinylated MAP capturing-specific peptides aMptD (GKNHHHQHH RPQ) and aMp3 (NYVIHDVPRHPA; Research Biochemicals, Cambridge, UK), a customized program was used for the automated separation (30 min mixing, followed by capture phase, two washes in 1 mL 0.05% PBS-Tween20 and final suspension in 500 μL 0.05% PBS-Tween20) (Foddai *et al.*, 2010).

PMA treatment

Bead suspensions obtained in the previous step were then submitted to PMA treatment as previously reported (Kralik *et al.*, 2010). PMA (Biotium, Inc., Hayward) was dissolved in 20% DMSO (Sigma-Aldrich) to obtain a stock concentration of 1 mM and stored at −20 °C in the dark. Twelve and half microliters of this solution was added to 500 μL of mycobacterial suspension bound to paramagnetic beads, in minimal light conditions. For the samples untreated with PMA, an equal volume of DMSO (20%) was added. After 5-min incubation in the dark in a rotary mixer, samples were exposed for 2 min to a 650-W halogen light source (Philips, Italy), placed *c.* at 15 cm from the tubes. During exposure, tubes were placed horizontally on ice to avoid excessive heating and to maximize light exposure. The complete step of 'dark incubation/photo induced cross-linking' was repeated after the addi-

tion of 12.5 μL of fresh PMA to a final concentration of 50 μM. The residual PMA, not linked to DNA, was removed by collecting the beads using a manual step of magnetic separation.

DNA extraction

Beads were suspended in 180 μL of freshly prepared lysis buffer containing 20 mg mL^{-1} of lysozyme (Roche, Monza, Italy), 20 mM Tris-HCl, 2 mM EDTA at pH 8.0 and 1.2% Triton (Sigma-Aldrich). The DNA was extracted following the protocol for Gram-positive bacteria (QIAmp DNA mini kit, Qiagen). A negative control containing only bulk milk was processed in parallel in each experiment.

Detection of MAP viability using qPCR

DNA was amplified using qPCR targeting the F57 sequence. The primer sequences were already described (Ricchi *et al.*, 2011), while the probe sequence was: FAM – 5′- TCCAGGAACGCTTGGCACTCG – 3′- TAMRA. An IS900 qPCR was also used to improve the limit of detection of the procedure (Donaghy *et al.*, 2011). In fact, only one copy of F57 is present in the genome of MAP, while IS900 is present in 14–18 copies. Some doubts about the analytical specificity of IS900-PCR have been raised due to the presence of IS900-like sequences in other mycobacteria. Nevertheless, the specificity of the IS900 qPCR used in the study was already tested by Donaghy (Donaghy *et al.*, 2011).

The amplification reactions were performed in triplicate for each sample, in a StepOne Plus System (Life Technologies) in 20 μL of master mix containing 300 nM of each primer, 6 nM of the probe and an internal positive control (TaqMan® Exogenous Internal Positive Control, Life Technologies) to avoid any possible false-negative results due to PCR inhibition. Moreover, a positive amplification control was added to all qPCR runs to check the efficiency of each reaction. All qPCRs were performed under the same conditions: 40 cycles of denaturation for 15 s at 95 °C and annealing/elongation for 60 s at 60 °C. All data, with the exception of those relative to the evaluation of limit of detection, are reported as the difference (ΔCq) between different treatments (with and without PMA) and viability status (live and dead bacteria) relative to each qPCR run, according to the following equations:

$$\Delta Cq_{wPMA} = Cq\,of\,100\%\,dead_{wPMA} - Cq100\%\,live_{wPMA} \quad (1)$$

$$\Delta Cq_{dead} = Cq\,of\,100\%\,dead_{wPMA} - Cq100\%\,dead_{w/oPMA} \quad (2)$$

$$\Delta Cq_{live} = Cq\,of\,100\%\,live_{wPMA} - Cq100\%\,live_{w/oPMA} \quad (3)$$

$$\Delta Cq_{w-w/oPMA} = Cq_{wPMA} - Cq_{w/oPMA} \quad (4)$$

Evaluation of the method in artificially contaminated raw milk after pasteurization

MAP-negative raw milk samples were spiked with live MAP cells in a final concentration of 10^4 CFU mL^{-1}. Half of the spiked milk aliquots were pasteurized while the other half were maintained at room temperature. At the end of pasteurization, both milk samples were submitted to PMS and then treated or not with PMA. Pasteurization was carried out by processing 200 mL of milk as described elsewhere (Grant *et al.*, 1996). Contaminated milk was maintained in agitation with a magnetic stirrer for 30 min at 63.5 °C, using a probe for temperature control. MAP cell quantification was performed using F57 and IS900 qPCR plasmid dilutions (see below). Two independent experiments were conducted.

Limits of detection and analytical specificity

Tenfold serial dilutions of a pure liquid culture of MAP ATCC 19698, ranging from *c.* 10^5 to 10^0 CFU mL^{-1} of both living and killed MAP cells, were used for spiking milk. The spiked samples were then submitted to PMS and PMA treatment. Absolute MAP quantity was determined by calibration curves generated using plasmid standards containing the F57 or the IS900 product inserts, as reported by Slana *et al.* (2008). These standard curves were derived from plasmid standard 10-fold dilutions. Each qPCR for the quantification of MAP was run with plasmid standard dilutions, and the number of MAP cells was calculated on the basis of the Cq obtained for the plasmid standards. MAP concentration was expressed as MAP cells mL^{-1}. Only the number of MAP mL^{-1} of milk relative to samples containing 100% live cells without PMA treatment was reported (see Supporting Information, Table S1). Three independent experiments were conducted.

The analytical specificity of the method was expected to be very high, because the entire procedure encompasses two steps (PMS and qPCR), whose analytical specificities have already been independently tested in other studies (Foddai *et al.*, 2010; Donaghy *et al.*, 2011; Ricchi *et al.*, 2011).

Statistical analysis

Statistical analysis was performed using Kruskall–Wallis test followed by the Mann–Whitney *U*-test (STATA 12, Texas). The Ordinal Multinomial Logistic Regression was

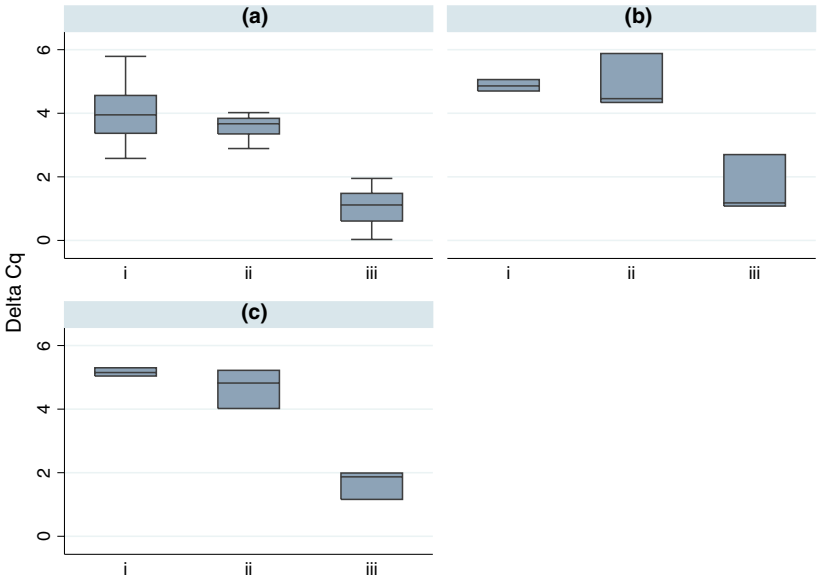

Fig. 1. Box and whisker plots of the differences in Cqs (△Cq) relative to the difference between 100% dead w PMA – 100% live w PMA (i), 100% dead w PMA−100% dead w/o PMA (ii) and 100% live w PMA−100% live w/o PMA (iii) for three different strains. The data confirmed the inhibitory effect of PMA on PCR in dead cells even after the PMS phase. (a) Box and whisker plot relative to ATCC 19698 strain (n = 10). The analysis using Kruskall–Wallis test ($P < 0.000$) followed by Mann–Whitney U–test showed the △Cq live (Eqn. 3; iii) was statistically different from those relative to other differences ($P < 0.000$). (b) Box and whisker plot relative to IZSLER 635/11 (n = 3) strain (Kruskall–Wallis test $P = 0.051$). (c) Box and whisker plot relative to IZSLER 917/11 strain (n = 3) (Kruskall–Wallis test $P = 0.061$).

performed using STATISTICA 9 software (StatSoft Inc., Tulsa, OK). A $P < 0.05$ was considered to be statistically significant.

Results and discussion

The conditions for the PMA treatment reported elsewhere (Kralik *et al.*, 2010) were checked to ascertain if they were applicable even after the PMS process. For this purpose, three different strains of MAP (ATCC 19698 and two field isolates, IZSLER 653/11 and IZSLER 917/11) were used. As Kralik previously reported (2010), the treatment with PMA, even after PMS capture, induced a consistent increase in the quantification cycle (Cq) only in the samples containing 100% dead cells. Figure 1 shows the difference in Cq (ΔCq) obtained in F57-qPCR for the DNA isolated from dead and live cells treated or untreated with PMA after the PMS capture for all MAP strains (ATCC 19698 and both field MAP isolates IZSLER 917 and 635). The $\Delta Cq_{\text{w PMA}}$ (Eqn. 1) was *c.* 4–5 cycles (i) and a similar result was achieved for ΔCq_{dead} (Eqn. 2; (ii). Taken together, the data confirmed the specific inhibitory effect of PMA in qPCR of dead cells even after the PMS process. Similar studies (Nocker *et al.*, 2006; Kralik *et al.*, 2010) have reported that *M. avium* subsp. *avium* and MAP showed the lowest signal reduction for comparing dead and live cells among the bacteria species tested. This could be due to the robustness of the MAP cell wall, a feature shared with other members of the genera *Mycobacteria,* which results in lower penetration of PMA even into dead cells. As Kralik reported (2010), we also found the use of lower concentrations of PMA (2 and 5 μM) resulted in a decrease in the ΔCq between dead and live cells ($\Delta Cq_{\text{w PMA}}$), making the assay less useful for the viability assessment.

Table 1. Statistical comparison of the mixed ratios of dead and live MAP cells

		ΔCq*			
Cell status	Percentage dead vs. live cells	Median	25th Perc.	75th Perc.	P values†
All dead	100 vs. 0	4.67	3.92	5.39	0.0026‡
	75 vs. 25	3.32	3.01	3.34	
Mixed population	50 vs. 50	2.87	2.78	2.94	0.0145§
	25 vs. 75	2.43	2.19	2.94	
All alive	0 vs. 100	1.75	1.56	2.08	0.0090¶

*$\Delta Cq = Cq_{\text{w PMA}} - Cq_{\text{w/o PMA}}$ relative to each single experiment (n = 5).

†Statistical analysis was performed considering cell status groups using the Kruskall–Wallis test ($P = 0.0014$) followed by *post hoc* Mann–Whitney U-test analysis (P values shown in table). The difference between cell status groups was considered statistically significant for $P \leq 0.0167$.

‡All dead vs. mixed population.

§Mixed population vs. all alive.

¶All alive vs. all dead.

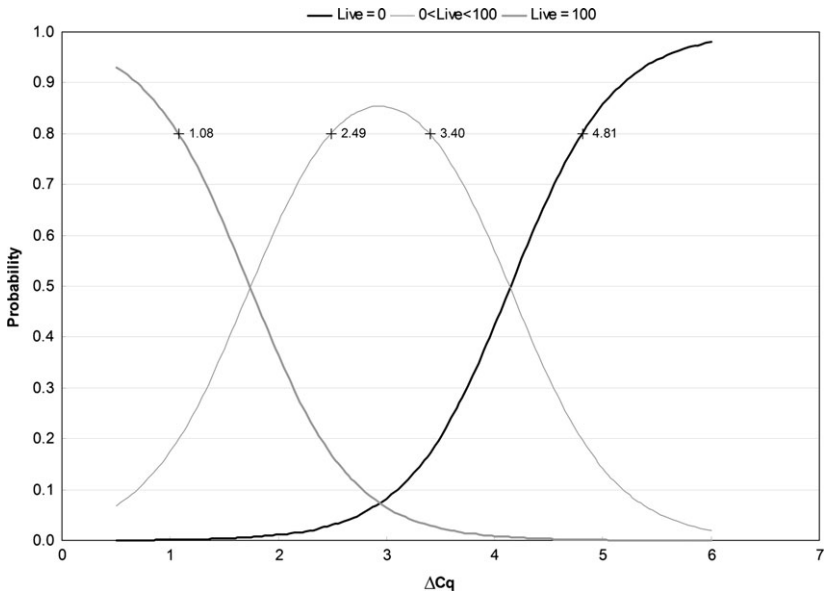

Fig. 2. Plot showing the probability of a sample belonging to different groups differentiated according to *Mycobacterium avium* subsp. *paratuberculosis* viability.

In contrast, the ΔCq_{live} (Eqn. 3) was lower with respect to the other two ΔCq results (iii). This last value should have been approximately around zero because the inhibitory effect of the PMA on qPCR should be specific only for dead cells. However, as previously reported not only for MAP (Kralik *et al.*, 2010) but also for other bacteria (Nocker *et al.*, 2006), this difference was around 1–2 Cq.

This method was then checked for its ability to discriminate among artificially spiked mixed populations (containing dead/live cells), with different percentages of dead and live cells. Cq values obtained in samples without PMA treatment were subtracted from those obtained in the same kind of sample treated with PMA ($\Delta Cq_{w-w/o\ PMA}$, Eqn. 4) and achieved by targeting F57 (Table 1). Overall, we found the method can discriminate the viability status of MAP cells, according to the percentage of dead cells present in the mixed population, showing a correlated trend in the ΔCq values. Moreover, by grouping the different percentages of dead and live cells into three groups (all dead, all live and mixed population), the above-mentioned $\Delta Cq_{w-w/o\ PMA}$ values were statistically different. Based on these data, an Ordinal Multinomial Logistic Regression analysis allowed us to generate a model to determine the probability that a sample will contain different populations of MAP (Fig. 2). Using this model to compare the results of different proportions of viable and dead cells, we were able to estimate the probability that a given sample contains a certain MAP cell population (dead, live or mixed). Basically, if the $\Delta Cq_{w-w/o\ PMA}$ value is lower than 1.08 cycles, there is at least an 80% probability that the sample contains only live MAP cells; if the $\Delta Cq_{w-w/o\ PMA}$ value is higher than 4.81, there is at least an 80% probability that the sample contains only dead MAP cells; and if the $\Delta Cq_{w-w/o\ PMA}$ value is between 2.49 and 3.4, there is at least an 80% probability that the sample contains both viable and dead MAP cells. The MAP cell population with the highest probability of occurrence

Table 2. Probability of milk samples before (raw milk) and after pasteurization (pausterized milk) to contain different population of only dead, only live and mixed MAP cells basing on their ΔCq

	F57 target		IS900 target	
	pasteurized milk	Raw milk	Pasteurized milk	Raw milk
ΔCq* (Median, 25th–75th Perc.)	3.93 (2.89–4.79)	1.60 (1.46–1.91)	3.09 (2.85–3.25)	1.40 (1.05–1.84)
Probability of detection of dead cells only†	38.3%	0.5%	9.8%	0.3%
Probability of detection of live cells only†	1.0%	57.0%	5.5%	66.9%
Probability of detection of mixed cells†	60.2%	42.5%	84.8%	32.8%

Theoretical input was 5×10^4 CFU mL^{-1} (see Materials and methods).

*ΔCq = Milk sample $Cq_{w\ PMA}$−Milk sample $Cq_{w/o\ PMA}$.

†Based on model obtained by Ordinal Multinomial Logistic Regression analysis.

should be subsequently used for the final interpretation of results. A very similar interpretation of the $\Delta Cq_{w-w/o\ PMA}$ values was proposed by Kobayashi *et al.*, 2010 for the evaluation of antibiotic activity on *Staphylococcus* spp. Fittipaldi *et al.*, 2011, evaluated the percentage of viable cells considering not only the $\Delta Cq_{w-w/o\ PMA}$ (Eqn. 4), but also a supplemental qPCR performed by inducing the death of all cells present in the sample analyzed before the treatment with PMA. This last qPCR was used to detect the false-positive viable cells eventually present because not all the DNA from dead cells is inactivated by PMA. However, the same paper showed that the percentages of false-positive cells with respect to the total population screened are generally low (< 1%).

The above-mentioned model was then used for testing spiked raw milk before and after pasteurization (Table 2). The $\Delta Cq_{w-w/o\ PMA}$ (Eqn. 4) for F57 and IS900 showed that with the highest probability, the conditions of pasteurization were not stringent enough to completely devitalize all MAP cells in the milk (Table 2). In fact, for nonpasteurized milk, the highest probability refers to the presence of only viable MAP cells (57.0% for F57 and 66.9% for IS900 assay), while for the pasteurized milk, the highest probability corresponds with the presence of a mixed population of live and dead cells (60.2% for F57 and 84.4% for IS900 assay; Table 2). This result essentially confirms other studies about the resistance of MAP to the pasteurization process (Grant *et al.*, 1996, 2005; Collins, 2011).

MAP concentration in both individual and bulk tank milk rarely exceeded 10^2 cells mL^{-1} (Herthnek *et al.*, 2008; Slana *et al.*, 2008). After PMA treatment of samples containing 100% dead cells, our method was able to consistently detect up to 5.0×10^2 CFU mL^{-1} (IS900 target, see Table S1). For this reason, we recognize the analytical sensitivity of the method needs be improved before its use on commercial milk.

In conclusion, the combination of PMS and PMA-qPCR allowed the detection of viable MAP cells in milk samples. The Ordinal Multinomial Logistic Regression model allows estimating the probability that a given sample contains different population of viable, mixed or dead MAP cells.

Further studies are necessary to improve the analytical sensitivity of the assay.

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Author contribution

M.R. and C.D.C. contributed equally to this work.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Limits of detection of the PMS and PMA-qPCR in milk.

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ORIGINAL RESEARCH
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The Impact of the Antimicrobial Compounds Produced by Lactic Acid Bacteria on the Growth Performance of *Mycobacterium avium* subsp. *paratuberculosis*

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Cell-free supernatants (CFSs) extracted from various lactic acid bacteria (LAB) cultures were applied to *Mycobacterium avium* subsp. *paratuberculosis* (MAP) cells to determine their effect on MAP viability. In addition, 5% lactic acid (LA; pH 3) and commercially synthetized nisin bacteriocin were also tested. This procedure was chosen in order to mimic the influence of LAB compounds during the production and storage of fermented milk products, which can be contaminated by MAP. Its presence in milk and milk products is of public concern due to the possible ingestion of MAP by consumers and the discussed role of MAP in Crohn's disease. Propidium monoazide real-time PCR (PMA qPCR) was used for viability determination. Although all CFS showed significant effects on MAP viability, two distinct groups of CFS – effective and less effective – could be distinguished. The effective CFSs were extracted from various lactobacilli cultures, their pH values were mostly lower than 4.5, and their application resulted in >2 $\log_{10}$ reductions in MAP viability. The group of less effective CFS were filtered from *Lactococcus* and enterococci cultures, their pH values were higher than 4.5, and their effect on MAP viability was <2 $\log_{10}$. LA elicited a reduction in MAP viability that was similar to that of the group of less effective CFS. Almost no effect was found when using commercially synthetized nisin at concentrations of 0.1–1000 μg/ml. A combination of the influence of the type of bacteriocin, the length of its action, bacteriocin production strain, and pH are all probably required for a successful reduction in MAP viability. However, certain bacteriocins and their respective LAB strains (*Lactobacillus* sp.) appear to play a greater role in reducing the viability of MAP than pH.

Keywords: bacteriocin, cell-free supernatant, paratuberculosis, pH, lactic acid, viability, propidium monoazide, real-time PCR

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INTRODUCTION

A variety of Gram-positive and Gram-negative bacteria are capable of producing compounds with antimicrobial activity that inhibits the growth of other bacteria – bacteriocins. Simple bacteriocins are chemically small- or medium-sized peptides or proteins. They are ribosomally synthetized and usually undergo post-translational modification. Their activity is directed against related species or

even against bacteria of the same strain (Zacharof and Lovitt, 2012). Bacteriocins are synthetized by many bacterial species, including lactic acid bacteria (LAB). Apart from bacteriocins, LAB produce organic acids, such as lactic and acetic acids, hydrogen peroxide, or diacetyl. All these compounds have antimicrobial properties, which underlies the use of LAB in the food industry as inhibitors of food-borne pathogens (probiotic cultures). LAB bacteriocins have the potential to be used in food biopreservation (Galvez et al., 2007). At this time, only nisin, as the most well-studied and best-known bacteriocin, has been approved for use in the food industry (O'Sullivan et al., 2002).

Mycobacterium avium subsp. *paratuberculosis* (MAP) causes an infectious and chronic disease in ruminants called paratuberculosis. The clinical phase is characterized by diarrhea, emaciation, and low milk yield, and is preceded by a long, latent phase (years) during which other, healthy animals can be infected. Therefore, the disease is mostly of importance in cattle herds. Infected animals can shed MAP in milk (Slana et al., 2008). This is of public concern due to the possible ingestion of MAP and its potential link to Crohn's disease (Hermon-Taylor, 2009; Chiodini et al., 2012). In the past, many studies investigated the ability of MAP to survive unfavorable conditions in dairy products; however, their results were not always consistent. Ability of MAP to survive pasteurization process was demonstrated in many studies (Grant et al., 2002; Carvalho et al., 2012), but the others did not confirm it (Serraino et al., 2017). Similarly, it applies to the fermentation process. MAP is capable to surive fermentation (Donaghy et al., 2004; Hanifian, 2014), but on the other hand, it is evident that its growth is suppressed during the fermentation and storage of milk products such as cheese (Spahr and Schafroth, 2001; Donaghy et al., 2004; Hanifian, 2014) or yogurts (Van Brandt et al., 2011; Klanicova et al., 2012). As the role of MAP in Crohn's disease is still not clear, reducing exposure of consumers thought reducing MAP contamination in food is desirable.

Utilization of propidium monoazide treatment followed by real-time PCR based on the amplification of the *F57* fragment (PMA qPCR) was previously employed to study MAP viability after exposure to diverse conditions (Pribylova et al., 2012; Kralik et al., 2014). The method combines the advantages of cultivation and qPCR. Results are obtained quickly, and the method is sensitive, specific, and provides quantitative data. The PMA dye selectively penetrates dead cells with compromised membranes and covalently binds to its DNA. After photoactivation of the dye (exposure to bright light), it forms insoluble complex with DNA and prevents its amplification in subsequent PCR. At the same time, the excess dye, which has not penetrated into the damaged cells, is inactivated by light. The portion of viable cells is then calculated as the quotient of PMA-treated stressed cells and the control (Nocker et al., 2006; Kralik et al., 2010). Previous reports have shown that the experimentally determined difference between completely live and dead (heat-treated) MAP cells using PMA qPCR is about 2 $\log_{10}$ (Kralik et al., 2010). As viability determination using culture and PMA qPCR has been described to yield comparable results, the latter can be applied for the determination of MAP viability as the sole method (Pribylova et al., 2012).

In previous publications, the effect of whole milk products (yogurts, acidophilus milk, etc.) containing LAB and the products of their metabolism on the viability of mycobacteria was studied (Van Brandt et al., 2011; Klanicova et al., 2012). The aim of this current study was to assess the viability of three MAP isolates after exposure to filtered cell-free supernatants (CFSs) acquired by the propagation of LAB cultures in their respective media in accordance with standard culture procedures. This procedure was chosen in order to mimic the influence of LAB compounds during the production and storage of fermented products. In addition to CFS, the effects of different concentrations of commercially synthetized nisin and one concentration of lactic acid (LA) on MAP viability were assessed. The viability of MAP strains and isolates was measured using the culture-independent method consisting of treatment with PMA combined with specific qPCR.

MATERIALS AND METHODS

Bacterial Isolates and Culture Conditions

The MAP laboratory strain CAPM 6381 (The Collection of Animal Pathogenic Microorganisms, Veterinary Research Institute, Czechia) and field isolates 7082 (white deer) and NL-3 (cattle) were used throughout the whole study. All strains and isolates were of RFLP type C1.

All strains and isolates were grown on Herrold's egg yolk medium (HEYM) with penicillin G, chloramphenicol, and amphotericin B (Becton Dickinson, Franklin Lakes, NJ, United States), supplemented with 2 μg/ml Mycobactin J (Allied Monitor, Fayette, MO, United States) at 37°C for 2–3 months until visible colonies were observed. Afterward, grown colonies were harvested using a loop and resuspended in 1.5 ml of Middlebrook 7H9 (M7H9) broth supplemented with 10% (vol/vol) Middlebrook OADC enrichment (both Becton Dickinson, Franklin Lakes, NJ, United States). In order to homogenize the MAP suspensions, 12 1-mm zirconia/silica beads (Biospec, Bartlesville, OK, United States) were added followed by vortexing for 30 s. To remove the MAP clumps, the suspension was centrifuged at 100 × *g* for 30 s and the upper cell fraction was resuspended in fresh M7H9 broth. The suspension was diluted to $OD_{600} \approx 0.15–0.20$, which corresponds to approximately 10^8 MAP cells/ml of suspension (Kralik et al., 2011).

Preparation of Milk Starter Cultures for CFS

Nine bacterial strains used as milk starter cultures originating from the Lactoflora® collection of milk cultures (Milcom, Prague, Czechia) were propagated in their respective media – M17 and MRS broth (both Oxoid CZ, Brno, Czechia). Each culture was characterized in detail with respect to its culture conditions, active acidity, and production of bacteriocins (**Table 1**). The type of bacteriocin was determined by the well method, PCR, or a combination of both. Each milk starter culture was cultured in 250 ml of its respective medium for 18 h. Afterward, the culture of starter bacteria was filtered through a 0.22-μm microbiological filter to prepare CFS. The sterilized, filtered

TABLE 1 | Characteristics of milk starter cultures used in this study.

No.	Strain	Species	Culture conditions		Active acidity	Bacteriocin production
S1	CCDM 731	*Lactococcus lactis* subsp. *Lactis*	37°C 18 h	M17 broth	4.65	Nisin
S2	CCDM 857	*Enterococcus faecalis*		M17 broth	4.51	Enterocin
S3	CCDM 108	*Lactobacillus helveticus*		MRS broth	4.14	Enterocin
S4	RL 26-P	*Lactobacillus plantarum*		MRS broth	3.67	Plantaricin
S5	CCM 7165	*Enterococcus faecium*		M17 broth	4.68	Enterocin
S6	RL 23-P	*Lactobacillus* sp.		MRS broth	4.11	Plantaricin
S7	CCDM 768	*Lactobacillus helveticus*		MRS broth	4.07	Enterocin
S8	CCDM 182	*Lactobacillus plantarum*		MRS broth	3.81	Plantaricin
S9	RL 22-P	*Lactobacillus gasseri*		MRS broth	4.51	Plantaricin

CFSs were subsequently applied to MAP strains and isolates to determine their influence on MAP viability over time.

Nisin and LA Preparation

In order to mimic the effect of bacteriocins and reduced pH separately, MAP suspensions were exposed to nisin and solutions of LA. Commercially available nisin (Sigma, St. Louis, MO, United States) was diluted in water and added to the M7H9 media to prepare solutions with final concentrations of 0.1, 1, 10, 100, and 1000 μg of nisin/ml of M7H9. A solution of LA was diluted in M7H9 media to a concentration of 5% (v/v) and the pH was adjusted to 3. This value was chosen with respect to the pH of CFS and previous studies (Klanicova et al., 2012).

Exposure of Antimicrobial Substances on the Viability of MAP Strains and Isolates

On day 0, 6 ml of each of the three MAP strains and isolates prepared as described above was aliquoted into 16 tubes (nine milk culture starters, five concentrations of nisin, LA treatment, and control) in biological triplicates. All the tubes were centrifuged at 7000 × *g* for 3 min and the supernatant was replaced by 6 ml of filtered CFS, solutions of nisin, and LA (or respective medium – MRS or M17 broth as a control sample). All the samples were placed in an incubator set at 37°C. In order to mimic the influence of antimicrobial compounds on MAP viability during the storage of the fermented product, all samples were taken on days 4, 8, and 12.

PMA Treatment

The viability of MAP after exposure to conditions suppressing bacterial growth, which are induced by fermentation and last during the production and maturation of the fermented product, was determined by the PMA procedure combined with subsequent *F57* qPCR (Kralik et al., 2010, 2014; Pribylova et al., 2012). On each sampling day (days 4, 8, and 12), 3× 0.5-ml aliquots were taken from each sample (technical triplicates). All samples were centrifuged (7000 × *g* for 3 min) and the supernatant was replaced with fresh medium. To each 500 μl sample (including controls), 12.5 μl of 1 mM PMA (Biotium Inc., Hayward, CA, United States) stock solution dissolved in 20% DMSO was added (final concentration 25 μM). The mixture was incubated in the dark for 5 min with mixing at 20 Hz and then placed on ice and exposed to light from a 650-W halogen bulb (B&H Photo Video, New York, NY, United States) for 2 min. The whole process of incubation in the dark and light exposure was repeated once again with a freshly added aliquot of PMA. After the PMA treatment, the samples were centrifuged at 7000 × *g* for 3 min and the supernatant was replaced with 500 μl of Tris-EDTA (TE) buffer supplemented with 50 ng/μl of fish sperm DNA (both SERVA Electrophoresis, Heidelberg, Germany). The MAP cells were lysed by incubation at 100°C for 20 min, and after centrifugation at 18,000 × *g* for 5 min, the supernatant served as a template for the qPCR.

F57 qPCR

Quantification of MAP cells for the purposes of viability determination was performed using a qPCR assay amplifying the single copy fragment *F57* according to a calibration curve based on serial dilution of a plasmid gradient on a LightCycler 480 instrument (Roche Molecular Diagnostic, Germany; Slana et al., 2008). MAP viability was determined as the quotient of absolute numbers of PMA-exposed stress factor-treated cells (treated with CFS, nisin, or LA) and the PMA-exposed control (stress factor untreated; MAP in the respective broth) cells (Kralik et al., 2010, 2014; Pribylova et al., 2012).

Statistical Analysis

All the statistical calculations were performed using Statistica 13.0 software (StatSoft, Tulsa, OK, United States). Three-way ANOVA (with factors CFS, time, strain and CFS, time, concentration, respectively) was used for statistical evaluation of the effect of CFS and nisin, respectively, on MAP strain viability. For the determination of LA influence on MAP viability, two-way ANOVA was used (with factors time and strain). *P*-values <0.05 were considered statistically significant.

RESULTS

Effect of CFS on MAP Viability

Based on their impact on MAP viability, two groups of CFS could be distinguished. Application of S3, S4, S6, S7, S8, and S9 CFS caused a significant reduction in the number of surviving cells (at least 2 $\log_{10}$, i.e., below 1%), while in the case of the group containing S1, S2, and S5 CFS the viability reduction was about maximum 1 $\log_{10}$ (**Figure 1**). CFS could therefore be divided into

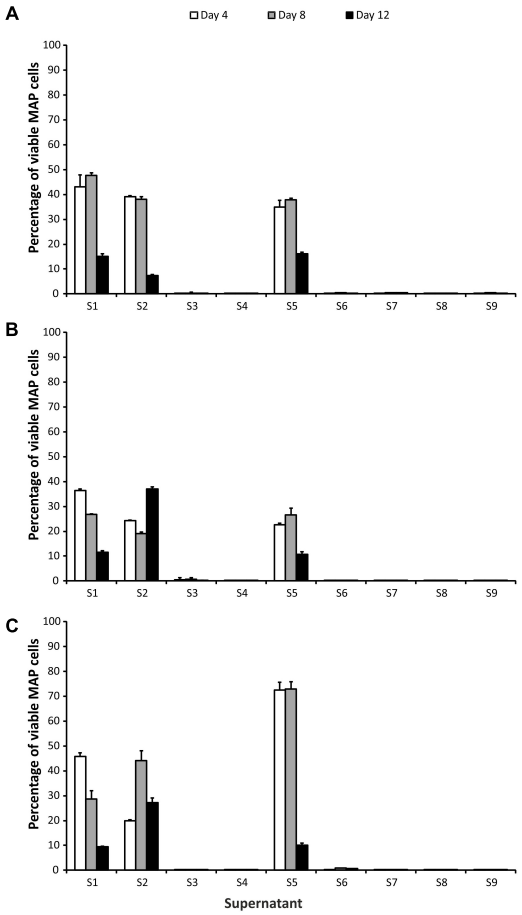

FIGURE 1 | Effect of supernatants on *Mycobacterium avium* subsp. *paratuberculosis* (MAP) viability. **(A)** Field isolate 7082 (white deer); **(B)** collection strain 6381; **(C)** field isolate NL-3 (cattle). Vertical bars represent sample standard deviations. The portion of viable cells is calculated as the quotient of PMA-treated stressed cells (supernatants) and the PMA-treated control (stress factor untreated; MAP in the respective broth).

effective (S3, S4, S6, S7, S8, and S9) and less effective (S1, S2, and S5), with a discriminatory value for classification into one or the other group of 1% (this corresponds to an average decrease in the number of surviving cells of 2 $\log_{10}$). To demonstrate the effect of effective and less effective CFS, ANOVA with three factors (CFS, time, and strain) was performed.

In the group of effective CFS, it was shown that while there was no statistically significant difference between the strains ($P > 0.05$, F-test), the CFS and the time factor were statistically significant variables ($P < 0.01$ and $P < 0.05$, respectively). The interaction of CFS and strain was also significant ($P < 0.05$). Subsequent testing confirmed that there was a significant difference between S4 and S6 ($P < 0.05$; Tukey's HSD test) and between S6 and S8 ($P < 0.05$). In the case of the time factor, a statistically significant difference between days 4 and 8 ($P < 0.05$) was demonstrated.

No difference was found among the CFS in the less effective group ($P > 0.05$; F-test). The strain and the time factor were statistically significant variables here (both $P < 0.01$) as well as all factor interactions. Significant differences between NL-3 and 6381 ($P < 0.01$; Tukey's HSD test) and 6381 and 7082 ($P < 0.05$) isolates were proven (**Figure 1**). Statistically significant differences between days 4 and 12 and between days 8 and 12 (in both cases $P < 0.01$) were also evident (**Figure 1**).

Effects of Nisin and LA on MAP Viability

Three-way ANOVA (time, strain, and concentration) was used to estimate the influence of nisin on MAP viability. There was no statistically significant impact of nisin exposure on any of the MAP isolates, despite the fact that the concentrations of nisin covered a larger than 5 $\log_{10}$ range (10^{-1}–10^{3} μg/ml; **Figure 2**).

The percentage of surviving MAP cells was reduced by less than 2 $\log_{10}$ after LA exposure. With respect to CFS, the LA would

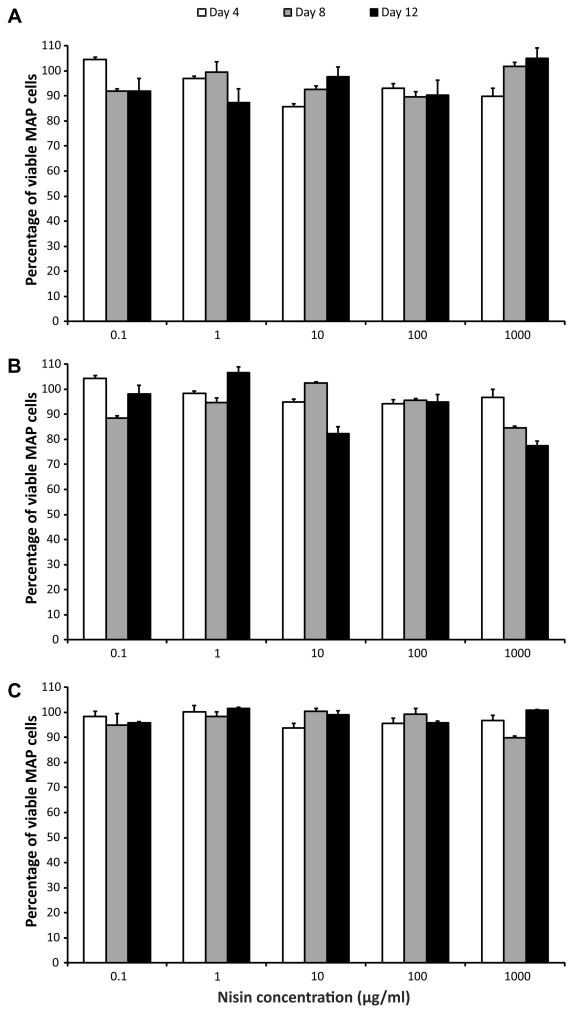

FIGURE 2 | Effect of nisin on MAP viability. **(A)** Field isolate 7082 (white deer); **(B)** collection strain 6381; **(C)** field isolate NL-3 (cattle). Vertical bars represent sample standard deviations. The portion of viable cells is calculated as the quotient of PMA-treated stressed cells (nisin) and the PMA-treated control (stress factor untreated; MAP in the respective broth).

thus be described as less effective. The percentage of surviving cells decreased over time for all strains (**Figure 3**). Both strain and time factors were statistically significant ($P < 0.01$; F-test). Difference between days 4 and 8 and between days 4 and 12 was confirmed ($P < 0.01$; Tukey's HSD test). The response of the NL-3 isolate was significantly different to those of CAPM 6381 and 7082 isolate ($P < 0.01$ for NL-3 and 7082). LA was approximately twice as effective in killing NL-3 MAP cells after 4 days compared with the other two isolates. At later stages of the experiment (days 8 and 12), the efficacy of MAP cell killing was equal in all strains (**Figure 3**).

DISCUSSION

All CFS exerted a significant effect on MAP viability. However, their effects were either more (S3, S4, S6, S7, S8, and S9) or less effective (S1, S2, and S5). No viable MAP were found after 4 days of culture with effective CFS. The effective CFSs were products of *Lactobacillus* sp., while enterococci and *Lactococcus lactis* subsp. *lactis* were producers of the less effective CFS group (**Table 1**). In the previous study, addition of CFS from a number of *Lb. paracasei* isolates delayed the growth of MAP. Application of CFS from *Lb. casei* and *Lb. rhamnosus* led to suppression of the increase in growth typical for MAP metabolic activity (Donaghy et al., 2005). Glass et al. (2004) showed CFS from *Lb. acidophilus* and *Lb. reuteri* significantly decreased viability of vegetative cells and spores of *Cryptosporidium parvum*. Gaggia et al. (2010) also tested the effect of various CFS derived from LAB on MAP viability; one strain of *Lb. plantarum* elicited a significant decrease in MAP intracellular pH and led to loss of its viability. The *Lb. plantarum* strain produced plantaricin, while the others, not effective LAB strains contained enterocins (Gaggia et al., 2010). In consistence with Gaggia et al. (2010), plantaricins were exclusively covered in the group of effective CFS in our case and showed significant decrease in MAP viability. On the other hand, the group of effective CFS also included enterocins, which were nevertheless covered in the group of less effective CFS as well (**Table 1**). It is known that different enterocins show different inhibitory spectra toward selected LAB and pathogens (Moreno et al., 2003). This could explain different effect of various enterocins on MAP viability within this study (effective and less effective CFS). The differing inhibitory effects of enterocins on MAP could probably be also explained by different LAB producers. While effective enterocins (and plantaricins as well) were produced only by lactobacilli (*Lb. helveticus*, *Lb. plantarum*, *L. gasseri*, and *Lb.* sp.), less effective enterocins and nisin were synthetized by *Lactococcus* and enterococci (**Table 1** and **Figure 1**).

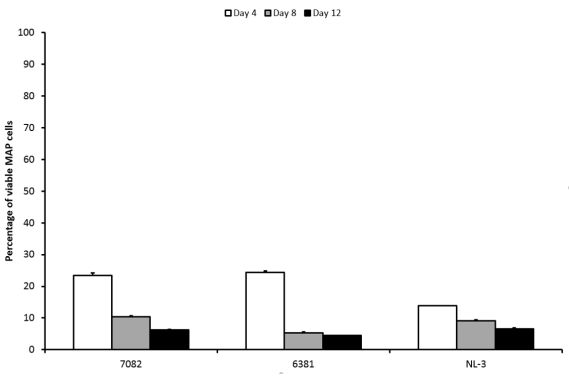

FIGURE 3 | Effect of LA on viability of three MAP isolates. Vertical bars represent sample standard deviations. The portion of viable cells is calculated as the quotient of PMA-treated stressed cells (LA) and the PMA-treated control (stress factor untreated; MAP in the respective broth).

pH values differed slightly between the two groups. Values of around 4 or slightly lower were observed in the effective group of CFS (except for S9 with its limit value), while values between 4.5 and 5.0 were measured in the less effective group. The correlation between pH and bacteriocin activity was observed previously by Moreno et al. (2003). They found that the activity and stability of enterocins are higher at low pH. This can be ascribed to the solubility of enterocins which is increasing with decreasing pH (Moreno et al., 2003). Klanicova et al. (2012) described a significant inhibition of MAP growth when pH values of fermented milk products dropped below 4. The production of organic acids and subsequent lowering of pH were shown to be the main inhibitory mechanism of bifidobacteria toward Gram-negative bacteria in another study. According to the authors, the bacteriocins of bifidobacteria have only negligible effect on Gram-negative bacteria compared to the production of organic acids and lowering of pH (Makras and De Vuyst, 2006). On the contrary, another study suggested that the inhibitory effect of lactobacilli themselves rather than acid production is responsible for MAP growth delay (Donaghy et al., 2005). The same observations were made by Van Brandt et al. (2011) who did not note any changes in MAP counts after storage of commercial yogurts (low pH) with inoculated MAP. Thus, the question of whether bacteriocins or pH have the critical impact on MAP viability is still open. Based on the results with CFS themselves, it seems that the type of bacteriocin, the length of its action, LAB production strain, and pH are all involved in the inhibition of MAP growth.

A vast range of nisin concentrations (0.1–1000 μg of nisin/ml media) was selected to cover the maximal range of concentrations. Nevertheless, there was almost no decrease in MAP viability at any concentration (**Figure 2**). In the study using alamarBlue reagent, the effect of various concentrations of nisin on *M. kansasii*, *M. tuberculosis*, and MAP viability was examined. Nisin showed good efficacy against MAP, but 60 μg/ml, the highest concentration tested, was below the MIC_{90} for MAP and *M. tuberculosis* (Carroll et al., 2010). Inhibition of *M. smegmatis* growth was observed at a nisin concentration of 10 μg/ml; a much higher concentration (2500 μg/ml) was needed for *M. tuberculosis* (Chung et al., 2000). By measuring intracellular pH, a large decrease in viable MAP cells using 2.5 kU/ml (recounted as 2500 μg/ml) of nisin was showed. After 30 min, no viable cells were detected (Gaggia et al., 2010). According to these previous findings, it seems that nisin concentrations markedly above 1000 μg/ml would be needed for any substantial decrease in MAP viability. However, taking into account the study of

Carroll et al. (2010), at least some reduction in MAP viability had to be attained, but this was not demonstrated at all (**Figure 2**). Thus, another interpretation can be used to explain the almost 100% maintenance of viability after several days of treatment with even high concentrations of nisin. The PMA qPCR method is based on the penetration of PMA through pores in bacterial membranes caused by the action of antimicrobial agents. It is known that nisin binds to cytoplasmic membranes and forms pores by inserting through membranes. It was found that pores formed by nisin in *M. bovis* BCG were large enough to leak protons, but too small for ATP (Chung et al., 2000). With respect to the relatively large size of the PMA molecule and the small pores formed by nisin, a possible inability of PMA to penetrate through these pores should be considered.

The mechanism of LA antimicrobial activity is based on the passing of its undissociated forms through the cell membrane. Increased cytoplasmic pH leads to the dissociation of the acid, the release of protons, and subsequent acidification of the cytoplasm (Cotter and Hill, 2003; Reis et al., 2012). It is known that mycobacteria have higher tolerance to acidic environments compared to other bacteria (Cotter and Hill, 2003). MAP cells remained culturable for at least 7 days at pH 4 (Cook et al., 2013). In fermented milk products with pH values of around 4, MAP remained culturable for even up to 6 weeks (Klanicova et al., 2012). In our study, 5% LA with pH 3 played a role in the reduction of MAP viability; however, it had only a moderate effect. This was evidenced by the presence of 14–25% viable MAP cells after 4 days and at least 5% of viable MAP after 12 days of LA treatment (**Figure 3**). With respect to CFS, the effect of LA was comparable to the group of less effective CFS. However, the pH of the LA (pH 3) was closer to the pH value of the effective CFS (below or around 4 mostly) than to the less effective ones (above 4.5). If the pH should have a decisive impact on reducing MAP viability, then its effect should be rather comparable to the group of effective CFS. Thus, it can be assumed that although pH plays an important role in reducing the viability of MAP, a significant decrease in viability below 1% is due to bacteriocins or LAB themselves than to pH.

The effect of LA on 6381 and 7082 strains was not as considerable as on NL-3 after the first 4 days of treatment (**Figure 3**). While NL-3 is a field isolate, 6381 is a laboratory strain. MAP 7082 has gone through many passages in the laboratory and therefore it can be expected that its response will rather correspond to the response of laboratory strain. In general, laboratory strains show higher tolerance to stress factors comparing to field isolates (Gumber and Whittington, 2007; Kralik et al., 2014), which could explain more successful decrease of viability in NL-3 compared to 6381 and 7082 (**Figure 3**). Totally different situation, however, occurred with the NL-3 after its exposure to the CFS No. 5. After 4 and 8 days of the treatment, CFS No. 5 showed negligible effect on NL-3 compared to other CFS from the less effective group (Nos. 1 and 2; **Figure 1**). Unexpectedly, NL-3 also showed significantly higher tolerance to CFS No. 5 compared to laboratory strain 6381 and high-passage strain 7082 (**Figure 1**). It is unclear why only the effect of CFS No. 5 on the NL-3 viability was so insignificant, and further investigation would be required to clarify this.

To sum up, the effect of filtered CFS acquired by the propagation of LAB cultures, commercially synthetized nisin, and one concentration of LA to the viability of MAP isolates was investigated. All nine CFS showed significant effects on MAP viability. Nevertheless, two distinct groups of effective and less effective CFS could be distinguished. The effective CFSs comprising of six bacteriocins (plantaricins and enterocins) were extracted from various lactobacilli cultures (*Lb. helveticus*, *Lb. plantarum*, *L. gasseri*, and *Lb*. sp.) and their pH values were mostly around 4 (3.6–4.5). Application of these CFS resulted in >2 $\log_{10}$ reduction in MAP viability (below 1% of surviving) and no viable MAP were observed after 4 days of treatment. The group of less effective CFS (enterocins and nisin) were filtered from *L. lactis* subsp. *lactis* and enterococci cultures (*E. faecalis* and *E. faecium*) and their pH values were higher than 4.5. Less effective CFS elicited a reduction in MAP viability of <2 $\log_{10}$. LA applied at a concentration of 5% (pH 3) resulted in a reduction in MAP viability similar to that of the less effective CFS group. After 4 days of treatment, about 14–25% of MAP cells survived; 5% of viable cells was still found after 12 days of LA treatment. On the contrary, almost no effect was found when using commercially synthetized nisin at concentrations of 0.1–1000 μg/ml. It seems that the type of bacteriocin, the length of its action, LAB production strain, and pH are probably all involved in determining the success of MAP viability reduction. Nevertheless, as the effect of LA was comparable to the group of less effective CFS than those effective, it can be assumed that certain bacteriocins and their LAB production strains could play more important role in reducing the viability of MAP than the pH.

AUTHOR CONTRIBUTIONS

PK was in charge of the whole project and participated in data production, data interpretation, and drafting the manuscript. VB designed and performed the statistical analysis and revised the paper critically. RD interpreted the data and wrote up the paper. All authors read and approved the final manuscript.

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A Novel Approach to the Viability Determination of *Mycobacterium avium* subsp. *paratuberculosis* Using Platinum Compounds in Combination With Quantitative PCR

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Mycobacterium avium subsp. *paratuberculosis* (MAP) represents a slow-growing bacterium causing paratuberculosis, especially in domestic and wild ruminants. Until recently, the assessment of MAP viability relied mainly on cultivation, which is very time consuming and is unable to detect viable but non-culturable cells. Subsequently, viability PCR, a method combining sample treatment with the DNA-modifying agent ethidium monoazide (EMA) or propidium monoazide (PMA) and quantitative PCR (qPCR), was developed, enabling the selective detection of MAP cells with an intact cell membrane. However, this technology requires a laborious procedure involving the need to work in the dark and on ice. In our study, a method based on a combination of platinum compound treatment and qPCR, which does not require such a demanding procedure, was investigated to determine mycobacterial cell viability. The conditions of platinum compound treatment were optimized for the fast-growing mycobacterium *M. smegmatis* using live and heat-killed cells. The optimal conditions consisting of a single treatment with 100 μM *cis*-dichlorodiammine platinum(II) for 60 min at 5°C resulted in a difference in quantification cycle (Cq) values between live and dead membrane-compromised mycobacterial cells of about 6 Cq corresponding to about 2 $\log_{10}$ units. This optimized viability assay was eventually applied to MAP cells and demonstrated a better ability to distinguish between live and heat-killed mycobacteria as compared to PMA. The viability assay combining the Pt treatment with qPCR thereby proved to be a promising method for the enumeration of viable MAP cells in foodstuffs, environmental, and clinical samples which could replace the time-consuming cultivation or laborious procedures required when using PMA.

Keywords: viability, qPCR, live-dead discrimination, platinum, mycobacteria, *Mycobacterium avium* subsp. *paratuberculosis*, propidium monoazide

INTRODUCTION

The detection of mycobacteria in recent years relied mainly on cultivation-based methods. However, these are only able to detect viable mycobacteria if they are cultivable, which may represent a substantial deficiency of these conventional methods. The number of mycobacteria detected by the culture may be reduced by those present in a dormant or viable non-culturable state (Elguezabal et al., 2011). In addition, the cultivation of slow-growing mycobacterial species such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), which is the causative agent of paratuberculosis, particularly in domestic and wild ruminants is very time consuming (several weeks to months). Another disadvantage of MAP cultivation is the need for chemical decontamination of the sample to suppress the growth of competitive microorganisms, which can result in a decrease in the sensitivity of detection (Slana et al., 2008). Moreover, the number of MAP cells may be underestimated due to the tendency of MAP cells to form clumps (Pickup et al., 2005).

The introduction of quantitative PCR (qPCR) has overcome these limitations, as qPCR is fast, more sensitive, and capable of detecting and quantifying even non-cultivable bacteria. qPCR systems targeting the multicopy element IS*900* and the single copy element *F57* have been developed for the specific detection and quantification of MAP, respectively (Slana et al., 2008). However, the disadvantage of qPCR is the inability to distinguish live bacteria from dead ones (live-dead discrimination), which makes this method unsuitable for assessing MAP viability. In past years, DNA intercalating dyes—ethidium monoazide (EMA) and propidium monoazide (PMA)—have been shown to enable viability determination in combination with PCR (Nogva et al., 2003; Nocker et al., 2006). These dyes penetrate membrane-compromised non-viable cells in which they form a covalent link to DNA, thereby suppressing the target nucleic acid amplification in qPCR following the cell treatment and genomic DNA extraction. Conversely, EMA and PMA do not permeate into live bacteria with an intact cell membrane, or do so to only a limited extent. An approach combining qPCR with the intercalation dyes EMA or PMA has already been applied to a wide range of gram-negative and gram-positive bacteria (Nocker and Camper, 2009). Furthermore, PMA-qPCR has also been used for the evaluation of the killing efficiency by disinfection or heat, both of which cause cell membrane damage (Nocker et al., 2007). PMA-qPCR has also been introduced for viable MAP cell quantification targeting *F57* (Kralik et al., 2010). The disadvantage of EMA/PMA-qPCR is the laborious procedure involving the need to work in the dark, since these compounds are activated by visible light. It is also necessary to keep the bacterial suspension on ice to avoid disruption of the cell wall or membrane of live cells due to an increase in temperature from light exposure with a halogen lamp, which may subsequently result in the undesirable penetration of the agents into live cells (Fittipaldi et al., 2012). This has led to the search for other compounds that enable live-dead discrimination without the need for such a demanding procedure.

Lately, platinum (Pt) and palladium (Pd) compounds—e.g., dichloro(ethylenediamine) platinum(II), *cis*-dichlorodiammineplatinum (II), platinum (IV) chloride, palladium(II) acetate, bis(benzonitrile) dichloropalladium(II), trans-diammine dichloropalladium(II)—have proven to be suitable candidates for viability assays. Their great advantage is that they are not as highly sensitive to visible light as EMA and PMA, meaning that working with them does not require a darkroom, while it is also not necessary to work on ice, and their effect is not conditioned by excitation by light. In addition, the Pt and Pd agents are less expensive than the monoazide dyes (Soejima and Iwatsuki, 2016; Soejima et al., 2016). These agents, like the aforementioned intercalating dyes, generally penetrate dead cells with a compromised membrane but not live ones, for which reason viability detection is also based on membrane integrity. Pt and Pd complexes within cells interact with DNA resulting in either intrastrand (between the bases on the same chain) or interstrand (between bases on opposite chains) cross-links, which subsequently interfere with the binding of DNA polymerase to the DNA and the amplification of the target nucleic acid sequence. Therefore, when detecting bacterial viability, only unmodified DNA derived from live cells is amplified during qPCR (Soejima et al., 2016). Pt and Pd compounds in conjunction with qPCR have already been applied in the detection and quantification of viable bacterial cells, *Escherichia coli* and *Cronobacter sakazakii* (Soejima and Iwatsuki, 2016; Soejima et al., 2016). Subsequently, these agents combined with reverse transcription qPCR (RT-qPCR) were also used for evaluation of viral infectivity, which is in turn based on capsid integrity, such as with human norovirus (NoV) and murine norovirus (MNV) (Fraisse et al., 2018), hepatitis E virus (HEV) and hepatitis A virus (HAV) (Randazzo et al., 2018), porcine epidemic diarrhea virus (PEDV) (Puente et al., 2020), and Aichi virus (Canh et al., 2019).

In this study, we evaluated the use of Pt compounds combined with qPCR for the discrimination of live and heat-killed (membrane-compromised) mycobacterial cells. To this end, five Pt compounds were screened, and treatment conditions were optimized for the fast-growing mycobacterium *M. smegmatis*. The optimized viability assay conditions using Pt compounds were finally applied to MAP and compared with PMA treatment as the reference method for MAP viability determination.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Optimization of treatment conditions with Pt compounds was performed with the fast-growing *M. smegmatis* strain (collection strain ATCC 700084). The optimized conditions were applied to slow-growing MAP (field isolate 7072). The strains were grown on Herrold's egg yolk medium (HEYM), for MAP in addition with 2 μg/ml of Mycobactin J (Allied Monitor, United States) (HEYM-MJ), and incubated at 37°C. Both mycobacterial cultures grown were then inoculated into liquid Middlebrook 7H9 broth (Difco, Livonia, United States), supplemented with Middlebrook OADC enrichment (Difco), and in the case of MAP also with the addition of 2 μg/ml of Mycobactin J, and cultured at 37°C with shaking to attain an optical density at 600 nm

(OD_{600}) of about 1.0 (BioPhotometer; Eppendorf, Germany). The bacterial cultures were centrifuged at 3,000 × g for 5 min, and the supernatant was discarded and replaced with ultrapure water (Top-Bio, Czech Republic). The bacterial suspensions were homogenized using vortex and 1 mm zirconia beads (BioSpec, United States) and centrifuged at 100 × g for 30 s to remove big clumps. The upper fraction of the cell suspension was diluted with ultrapure water to an OD_{600} of about 0.15, which corresponded to 10^7 cells/ml, as determined by subsequent qPCR. The bacterial suspension was distributed in an amount of 500 μl per microtube. One half of the 500 μl aliquots of bacterial suspension was heat-treated (see below) and represented "dead cells," while the other non-heat-treated half of the aliquots constituted "live cells."

The amount of viable *M. smegmatis* cells in the bacterial suspension was assessed by culturing, spreading 100 μl of an appropriate dilution of the suspension in triplicates on plates with the HEYM medium, and incubating at 37°C for 3 days until colony forming unit (CFU) counting.

Preparation of Dead Cells by Heat Treatment

Heat treatment was conducted for both *M. smegmatis* and MAP by exposing the 500 μl aliquots of bacterial suspensions to 100°C for 4 min with shaking (100 rpm) using heat block and immediately cooling on ice. Killing efficacy was verified by seeding the heat-treated *M. smegmatis* and MAP cells on HEYM and HEYM-MJ agar, respectively, and incubating at 37°C for 2 and 4 weeks, respectively. Based on no difference recorded in the Cq values of heat-killed and non-heat-treated (both Pt-untreated) *M. smegmatis* and MAP cells in qPCR, it was shown that the heat treatment did not cause cell rupture and, thereby, DNA release and loss. This dead cell preparation protocol was part of each of the experiments performed in section "Viability Assay on Mycobacterial Cells."

Preparation of Platinum Compounds and Propidium Monoazide

Five Pt compounds, previously described by Soejima et al. (2016), were used for viability testing: dichloro(ethylenediamine) platinum (II), *cis*-dichlorodiammineplatinum (II), tetrakis(triphenylphosphine) platinum (0), chloroplatinic acid hexahydrate, and platinum (IV) chloride (Sigma-Aldrich, United States). The chemicals were dissolved and diluted to appropriate concentrations (specified for individual experiments below) in physiological saline and left for around 1 h at 40°C with shaking (1,000 rpm) in the dark to promote dissolution of the compounds. Pt compounds were not dissolved in the organic solvent dimethyl sulfoxide (DMSO) as it is discouraged due to their possible interaction altering the structure of the Pt compound complexes and impairing their ability to interact with DNA (Yi and Bae, 2011; Hall et al., 2014).

PMA was prepared based on the previous study by Kralik et al. (2010), in which 1 mg of PMA (Biotium, United States) was dissolved in 1.9 ml of 20% DMSO (Sigma-Aldrich) to obtain a 1 mM stock solution.

Direct Effect of Platinum Compounds on *Mycobacterium avium* subsp. *paratuberculosis* DNA Amplification

One loop of grown MAP culture was resuspended in 200 μl of ultrapure water. MAP DNA was then isolated using a Quick-DNA Fecal/Soil Microbe Microprep kit (Zymo Research, Tustin, California, United States) according to manufacturer's protocol. A MagNA Lyser instrument (Roche Diagnostics GmbH, Mannheim, Germany) was used for disruption of the MAP cells, allowing the tubes to be shaken at 6,400 rpm for 1 min. The concentration of isolated DNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, United States). DNA purity was checked to assure that the ratio of the absorbance at 260 and 280 nm ($A_{260/280}$) was not higher than 1.8 in order to avoid significant contamination with RNA. The DNA was divided into two portions. The first was diluted with ultrapure water and the second with a Tris-ethylenediaminetetraacetic acid (EDTA; TE) buffer (Serva Electrophoresis GmbH, Germany) to a concentration of 1 ng/μl and distributed in an amount of 50 μl per 2 ml microtube. Ten microliter of each Pt compound solution diluted to the appropriate concentration (10, 100, and 1,000 μM in the final DNA solution) was then added individually to the microtube lids, which were subsequently gently closed, and the Pt compounds and DNA solutions were uniformly mixed by a short spin. After incubation with a particular agent for 30 min at 37°C with shaking (100 rpm), MAP DNA was purified using a DNeasy Blood & Tissue Kit (Qiagen, Germany), after which qPCR was performed. In the case of control samples, the same procedure was followed except for the addition of 10 μl of physiological saline instead of the Pt compound solution. The experiment was carried out in biological duplicates for each condition. A schematic overview of the optimization of Pt compound treatment is shown in **Figure 1**.

Viability Assay on Mycobacterial Cells

Optimization of the Viability Assay Using Platinum Compounds on *M. smegmatis*

Ten μl of Pt compound solutions diluted to appropriate concentrations corresponding to 50, 100, 200, and 300 μM in the final bacterial suspension was added individually to the lids of the microtubes with bacterial suspensions of live and dead cells (500 μl) prepared as described in section "Preparation of Dead Cells by Heat Treatment." For controls, an identical volume of physiological saline was added instead of the Pt compound solution. The microtube lids were gently closed, and the Pt-treated and non-treated bacterial suspensions were uniformly spun briefly and incubated for an appropriate time (15, 30, 45, or 60 min depending on the concentration used) at 37°C with shaking (100 rpm). All the microtubes were then centrifuged at 7,000 × g for 5 min. The supernatant was discarded and the cell pellet was subjected to DNA extraction (see below). Each tested condition was analyzed in biological duplicate.

In order to increase the difference in quantification cycle (Cq) values between live and dead cells, multiple treatments (twice and three times with intermediate harvesting of cells by

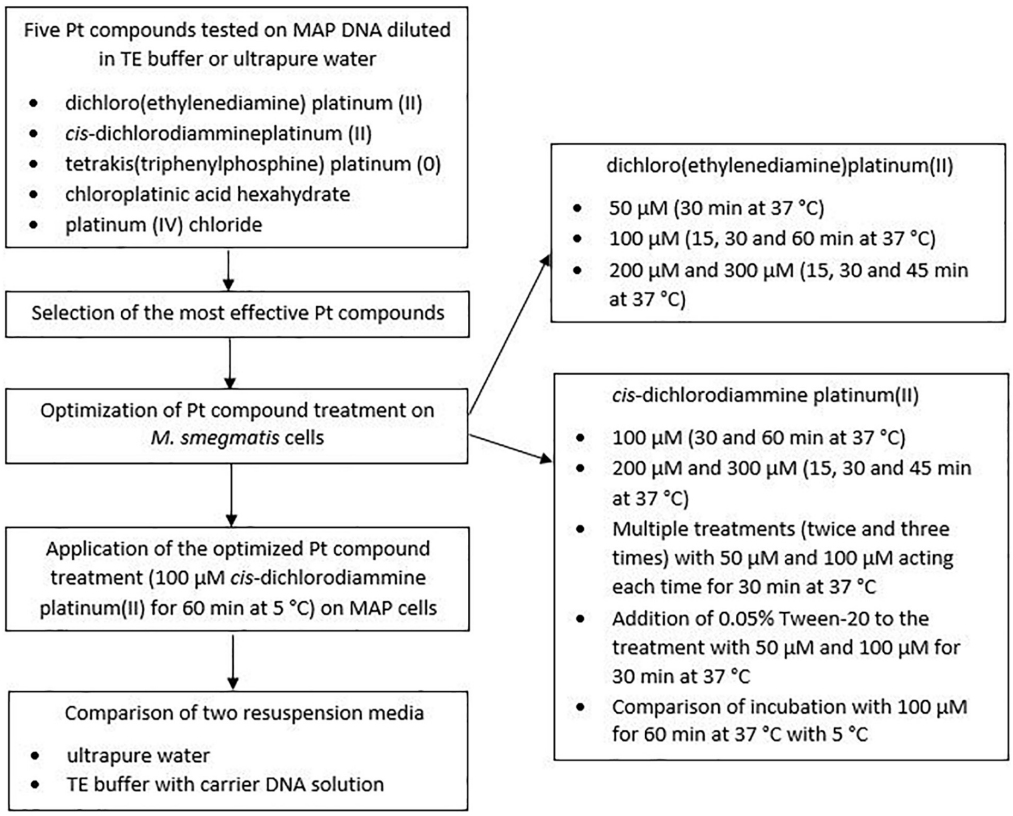

FIGURE 1 | Schematic overview of the optimization of Pt compound treatment.

centrifugation) with Pt compound solutions [50 and 100 µM *cis*-dichlorodiammine platinum(II) in the final bacterial suspension] acting each time for 30 min were also applied. Another factor investigated to increase the difference in Cq values between live and dead cells was the use of a surfactant (0.05% Tween-20; VWR Chemicals, United States) that was added to 500 µl aliquots of viable and dead cell suspensions immediately prior to the treatment with Pt compound solutions [50 and 100 µM *cis*-dichlorodiammine platinum(II) in the final bacterial suspension]. In both cases, all other steps were identical to the above procedure.

The last optimization experiment concerned testing the effect of incubation temperature during Pt treatment, comparing 37°C (with shaking at 100 rpm) with 5°C (manually shaken with all microtubes every 15 min during the incubation).

Application of the Optimized Viability Assay Using Platinum Compound on *Mycobacterium avium* subsp. *paratuberculosis*

The conditions optimized in the viability assay on *M. smegmatis* were subsequently applied to MAP. The optimized procedure consisted of a single treatment of live and dead MAP cells with 100 µM *cis*-dichlorodiammine platinum(II) for 60 min at 5°C (with occasional manual shaking) without the use of the surfactant followed by centrifugation at 7,000 × g for 5 min, removal of supernatant, and DNA extraction.

Viability Assay Using Propidium Monoazide

The procedure of PMA treatment was performed according to the study by Kralik et al. (2010). Briefly, under minimal light conditions, 12.5 µl of PMA was added to 500 µl aliquots of viable and dead MAP cell suspensions to attain a final concentration of 25 µM, the microtubes were briefly centrifuged and incubated at room temperature for 5 min with shaking (1,200 rpm). The microtubes were then placed horizontally on ice and exposed to light from a halogen lamp with a 650 W bulb from a distance of about 20 cm for 2 min. The PMA treatment step was repeated once more. Subsequently, the microtubes were centrifuged at 7,000 × g for 5 min, the supernatant was discarded, and the cell pellets subjected to DNA extraction. The same procedure was carried out for control samples, but instead of PMA the same volume of 20% DMSO was added to 500 µl aliquots of viable and dead MAP cell suspensions. Each tested condition was analyzed in biological duplicate.

DNA Extraction Following the Platinum and Propidium Monoazide Treatment on Mycobacterial Cells

Mycobacterial DNA was obtained as a crude lysate according to the study by Kralik et al. (2010) which reported that the crude lysate preparation proved to be a suitable alternative to

commercial DNA isolation kits. For *M. smegmatis*, pellets of Pt-treated and untreated cells (live and dead cells in both cases) were resuspended in 500 μl of ultrapure water. In the case of MAP, the effect of ultrapure water and TE buffer supplemented with carrier DNA solution (salmon sperm DNA, 50 ng/μl; Serva, Germany) that reduces DNA losses during manipulation (Slana et al., 2008) in a volume of 500 μl used to resuspend the pellets of Pt- or PMA-treated and untreated cells was monitored. The resuspended cells were then subjected to lysis at 100°C for 20 min. Subsequently, centrifugation at 14,000 × g for 5 min was performed and the supernatant containing the DNA was analyzed by qPCR.

Quantitative PCR and Data Analysis

DNA purification (in the case of the application of Pt compounds directly to MAP DNA) or extraction (in the viability assay on mycobacterial cells) were followed by qPCR. For *M. smegmatis*, a qPCR assay targeting the ITS (internal transcribed spacer) sequence was performed as previously described by Sevilla et al. (2015). qPCR targeting the *F57* sequence developed by Slana et al. (2008) was used for MAP. Both qPCR assays included an internal amplification control (IAC) that allowed false negative results to be detected. All qPCR reactions were performed using a LightCycler 480 (Roche Molecular Diagnostic, Germany). All qPCR samples were tested in technical duplicates.

Cq values were determined using the "Fit Point Analysis" option of the LightCycler 480 software (version 1.5.0.39). The differences between the Cq values (ΔCq) of isolated MAP DNA or live and dead cells treated with Pt compounds or PMA and the respective untreated control samples calculated from all biological and technical replicates were compared using the following formulas, which were taken from Kralik et al. (2010) and slightly modified:

$$\Delta Cq_{\text{DNA with Pt}-\text{DNA without Pt}} \quad (1)$$

$$\Delta Cq_{\text{dead with Pt/PMA}-\text{live with Pt/PMA}} \quad (2)$$

$$\Delta Cq_{\text{dead with Pt/PMA}-\text{dead without Pt/PMA}} \quad (3)$$

$$\Delta Cq_{\text{live with Pt/PMA}-\text{live without Pt/PMA}} \quad (4)$$

Equation (1) expresses the differences in target sequence amplification of Pt-treated and untreated DNA samples. The highest possible value is desirable, indicating that the Pt compound binds significantly to the target DNA sequence and thus prevents its amplification during qPCR. Equation (2) expresses the extent to which a particular Pt or PMA permeates dead cells while taking into account the undesired permeation of the agents into live cells. Equation (3) also expresses the extent to which Pt or PMA permeates dead cells, regardless of live cells. For both Equations (2) and (3), the highest possible value is desirable (ideally no amplification in dead Pt- or PMA-treated cells indicating that the agents bound to all target sequences and thus prevented their amplification in all dead cells). Equation (4) expresses the extent to which Pt or PMA permeates live cells, so the lowest possible value is required.

Statistical Analysis

Analysis of ΔCq was performed by one-way analysis of variance (ANOVA), two-way ANOVA, Welch's *t*-test, and Tukey's HSD test (detailed in the relevant figures). Data analysis was performed using the statistical software Statistica 13.2 (StatSoft Inc., Tulsa, OK, United States). *P*-values less than 0.05 were considered statistically significant.

RESULTS

Direct Effect of Platinum Compounds on *Mycobacterium avium* subsp. *paratuberculosis* DNA Amplification

First, the direct effect of five Pt compounds on the amplification of a target sequence of isolated MAP DNA was evaluated, wherein all Pt compounds were tested at three concentrations: 10, 100, and 1,000 μM in DNA solution (**Figure 2**). The aim of this experiment was to select a Pt compound that shows maximal binding to the target DNA sequences and thus prevents its amplification during qPCR without affecting the IAC. The differences in the Cq values of Pt-treated and untreated samples were expressed according to Equation (1). Significant suppression of MAP DNA amplification was observed for two Pt compounds: dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II). Reduction of the qPCR signals was, as expected, concentration-dependent. At a concentration of 1,000 μM, a significant signal reduction of about 16 Cq and complete amplification suppression was achieved for dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II), respectively, while a concentration of 100 μM already showed a lower reduction of about 6 and 7 Cq, respectively. The concentration of 10 μM was already insufficient for the Pt compound molecules to be chelated to a significant number of DNA template molecules. The other three Pt compounds [tetrakis(triphenylphosphine)platinum(0), chloroplatinic acid hexahydrate and platinum(IV) chloride] showed a limited effect on MAP DNA amplification ($\Delta Cq < 5$) even at the highest concentration tested (1,000 μM), for which reason the treatment with these compounds was excluded from the following optimization on mycobacterial cells.

In this experiment, the effect of TE buffer or ultrapure water on the activity of Pt compounds was also assessed (**Figure 2**). When TE buffer was used, a significantly lower reduction in amplification (lower $\Delta Cq_{\text{DNA with Pt}-\text{DNA without Pt}}$) of the target DNA sequence after treatment with 100 and 1,000 μM dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II) was observed compared to ultrapure water. Ultrapure water was, therefore, used in the following experiments as a dilution medium when diluting the mycobacterial cells.

After treatment of MAP DNA with Pt compounds, it was necessary to remove unbound Pt molecules from the DNA solution using a purification kit (DNeasy Blood & Tissue Kit) before performing qPCR. This necessity was demonstrated by

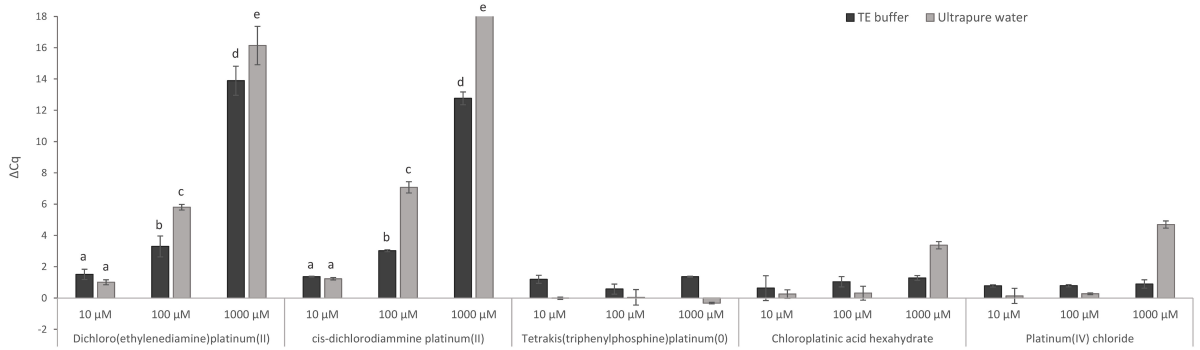

FIGURE 2 | Direct effect of Pt compound solutions at various concentrations on amplification of MAP DNA diluted with TE buffer or ultrapure water. The ΔCq values express the mean difference of individual Cq values of Pt-treated and mean Cq value of untreated control samples calculated from four replicates (biological and technical). The significance of differences between ΔCq was calculated by two-way ANOVA with factors of concentration and dilution medium and Tukey's HSD test. The same letters indicate statistically insignificant differences ($P > 0.05$) between ΔCq values, while different letters indicate significant differences ($P < 0.05$). Error bars represent standard deviations calculated from four replicates. The bar above value of 18 in ΔCq on the *y*-axis signifies that no amplification occurred.

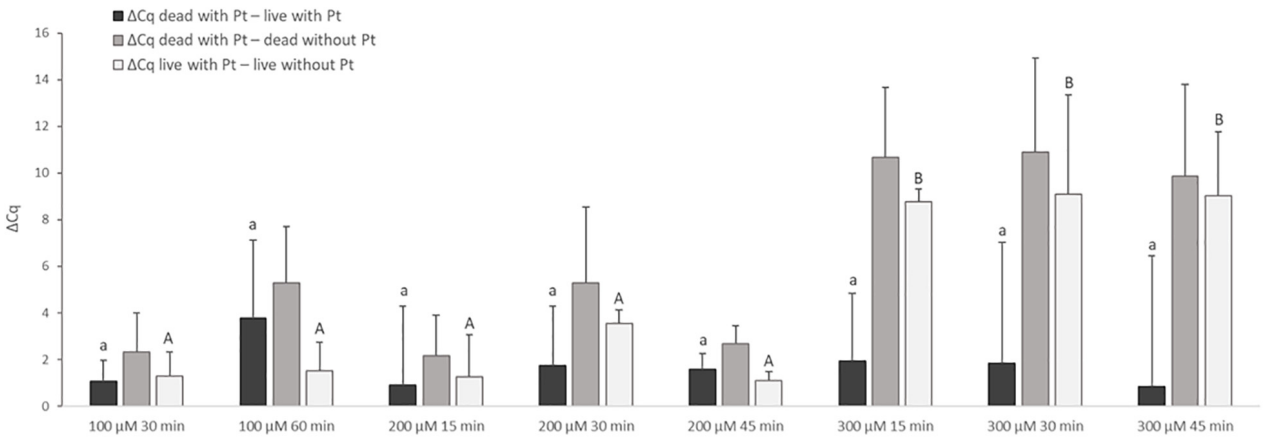

FIGURE 3 | Effect of treatment of *M. smegmatis* cells with *cis*-dichlorodiammine platinum(II) at different concentrations and for different times. The aim was to find the treatment condition providing the highest possible value for $\Delta Cq_{\text{dead with Pt – live with Pt}}$ and $\Delta Cq_{\text{dead with Pt – dead without Pt}}$ and the lowest possible value for $\Delta Cq_{\text{live with Pt – live without Pt}}$. The ΔCq values express the individual mean differences of Cq values calculated from four replicates (biological and technical). The significance of differences between ΔCq was calculated by one-way ANOVA and Tukey's HSD test. The same letters indicate statistically insignificant differences ($P > 0.05$) between ΔCq values, while different letters indicate significant differences ($P < 0.05$). The values of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ were marked with lowercase letters, and the values of $\Delta Cq_{\text{live with Pt – live without Pt}}$ were marked with capital letters, as it was evaluated separately. Error bars represent standard deviations calculated from four replicates.

the fact that the omission of the purification step considerably inhibited the amplification of IAC (data not shown).

Optimization of the Viability Assay Using Platinum Compounds on *M. smegmatis*

Treatment with Pt compounds that exhibited the greatest effect on MAP DNA amplification was subsequently optimized on live and dead *M. smegmatis* cells. The cells were treated with dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II) at three concentrations for different exposure times (100 μM for 30 and 60 min; 200 and 300 μM for 15, 30, and 45 min) at 37°C. The differences in Cq values of live and dead cells treated with Pt compounds and the respective untreated control samples were calculated according to Equation (2–4). Dichloro(ethylenediamine)platinum(II) penetrated considerably into live cells at all three concentrations tested, as shown by the calculation of $\Delta Cq_{\text{live with Pt – live without Pt}}$, whose lowest calculated value was about 7 Cq (data not shown). For *cis*-dichlorodiammine platinum(II), although not with a significant difference, the highest mean value of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ was recorded at 100 μM, to which the cells were exposed for 60 min (**Figure 3**). Treatment with both 200 and 300 μM *cis*-dichlorodiammine platinum(II) did not lead to a higher value of $\Delta Cq_{\text{dead with Pt – live with Pt}}$, and in addition the 300 μM concentration caused more significant penetration of the agent into live cells. Based on these data, the use of *cis*-dichlorodiammine platinum(II) at concentrations greater than 100 μM was not found to be beneficial.

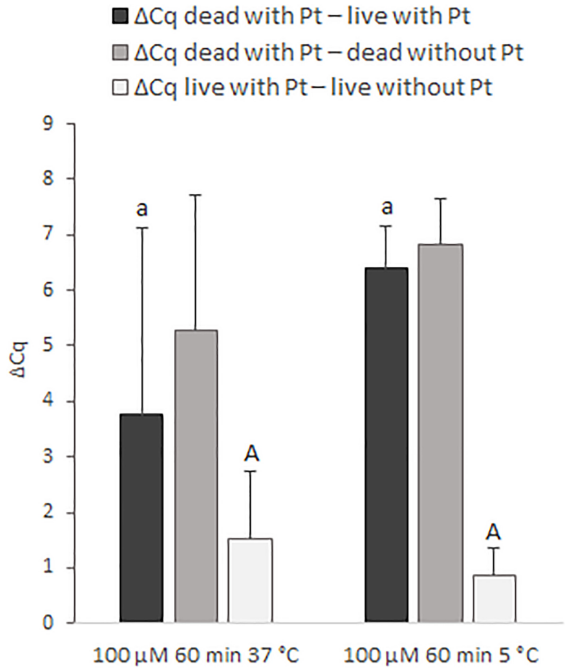

FIGURE 4 | Effect of different incubation temperatures (37 and 5°C) on the treatment of *M. smegmatis* cells with 100 µM cis-dichlorodiammine platinum(II) for 60 min. The aim was to find the incubation temperature providing the highest possible value for $\Delta Cq_{\text{dead with Pt – live with Pt}}$ and $\Delta Cq_{\text{dead with Pt – dead without Pt}}$ and the lowest possible value for $\Delta Cq_{\text{live with Pt – live without Pt}}$. The ΔCq values express the individual mean differences of Cq values calculated from four replicates (biological and technical). The significance of differences between ΔCq was calculated by Welch's *t*-test. The same letters indicate statistically insignificant differences ($P > 0.05$) between ΔCq values, while different letters indicate significant differences ($P < 0.05$). The values of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ were marked with lowercase letters, and the values of $\Delta Cq_{\text{live with Pt – live without Pt}}$ were marked with capital letters, as it was evaluated separately. Error bars represent standard deviations calculated from four replicates.

Since even a 100 µM concentration of dichloro(ethylenediamine)platinum(II) for 30 min considerably affected live cells, a lower concentration of 50 µM for 30 min and 100 µM for only 15 min was subsequently applied. Neither led to any improvement, i.e., lower penetration of this Pt compound into live cells (data not shown).

In order to try to increase ΔCq between live and dead cells, multiple treatments (twice and three times) with 50 and 100 µM *cis*-dichlorodiammine platinum(II) acting each time for 30 min were evaluated. In addition, the possibility of using a surfactant was also examined by adding 0.05% Tween-20 to the procedure involving a single exposure to 50 and 100 µM *cis*-dichlorodiammine platinum(II) for 30 min. However, both alterations repeatedly (in two independent experiments) resulted in an undesirable noticeable increase of Cq values for live cells (increase in $\Delta Cq_{\text{live with Pt–live without Pt}}$) (data not shown). Therefore, one exposure of the cells to 100 µM *cis*-dichlorodiammine platinum(II) for 60 min without the use of the surfactant was chosen for subsequent experiments.

In the next step, the effect of incubation temperature (37 and 5°C) on the discrimination of live and dead cells was assessed for 100 µM *cis*-dichlorodiammine platinum(II) (**Figure 4**). Incubation of the cells for 60 min with this Pt compound at 5°C provided higher mean values of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ (by about 2.7 Cq), as well as $\Delta Cq_{\text{dead with Pt – dead without Pt}}$ (by about 1.6 Cq), and a lower mean value of $\Delta Cq_{\text{live with Pt – live without Pt}}$ (by about 0.7 Cq) compared to incubation at 37°C. Although these differences in ΔCq values were not statistically significant, based on more favorable mean ΔCq values and standard deviations, it was evaluated that incubation at 5°C was superior to 37°C.

By evaluating all the conditions tested for *M. smegmatis*, it was concluded that a single treatment with 100 µM *cis*-dichlorodiammine platinum(II) for 60 min at 5°C without the use of the surfactant made it possible to attain the highest value of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ and at the same time the lowest value of $\Delta Cq_{\text{live with Pt – live without Pt}}$. Finally, the optimized procedure for this Pt compound was applied to MAP.

Application of the Optimized Viability Assay Using Platinum Compounds on *Mycobacterium avium* subsp. *paratuberculosis*

Live and dead MAP suspensions were subjected to the optimized Pt treatment condition as described above, which was compared with PMA treatment as the reference method for MAP viability determination (**Figure 5**). The differences in Cq values of live and dead cells treated with Pt or PMA compounds and the respective untreated control samples were calculated according to Equations (2–4).

For MAP, when *cis*-dichlorodiammine platinum(II) was applied and ultrapure water was used as the resuspension medium, $\Delta Cq_{\text{dead with Pt – live with Pt}}$ gave a result of slightly above 6 Cq and $\Delta Cq_{\text{live with Pt – live without Pt}}$ of about 1.5 Cq, which corresponded approximately to the values obtained with *M. smegmatis*. However, a markedly low $\Delta Cq_{\text{dead with Pt – dead without Pt}}$ was noted, which stemmed from lower DNA amplification in a control sample of dead cells, which was not observed in any of the optimization experiments in *M. smegmatis*. The cause could not be the rupture of the cells during the heat treatment, as in the case of control samples of dead cells in which TE buffer with carrier DNA solution was used as the resuspension medium, the same initial steps including heat treatment were applied and the amplification did not decrease. On the other hand, the use of TE buffer with carrier DNA solution in the Pt treatment of cells led to a significant undesirable decrease in the value of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ by about 6 Cq to almost zero compared to the use of ultrapure water. This result showed that the use of TE buffer with carrier DNA solution as a resuspension medium is not suitable in the treatment of cells with Pt compounds.

Regarding the use of PMA and a comparison of both resuspension media mentioned, both $\Delta Cq_{\text{dead with Pt – live with Pt}}$ and $\Delta Cq_{\text{dead with Pt – dead without Pt}}$ showed higher mean values with lower standard deviations when TE buffer with carrier DNA

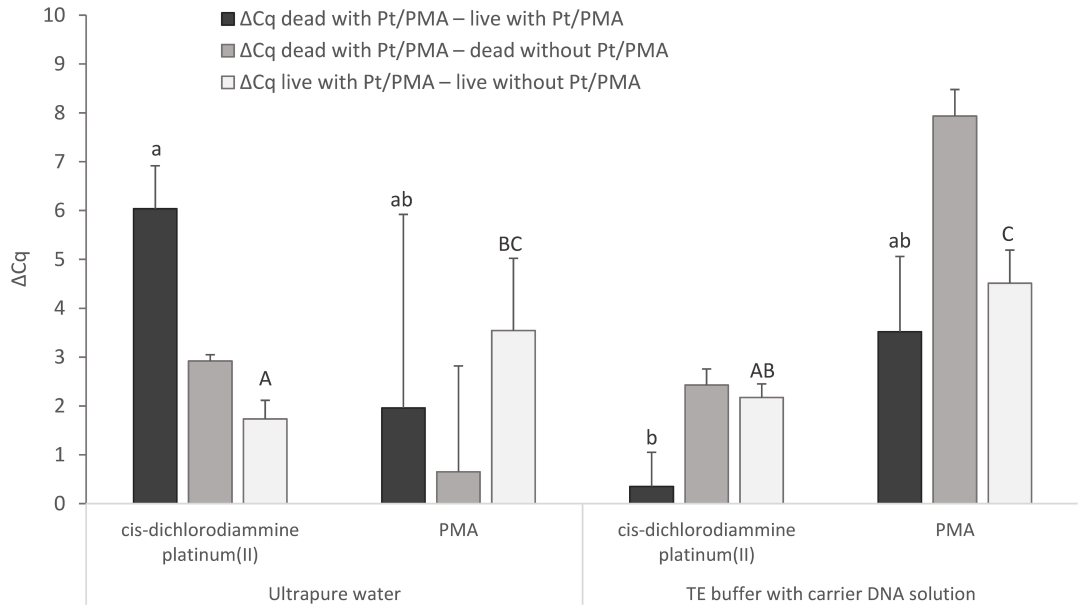

FIGURE 5 | Comparison of Pt and PMA treatment of MAP cells and the effect of different resuspension media (ultrapure water or TE buffer with carrier DNA solution) used to resuspend the cells after the treatment. The aim was to determine the agent showing the highest possible value for $\Delta Cq_{\text{dead with Pt/PMA – live with Pt/PMA}}$ and $\Delta Cq_{\text{dead with Pt/PMA – dead without Pt/PMA}}$ and the lowest possible value for $\Delta Cq_{\text{live with Pt/PMA – live without Pt/PMA}}$. The ΔCq values express the individual mean differences of Cq values calculated from four replicates (biological and technical). The significance of differences between ΔCq was calculated by two-way ANOVA with factors of resuspension medium and agent used, and Tukey's HSD test. The same letters indicate statistically insignificant differences ($P > 0.05$) between ΔCq values, while different letters indicate significant differences ($P < 0.05$). The values of $\Delta Cq_{\text{dead with Pt – live with Pt}}$ were marked with lowercase letters, and the values of $\Delta Cq_{\text{live with Pt – live without Pt}}$ were marked with capital letters, as it was evaluated separately. Error bars represent standard deviations calculated from four replicates.

solution was used. However, significant undesired penetration of PMA into viable MAP cells was recorded in both cases, as shown by $\Delta Cq_{\text{live with Pt – live without Pt}}$ (about 4 Cq). Based on these data, it can be suggested that *cis*-dichlorodiammine platinum(II) appeared to be more suitable for distinguishing between viable and dead membrane-compromised MAP cells than PMA.

DISCUSSION

The present study aimed to optimize the viability determination of mycobacterial cells using Pt compounds in combination with qPCR. The procedure of Pt compound treatment is conditioned by disruption of cell membranes in dead cells (e.g., by high temperature) into which the agents can penetrate, while viable cells with intact membranes remain unaffected. Inside the membrane-compromised dead cells, the Pt compounds interact with DNA and subsequently interfere with the amplification of the target nucleic acid sequence during qPCR. qPCR, which follows the Pt-treatment, thereby enables the evaluation of the differences between Pt-treated and untreated control samples through measured Cq values. The viability assay combining the sample treatment with Pt compounds and qPCR is able to detect the viable mycobacterial cells within 1 day and is therefore clearly faster than cultivation, a gold standard for viability evaluation, which is very challenging for slow-growing bacteria such as MAP.

In our study, five Pt compounds were first assessed to determine chelating ability with purified MAP DNA (**Figure 2**). The greatest suppression of MAP DNA amplification was observed for the Pt compounds dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II), so these two Pt compounds were selected for further experiments. The Pt compounds dichloro(ethylenediamine)platinum(II) and *cis*-dichlorodiammine platinum(II) have already been confirmed to prevent nucleic acid amplification by the study by Soejima et al. (2016), in which these agents were applied to extracted DNA from *C. sakazakii*. Another study also examined the effect of these Pt compounds on purified RNA from NoV and MNV, and revealed that *cis*-dichlorodiammine platinum(II), platinum(IV) chloride, and tetrakis(triphenylphosphine)platinum(0) reduced nucleic acid amplification, while dichloro(ethylenediamine)platinum(II) had a very limited effect (Fraisse et al., 2018). Further studies have reported that platinum(IV) chloride can also bind to purified RNA from the HEV virus (Randazzo et al., 2018), and both platinum(IV) chloride and *cis*-dichlorodiammine platinum(II) can be chelated to purified RNA from PEDV (Puente et al., 2020).

Further, we investigated whether the medium used to dilute MAP DNA can affect the activity of Pt compounds, comparing TE buffer and ultrapure water (**Figure 2**). Ultrapure water enabled a significantly greater reduction of qPCR signal compared to TE buffer for both selected Pt compounds at concentrations of 100 and 1,000 μM. Thus, as

expected based on previous findings for other Pt compounds (Busch and Bailar, 1956; Appleton et al., 1982), TE buffer consisting of chelating agent EDTA can chelate Pt compounds used in our study, which subsequently do not bind to DNA and interfere with qPCR. These data demonstrated that EDTA-containing TE buffer should not therefore be used in viability assays utilizing Pt compounds screened herein. As a more suitable dilution medium, we evaluated water which was also used in the study by Soejima et al. (2016), in which DNA as well as a bacterial suspension was diluted with water prior to Pt treatment.

The treatment with two Pt compounds that was shown to suppress amplification of the target sequence of MAP DNA was first optimized for fast-growing *M. smegmatis* as a model for mycobacterial species due to its shorter cultivation time, and finally applied to slow-growing MAP. Based on optimization experiments investigating two Pt compounds at various concentrations, incubation times and temperatures, surfactant utilization and repeated exposures (**Figures 3**, **4**), a procedure for Pt treatment which exhibited maximal suppression of DNA amplification in dead cells and at the same time a minimal impact on the signal from live cells was compiled. This procedure consisted of a single treatment with 100 μM *cis*-dichlorodiammine platinum(II) for 60 min at 5°C. An incubation temperature of 5°C was considered to be more suitable for Pt treatment of mycobacteria than 37°C, which corresponds with the findings of Fraisse et al. (2018), in which the effect of incubation temperature on the treatment of noroviruses with platinum(IV)chloride has also been demonstrated.

The maximum differences achieved between Pt-treated live and dead *M. smegmatis* as well as MAP cells were slightly above 6 Cq (**Figures 4**, **5**), indicating that the Pt treatment did not completely eliminate amplification of the target sequence in dead cells. As in our study, incomplete signal reduction in heat-killed MAP cells (a similar difference of about 7 Cq compared to Pt-treated viable cells) has been reported by Kralik et al. (2010) in a viability assay using PMA dye. In contrast, complete suppression of DNA amplification was achieved using *cis*-dichlorodiammine platinum(II) by previous investigations in enterobacteria by Soejima et al. (2016). The limited effect of both Pt and PMA treatments on mycobacteria could be due to their thicker hydrophobic cell wall rich in mycolic acids responsible for lower penetration of the agents even into dead membrane-compromised cells. Another explanation for the incomplete suppression of DNA amplification in dead mycobacterial cells could be that short DNA sequences (<150 bp) were targeted in qPCR, as compared to the 424 bp amplicon used by Soejima et al. (2016). It has already been reported that the signal suppression from dead cells in viability PCR depends on the amplicon length, i.e., the longer the amplicon size, the more efficient the elimination of the PCR signal (Fittipaldi et al., 2012). On the other hand, a slight qPCR signal reduction after Pt treatment was also observed for non-heat-treated mycobacteria. This can be explained as in the publication by Kralik et al. (2010), in which PMA was used, by the presence of membrane-compromised cells even in an otherwise viable population or by the permeation of Pt compounds into live cells to a limited extent. In addition, Pt molecules are not inactivated after the treatment as occurs with PMA after irradiation and reaction with water molecules (Nocker and Camper, 2009). For this reason, the slight signal reduction for live cells could also be due to the fact that the washing step did not remove all of the Pt molecules contained in the supernatant and these may modify the DNA during the extraction procedure. Our efforts to quantify the initial bacterial suspension of *M. smegmatis* by cultivation were related to this, although the determined cell number varied by about 1.5–2 $\log_{10}$ units compared to qPCR, which corresponds to the findings of Kralik et al. (2012). The reduced CFU counts are attributed to the tendency of mycobacterial cells to clump, i.e., one CFU can arise from a cluster of several cells (Pickup et al., 2005). Therefore, the initial cell numbers of *M. smegmatis* as well as MAP were estimated only retrospectively by qPCR.

Since complete suppression of the qPCR signal after Pt treatment for dead cells was not achieved, we investigated whether the use of the surfactant Tween-20 known to affect biological membranes (Burden, 2012) would lead to an increase in signal reduction. Although 0.05% Tween-20 increased the qPCR signal reduction for dead *M. smegmatis* cells, viable cells were also significantly influenced which was an undesirable effect (data not shown). Another study also evaluated the use of Tween-20 (0.5%) in a viral infectivity assay to increase the efficiency of platinum(IV)chloride treatment for noroviruses, but showed no effect (Fraisse et al., 2018).

The mentioned PMA dye was also used in our final experiment, in which its efficiency for distinguishing between viable and dead MAP cells was compared with that of *cis*-dichlorodiammine platinum(II) (**Figure 5**). According to this experiment, *cis*-dichlorodiammine platinum(II) allowed the discrimination of the MAP cells more efficiently than PMA, which penetrated more markedly into the viable cells. The difference between PMA-treated and untreated viable MAP cells was around 4 Cq, which was remarkably higher compared to the results achieved by Kralik et al. (2010) using the same PMA treatment. The dissimilarity between the results we obtained and those in the above study can probably be explained by the fact that the MAP strain used was more sensitive to PMA treatment. Pt compounds were also evaluated as superior to PMA for discrimination between live and dead bacteria, specifically enterobacteria, by Soejima et al. (2016). Lower cost compared to PMA and no need for activation by light are considered the other advantages of Pt compounds (Soejima et al., 2016).

The last experiment also investigated the effect of the resuspension medium used after Pt and PMA treatment prior to exposing the MAP cells to thermal lysis. Since a DNA amplification decrease was noted for the control sample of dead MAP cells when using ultrapure water as a resuspension medium, we tested TE buffer supplemented with carrier DNA solution (**Figure 5**), which is known to stabilize DNA and reduce losses during nucleic acid manipulation (Slana et al., 2008), as an additional medium. The use of TE buffer with carrier DNA solution resulted in no difference in Cq values between control samples of live and dead cells, although it did result in a significantly lower difference in Cq values between live

and dead MAP cells treated with Pt compound compared to ultrapure water. TE buffer is not, therefore, suitable for use in a viability assay utilizing Pt compounds either as a resuspension medium or as a dilution medium for diluting the cell suspension, as was found in the initial experiment. Heat-killed MAP cells could not rupture during the heat treatment, as this would have the same effect on the cells resuspended with TE buffer with carrier DNA solution, because in both cases the same initial steps were followed, including the same dilution medium. A possible explanation could be that the ultrapure water used does not have the above-mentioned ability to stabilize DNA and reduce DNA losses during manipulation as TE buffer which suppresses the undesired activity of DNases and maintains a stable pH of the solution. Surprisingly, the same effect on the control samples of dead cells as for MAP was not observed for *M. smegmatis*. Further investigation of this matter will, therefore, be needed. However, this outcome does not contradict the desired effect of the Pt compound, i.e., the reduction of the qPCR signal for dead cells with minimal impact on viable cells, as evidenced by the achievement of a comparable $\Delta Cq_{\text{dead with Pt}-\text{live with Pt}}$ as in *M. smegmatis*. Similarly, water (sterile) was also used by Soejima et al. (2016) in a viability assay to dilute cell suspensions of enterobacteria and to dissolve the purified DNA. Furthermore, *E. coli* cells were also washed and resuspended in water (distilled) in a viability assay utilizing EMA (Shi et al., 2011). In contrast, ultrapure water was evaluated as an unsuitable dilution medium in the Pt treatment of noroviruses (Fraisse et al., 2018).

CONCLUSION

In conclusion, we optimized Pt treatment to assess the viability of mycobacterial cells. The optimal conditions consisted of a single treatment with 100 μM *cis*-dichlorodiammine platinum(II) for 60 min at 5°C. The Pt compound treatment in combination with qPCR demonstrated a better ability to distinguish between viable and dead membrane-compromised mycobacteria suspended in water as compared to PMA. Pt-qPCR therefore proved to be a promising method for the viability determination of MAP cells in foodstuffs and environmental and clinical samples, which could replace time-consuming cultivation or laborious procedures required when using PMA. Further studies could investigate the applicability of the method to other matrices (e.g., milk). As our study addressed only one MAP strain, the following studies could also focus on verifying the optimized procedure in other cattle as well as sheep strains of MAP. It is also necessary to overcome the shortcoming associated with a possible overestimation of viable mycobacterial cell numbers resulting from incomplete elimination of the target sequence amplification in Pt-treated dead cells.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MC designed and performed all the experiments, analyzed and interpreted the data, and wrote the manuscript. MB participated in the initial optimization experiments. VB performed the statistical analysis. IS revised the manuscript. PK designed the study and revised the manuscript. All authors contributed to the article and approved the submitted version.

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A viability assay combining palladium compound treatment with quantitative PCR to detect viable *Mycobacterium avium* subsp. *paratuberculosis* cells

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***Mycobacterium avium* subsp. *paratuberculosis* (MAP) is a pathogenic bacterium causing the paratuberculosis, chronic and infectious disease common particularly in wild and domestic ruminants. Currently, culture techniques to detect viable MAP are still used most commonly, although these require a long incubation period. Consequently, a faster molecular method for assessing MAP cell viability based on cell membrane integrity was introduced consisting of sample treatment with the intercalation dye propidium monoazide (PMA) followed by quantitative PCR (qPCR). However, the PMA-qPCR assay is complicated by demanding procedures involving work in a darkroom and on ice. In this study, we therefore optimized a viability assay combining sample treatment with palladium (Pd) compounds as an alternative viability marker to PMA, which does not require such laborious procedures, with subsequent qPCR. The optimized Pd-qPCR conditions consisting of 90 min exposure to 30 µM bis(benzonitrile)dichloropalladium(II) or 30 µM palladium(II)acetate at 5 °C and using ultrapure water as a resuspension medium resulted in differences in quantification cycle (Cq) values between treated live and dead MAP cells of 8.5 and 7.9, respectively, corresponding to approximately 2.5 log units. In addition, Pd-qPCR proved to be superior to PMA-qPCR in distinguishing between live and dead MAP cells. The Pd-qPCR viability assay thus has the potential to replace time-consuming culture methods and demanding PMA-qPCR in the detection and quantification of viable MAP cells with possible application in food, feed, clinical and environmental samples.**

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a slow-growing and highly resistant bacterium causing paratuberculosis, particularly in wild and domestic ruminants, e.g. deer, sheep, cattle and goats[1]. Clinical symptoms of this chronic and infectious disease appearing after a years-long latent period include diarrhea, reduced milk production, weight lost and exhaustion leading to the death of infected individuals[2]. The commonest sources of infection are feces and milk from MAP-infected animals[3], though MAP can also be transmitted vertically to the fetus during an intrauterine infection[4]. MAP is also likely to participate in the development of Crohn's disease in humans who may be exposed to it through contaminated milk, meat and water[5].

Cultivation is one of the commonest methods used for the direct detection of viable MAP cells, though its disadvantages are the long incubation period (at least 12 weeks) and the need for preliminary chemical decontamination, which can reduce its sensitivity[6]. In addition, cultivation is not able to detect MAP cells present in a dormant or viable non-culturable state[1], and the clumping of MAP cells increases the variability and decreases the accuracy of the data determined by this method[7]. In order to overcome these shortcomings, a molecular technique combining the treatment of the sample with the intercalating dye propidium monoazide (PMA) with quantitative PCR (qPCR) of the single-copy sequence *F57* (PMA-qPCR), has been introduced to assess the MAP viability[8]. The discrimination between viable and dead cells is based on membrane integrity. PMA dye penetrates selectively into the dead membrane-compromised cells, but not into viable cells with intact cell membranes. PMA molecules inside the cell intercalate into the DNA, whose modifications are irreversible after irradiation with

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visible light, which interferes with the subsequent amplification of the target sequence by qPCR. The advantages of PMA-qPCR over cultivation are time saving and higher sensitivity, although a demanding procedure requiring manipulation in a darkroom is a major limitation. In addition, bacterial suspensions need to be kept on ice during irradiation when a rise in temperature could disrupt the cell membranes of viable cells, into which the dye could subsequently penetrate[9].

Platinum (Pt) and palladium (Pd) compounds proved to be suitable alternative viability markers to PMA dye, showing a similar potential to distinguish between viable and dead cells or infectious and non-infectious virus particles based on cell membrane integrity[10,11] or capsid integrity[12,13]. In contrast to PMA, Pt and Pd compounds are not sensitive to visible light, therefore they do not require manipulation in a darkroom or on ice, their effect is not conditioned by excitation by light, and they are less expensive. Regarding comparison of these two metals, Pd compounds have been shown to be more convenient to use than Pt compounds as lower concentrations are needed for the viability assay, resulting in lower reagent costs[10].

In the present study, we therefore aimed to optimize a viability assay combining Pd compound treatment with qPCR (Pd-qPCR) for selective detection of viable mycobacterial cells, as an alternative approach to Pt-qPCR addressed in a previous study[14]. First, we evaluated the effect of four Pd compounds on MAP DNA, selecting for the compound that binds the most to the target MAP sequence and prevents its amplification by qPCR. Subsequently, the most suitable concentrations, exposure time, temperature and resuspension medium were optimized for selected Pd compounds on *M. smegmatis*, as a fast-growing model mycobacterium with a continuation on the target slow-growing mycobacterium MAP. Finally, we compared the optimized Pd-qPCR protocol with the reference PMA-qPCR to reveal which viability assay allows more accurate distinction between viable and dead MAP cells.

Methods

Preparation of mycobacterial cultures. Mycobacterial cultures were prepared in the same way as in the previous study by Cechova et al.[14]. Fast-growing *M. smegmatis* (collection strain ATCC 700084) was used for initial optimization of Pd compound treatment, and slow-growing MAP (field isolate 7072) was subsequently exposed to the optimized conditions. Both mycobacterial strains were first grown on Herrold's egg yolk medium (HEYM), with the addition of 2 µg/ml of Mycobactin J (Allied Monitor, USA) (HEYM-MJ) in the case of MAP, at 37 °C. The grown cultures were then inoculated into Middlebrook 7H9 broth (Difco, Livonia, USA) supplemented with Middlebrook OADC enrichment (Difco), and with the addition of 2 µg/ml of Mycobactin J for MAP, and incubated at 37 °C with shaking until an optical density at 600 nm (OD_{600}) of about 1.0 (BioPhotometer; Eppendorf, Germany) was attained. The cultures were washed with ultrapure water (Top-Bio, Czech Republic), homogenized by vortexing with 1 mm zirconia beads (BioSpec, USA), and centrifuged at 100 × g for 30 s to eliminate big cell clumps. The upper portion of the bacterial suspension was diluted with ultrapure water to an OD_{600} of 0.15, which was determined as 10^7 copies/ml by subsequent qPCR, and distributed in a volume of 500 µl per microtube.

In addition, the cultivation was used as a reference method for the quantification of viable *M. smegmatis* cells in the suspension, dispensing 100 µl of serial dilutions in triplicates on plates containing HEYM medium and incubating for 3 days at 37 °C until colony-forming units (CFU) were calculated.

Preparation of dead mycobacterial cells. Dead cells were prepared in the same manner as in the previous study by Cechova et al.[14] by placing 500 µl aliquots of bacterial suspension in a thermoblock tempered at 100 °C for 4 min (with shaking at 100 rpm) and then cooling them immediately on ice. To verify cell killing, heat-treated cell suspensions of *M. smegmatis* and MAP were seeded on HEYM and HEYM-MJ agar, respectively, and incubated at 37 °C for 2 weeks and 4 weeks, respectively. Cell rupture and DNA release during the heat treatment for the killed cells (*M. smegmatis* as well as MAP) was ruled out on the basis of the fact that no differences in Cq values were noticed between the heat-killed and non-heat-treated (both unexposed to Pd compounds) cells in qPCR.

Preparation of palladium compounds and propidium monoazide. Four Pd compounds, previously reported in the study by Soejima and Iwatsuki[10], were used to assess mycobacterial cell viability: dichloro(n-cycloocta-1,5-diene)palladium(II), bis(benzonitrile)dichloropalladium(II), palladium(II)acetate (Sigma-Aldrich, USA) and trans-diammine dichloropalladium(II) (Alfa Aesar, USA). The compounds were dissolved to the appropriate concentration (see below) in physiological saline solution with heating at 40 °C for approximately 1 h with shaking (1,000 rpm). PMA was prepared by the dissolution of 1 mg of PMA (Biotium, USA) in 1.9 ml of 20% dimethyl sulfoxide (DMSO; Sigma-Aldrich) to obtain a 1 mM solution[8].

The effect of palladium compounds on DNA amplification. The same initial procedure as in the previous study[14] was followed. An MAP cell suspension, prepared by resuspension of one loop of MAP culture in 200 µl of ultrapure water, was subjected to DNA isolation using a Quick-DNA Fecal/Soil Microbe Microprep kit (Zymo Research, Tustin, California, USA) according to the manufacturer's protocol. Additionally, the original protocol was upgraded with the mechanical homogenization of MAP with 0.1 mm zirconia silica beads (Biospec) in a MagNA Lyser instrument (Roche Diagnostics GmbH, Mannheim, Germany) at 6,400 rpm for 1 min. The concentration of isolated DNA was determined using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA), and subsequently the DNA was divided into two parts, with the first diluted with ultrapure water and the second with tris-ethylenediaminetetraacetic acid (EDTA; TE) buffer (Serva Electrophoresis GmbH, Germany) to 1 ng/µl, and dispensed at 50 µl per microtube. Each Pd compound solution was added individually to the caps of the microtubes filled with the DNA solution, which were subsequently closed

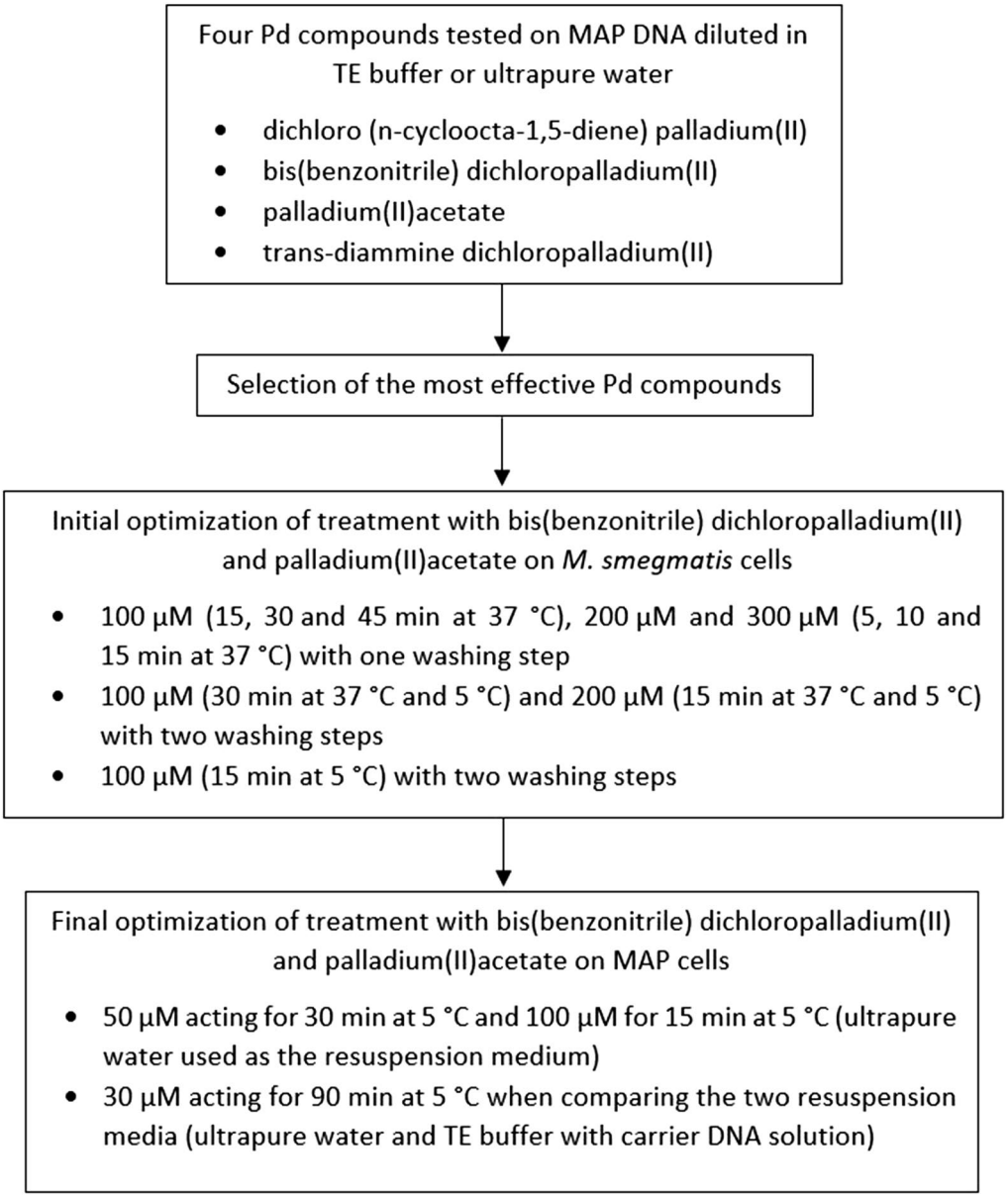

Figure 1. Schematic overview of the optimization of Pd compound treatment.

and uniformly spun to mix the Pd compounds and DNA solutions to reach a 10 µM, 100 µM and 1000 µM concentration. Incubation with a particular Pd compound for 30 min at 37 °C with shaking at 100 rpm was followed by DNA purification using a DNeasy Blood & Tissue Kit (Qiagen, Germany), and the DNA was subjected to qPCR. The identical procedure was performed for control samples with the exception that physiological saline solution was added instead of the Pd compound solution. Each condition was tested in biological duplicate. A schematic overview of the optimization of the treatment with Pd compounds is shown in Fig. 1.

Initial optimization of palladium compound treatment conditions on *M. smegmatis* cells. Solutions of bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate were added to the caps of the microtubes with 500 µl of viable and heat-killed cell suspensions to reach a 100 µM, 200 µM and 300 µM final concentration after mixing (Fig. 1). Physiological saline solution was added instead of the Pd compound solution in the case of control samples. The microtube caps were carefully closed, and the Pd-treated and untreated cell suspensions were uniformly spun and incubated at 37 °C with shaking at 100 rpm or 5 °C with manual shaking every 10 min for 5, 10, 15, 30 or 60 min depending on the concentration applied. The microtubes were centrifuged at 7,000 × g for 5 min, and the supernatant was removed. Then, either DNA extraction (see below) from the cell pellet was performed immediately or the cells were additionally washed with ultrapure water before extraction. Each condition was tested in biological duplicate.

Application of the palladium compound treatments on MAP cells. After initial optimization on *M. smegmatis* cells, optimization of the viability assay continued on the target MAP cells. Live and dead MAP cell suspensions were treated with 30 µM (acting for 90 min), 50 µM (30 min) and 100 µM (15 min) bis(benzonitrile) dichloropalladium(II) and palladium(II)acetate at 5 °C (with manual shaking about every 10 min) followed by centrifugation at 7,000 × g for 5 min, washing with ultrapure water and DNA extraction (Fig. 1).

Viability assay utilizing propidium monoazide. The procedure previously described in the study by Kralik et al.[8] was followed for PMA treatment. In brief, 12.5 µl of PMA was added to 500 µl aliquots of live and dead MAP cells to achieve a final 25 µM concentration. The microtubes were briefly spun and incubated for 5 min at room temperature with shaking at 1,200 rpm under minimal light conditions. Subsequently, the microtubes placed horizontally on ice were irradiated with light (a halogen lamp with a 650 W bulb) for 2 min from a distance of 20 cm. This treatment with PMA was repeated once more, and the microtubes were then centrifuged at 7,000 × g for 5 min. The supernatant was removed, followed by DNA extraction from the cell pellets. The identical procedure was performed in the case of controls except that 20% DMSO was added instead of PMA. This experiment was carried out in biological duplicate.

DNA extraction after treatment with palladium compounds and propidium monoazide. DNA was extracted as a crude lysate as described in the study by Kralik et al.[8], in which this procedure showed comparable outcomes to DNA isolation kits. The cell pellets were resuspended in 500 µl of ultrapure water in the case of *M. smegmatis*. For MAP, only ultrapure water was used at the initial treatment with 50 and 100 µM Pd compounds, while ultrapure water and TE buffer supplemented with carrier DNA solution (salmon sperm DNA, 50 ng/µl, Serva, Germany), both in a volume of 500 µl, were compared as the media used to resuspend the cell pellets in the case of subsequent treatment with 30 µM. Only TE buffer with carrier DNA solution was used in the case of treatment with PMA. The resuspended cells were lysed in a thermoblock at 100 °C for 20 min, then centrifuged at 14,000 × g for 5 min, and the supernatant containing DNA was collected and analyzed by qPCR.

Quantitative PCR, data analysis and statistics. qPCR assay targeted the ITS (internal transcribed spacer) sequence for *M. smegmatis*[15] and the *F57* sequence for MAP[16]. An internal amplification control (IAC) was included in both qPCR assays. All samples were analyzed in technical duplicates using a LightCycler 480 (Roche Molecular Diagnostics, Germany).

The "Fit Point Analysis" of the LightCycler 480 software (version 1.5.0.39) was used to determine Cq values, from which the differences (ΔCq) between purified MAP DNA or live and dead mycobacterial cells treated with Pd compounds or PMA and the respective control (untreated) samples were calculated. The following formulas for calculating the ΔCq were, similarly to the previous study[14], taken with slight modifications from the study by Kralik et al.[8]:

$$\Delta Cq_{\text{DNA with Pd}-\text{DNA without Pd}} \quad (1)$$

$$\Delta Cq_{\text{dead with Pd/PMA}-\text{live with Pd/PMA}} \quad (2)$$

$$\Delta Cq_{\text{dead with Pd/PMA}-\text{dead without Pd/PMA}} \quad (3)$$

$$\Delta Cq_{\text{live with Pd/PMA}-\text{live without Pd/PMA}} \quad (4)$$

Equation (1) describes the difference in amplification of the target sequence of Pd-treated and untreated MAP DNA samples. The highest possible ΔCq value is desirable, i.e. the greatest possible suppression of target sequence amplification by the Pd compound (without affecting the IAC). Equation (2) indicates the extent to which Pd or PMA suppresses target sequence amplification in dead cells, with the resulting value being reduced by the extent to which the agent undesirably suppresses amplification in live cells. Equation (3) also indicates the extent to which Pd or PMA suppresses amplification in dead cells, though regardless of live cells. For both Eqs. (2) and (3) the highest possible ΔCq value is desirable. Equation (4) indicates the extent to which Pd or PMA undesirably suppresses target sequence amplification in live cells, i.e. the lowest possible ΔCq value is required.

In the statistical analysis of ΔCq, one-way and two-way analysis of variance (ANOVA) and Tukey's HSD test were applied using the statistical software Statistica 13.2 (StatSoft Inc., Tulsa, OK, USA). P-values less than 0.05 were considered statistically significant.

Results

The effect of palladium compounds on DNA amplification. In the first step, the binding ability of four Pd compounds to purified MAP DNA was evaluated (Fig. 2). The ΔCq values of Pd-treated and untreated control samples were calculated according to Eq. (1). The highest suppression of amplification was found for bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate, in which complete qPCR signal eliminations were achieved at a concentration of 1,000 µM. This experiment also examined the impact of two different media (TE buffer and ultrapure water) used to dilute the DNA on the ability of Pd compounds to chelate the target DNA sequence (Fig. 2). TE buffer proved to be an unsuitable dilution medium, as its utilization resulted in a significantly lower reduction in DNA amplification as compared to ultrapure water. Based on these results, bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate were selected for subsequent optimization using live and heat-killed *M. smegmatis* and MAP cells diluted in ultrapure water.

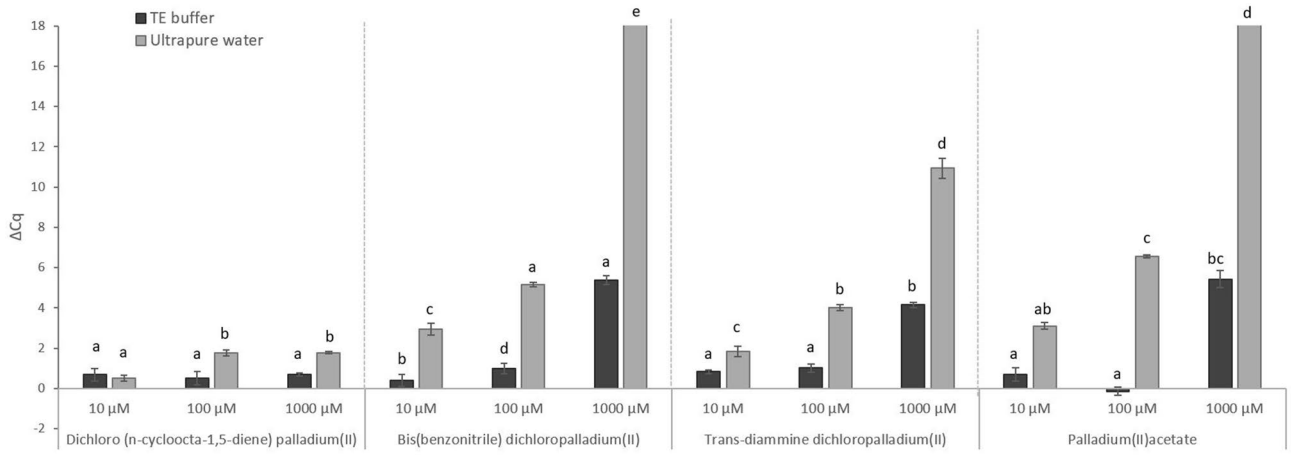

Figure 2. The effect of Pd compound solutions at three concentrations on the amplification of MAP DNA diluted with TE buffer or ultrapure water. The ΔCq values represent the mean difference of Cq values of Pd-treated and mean Cq value of Pd-untreated control samples counted from four biological and technical replicates. Two-way ANOVA with factors of concentration (10, 100, 1000 µM) and dilution medium (TE buffer, ultrapure water) and Tukey´s HSD test were used to evaluate the significance of differences between ΔCq, with all Pd compounds evaluated separately. Identical letters signify statistically insignificant differences ($P > 0.05$) between ΔCq values, and different letters signify significant differences ($P < 0.05$). Error bars express standard deviations counted from four replicates. The bars with ΔCq value above 18 indicate that no amplification occurred.

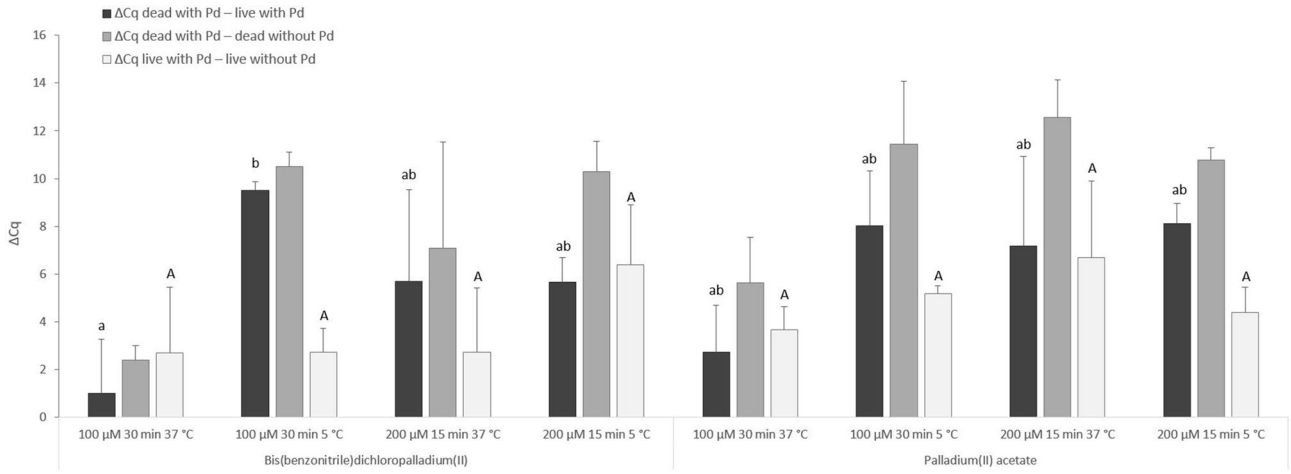

Figure 3. The effect of treatment of *M. smegmatis* cells with bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate at two different concentrations (100 µM, 200 µM), times (30 min, 15 min) and temperatures (37 °C, 5 °C). The ΔCq values represent the individual mean differences of Cq values counted from four biological and technical replicates. One-way ANOVA and Tukey´s HSD test were used to evaluate the significance of differences between ΔCq. Identical letters signify statistically insignificant differences ($P > 0.05$) between ΔCq values, and different letters signify significant differences ($P < 0.05$). The values of $\Delta Cq_{\text{dead with Pd – live with Pd}}$ were labeled with lowercase letters, and the values of $\Delta Cq_{\text{live with Pd – live without Pd}}$ were labeled with capital letters, since they were assessed separately. Error bars express standard deviations counted from four replicates.

Initial optimization of palladium compound treatment conditions on *M. smegmatis* cells. Bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate at concentrations of 100 µM, 200 µM and 300 µM were used for the initial treatment of live and heat-killed cell suspensions at 37 °C. Since substantial inhibitions of IAC amplification occurred in qPCR, an additional washing step was added in the following experiment, in which only concentrations of 100 µM acting for 30 min and 200 µM for 15 min were applied (Fig. 3). In addition, the effect of temperature (37 °C and 5 °C) during the treatment with the two Pd compounds was investigated in this experiment. For both Pd compounds, a concentration of 100 µM acting for 30 min at 5 °C appeared to be the most suitable of the conditions tested (the highest values of $\Delta Cq_{\text{dead with Pd – live with Pd}}$ and $\Delta Cq_{\text{dead with Pd – dead without Pd}}$), although a high level of undesirable penetration of both Pd compounds into live cells was observed (a value of $\Delta Cq_{\text{live with Pd – live without Pd}}$ greater than 2 Cq). Thereafter, exposure to Pd compounds at a

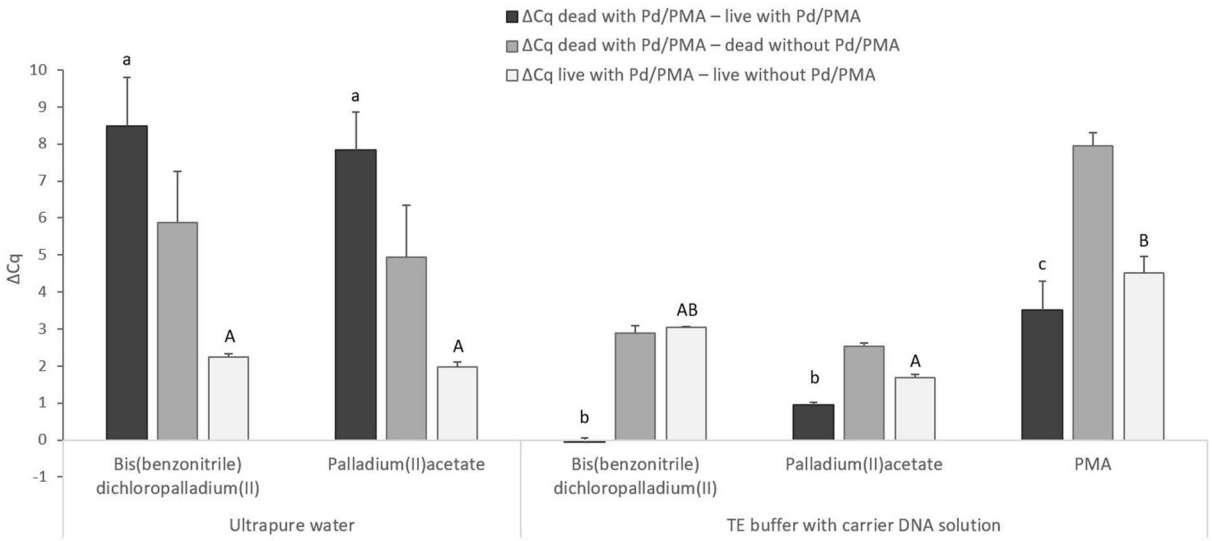

Figure 4. Comparison of treatment of MAP cells with bis(benzonitrile)dichloropalladium(II), palladium(II) acetate and PMA, and the effect of two different resuspension media (ultrapure water, TE buffer with carrier DNA solution) used after the Pd treatment. The ΔCq values represent the individual mean differences of Cq values counted from four biological and technical replicates. Two-way ANOVA with factors of agents used and resuspension medium and Tukey´s HSD test were used to evaluate the significance of differences between ΔCq. Identical letters signify statistically insignificant differences ($P > 0.05$) between ΔCq values, and different letters signify significant differences ($P < 0.05$). The values of $\Delta Cq_{\text{dead with Pd/PMA – live with Pd/PMA}}$ were labeled with lowercase letters, and the values of $\Delta Cq_{\text{live with Pd/PMA – live without Pd/PMA}}$ were labeled with capital letters, since they were assessed separately. Error bars express standard deviations counted from four replicates.

concentration of 100 µM was retested for 15 min, although at 5 °C with double washing. This did not, however, result in lower permeation of the Pd compounds into live cells (data not shown).

Application of the palladium compound treatments on MAP cells. Since a high degree of permeation of both bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate into live *M. smegmatis* cells was observed at a concentration of 100 µM, a lower concentration of 50 µM (30 min at 5 °C) was tested in addition to 100 µM (15 min at 5 °C) when applying the viability assay to MAP cells. However, a high value of $\Delta Cq_{\text{live with Pd – live without Pd}}$ of about 3 Cq was still recorded for both conditions (data not shown). The live and heat-killed MAP cells were subsequently exposed to an even lower concentration of 30 µM for an extended period of time (90 min) at 5 °C and compared to a reference viability assay using PMA. For Pd compounds, the effect of ultrapure water and TE buffer with carrier DNA solution used to resuspend cell pellets after the first wash was also evaluated (Fig. 4). When using ultrapure water, the viability assay utilizing bis(benzonitrile) dichloropalladium(II) and palladium(II)acetate attained differences in Cq values between treated live and dead MAP cells of 8.5 and 7.9, respectively, corresponding to approximately 2.5 log units. Regarding the permeation of Pd compounds into live MAP cells, values of $\Delta Cq_{\text{live with Pd– live without Pd}}$ of slightly above and slightly below 2 Cq were recorded for bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate, respectively. The second resuspension medium tested – TE buffer with carrier DNA solution – showed a significant drop in the ΔCq values of Pd-treated live and dead MAP cells below 1 Cq for both Pd compounds compared to ultrapure water. TE buffer with carrier DNA solution is therefore not a suitable medium in a viability assay using these Pd compounds. Nevertheless, it should be noted that ultrapure water is probably not a suitable medium either, as slightly reduced DNA amplification in the dead-cell control was recorded in MAP, though not detected in *M. smegmatis*. In the viability PCR using PMA, the difference in Cq values between PMA-treated live and dead MAP cells attained a value of about 3.5, which was significantly lower compared to both Pd compounds and, in addition, PMA penetrated more significantly into live MAP cells (Fig. 4).

Discussion

Cultivation is currently a standard method for assessing MAP viability, but it is very time-consuming (up to several months). In contrast, viability PCR is capable of viability evaluation within one day. However, the currently used PMA-qPCR requires demanding procedures involving manipulation in a darkroom and on ice. The aim of this study was to optimize a viability assay utilizing Pd compounds in combination with qPCR for the selective detection of live mycobacterial cells, specifically MAP, whose procedure is less demanding compared to PMA-qPCR and also less costly than both PMA-qPCR and the recently introduced Pt-qPCR[10].

Four Pd compounds were tested for their ability to chelate MAP DNA manifested in a decrease in DNA amplification (Fig. 2). Bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate showed the greatest suppression of DNA amplification. The direct effect of these Pd compounds on DNA amplification was also evaluated

in the study by Soejima and Iwatsuki[10] using purified *Cronobacter sakazakii* DNA. The same Pd compounds did not show any effect on norovirus RNA in another study addressing the detection of infectious noroviruses[12].

A significant difference between the use of TE buffer and ultrapure water was demonstrated when a comparison of the two DNA dilution media was made (Fig. 2). Higher ΔCq values between Pd-treated and untreated DNA samples were recorded when using ultrapure water as compared to TE buffer. The reason for the lower reduction in amplification in the case of TE buffer was probably the fact that the Pd compounds used in our study apparently formed chelates with the EDTA, as is confirmed by previous studies on Pd complexes[17,18], and these were consequently unable to bind to DNA. Ultrapure water was therefore used to dilute mycobacterial cell suspensions in subsequent viability assays.

The treatment with the Pd compounds bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate, which showed the greatest reduction in the qPCR signal when evaluated for the direct effect on MAP DNA, was then optimized on mycobacterial cells. Initial optimization steps were performed on *M. smegmatis*, as a model mycobacterium due to its short incubation time, with a continuation on the target slow-growing MAP. Based on the optimization procedures examining various concentrations, exposure times and temperatures, the optimal conditions for the Pd treatment of MAP cells were defined as 90 min exposure to a 30 µM concentration at 5 °C (Figs. 3 and 4). The differences noticed between Pd-treated live and dead MAP cells under these optimized conditions were 8.5 and 7.9 Cq for bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate, respectively, i.e. about 2.5 log units. The results achieved in the present study confirmed the findings of the previous study by Soejima and Iwatsuki[10] that Pd compounds are effective at much lower concentration than Pt compounds that have recently been applied to MAP[14], which is important in terms of cost reduction. The maximum value of the difference between the Cq of Pt-treated live and dead MAP cells achieved in the above study was slightly above 6 Cq when treated with 100 µM *cis*-dichlorodiammine platinum(II), which is lower by about 2.5 Cq and 2 Cq compared to bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate, respectively, at a concentration of 30 µM, as evaluated in the present study. Based on these findings, Pd compounds seems to be superior to Pt compounds in viability PCR applied to MAP. To date, the Pd compounds have been used to assess viability only in the enterobacteria *Escherichia coli* and *C. sakazakii*[10], in which complete suppression of the qPCR signal of dead cells was achieved. In our study, absolute elimination of the qPCR signal was not attained for either dead MAP or *M. smegmatis* cells. This is probably caused by a more complex mycobacterial cell wall, through which Pd compounds are less permeable even in membrane-compromised dead cells. Another probable factor could be the use of a short amplicon (< 150 bp), which made it impossible to eliminate the qPCR signal more efficiently[9]. Furthermore, the efficient chelation of the Pd compound molecules with the target DNA could be reduced, as it has been suggested the Pd molecules possibly also adsorb to cell wall transmembrane proteins and DNA-binding proteins[10]. Regarding the undesirable impact of Pd compounds on live MAP cells, the differences observed between Pd-treated and untreated live MAP cells were around 2 Cq for both bis(benzonitrile) dichloropalladium(II) and palladium(II)acetate. Similarly, in a study by Soejima and Iwatsuki[10], differences between Pd-treated and untreated live enterobacteria of up to 2.1 Cq were recorded, which was evaluated by the authors as an insignificant permeation. In addition to the possible limited penetration of Pd molecules into live cells, which, as in the case of dead cells, may result in suppression of target DNA amplification, the slight increase in Cq values for non-heat-treated cells after Pd-treatment could also be caused by the presence of membrane-compromised cells naturally occurring in the viable cell suspension. We tried to estimate the number of viable cells in the initial *M. smegmatis* suspension retrospectively by calculating CFU on plated serial dilutions. However, it greatly underestimated (by about 1.5–2 log units) the cell concentrations due to the clustering of mycobacteria as compared with the determination by qPCR (data not shown). These results are consistent with the study by Elguezabal et al.[7], who evaluated that the qPCR of a single-copy gene is a more reliable method for determining mycobacterial numbers than cultivation, which we followed in our study for both *M. smegmatis* and MAP.

The final experiment also demonstrated the effect of the resuspension medium used after the first washing of the cells following the Pd-treatment on the viability assay (Fig. 4). Since heat-killed Pd-untreated MAP cells showed a decrease in the target sequence amplification when using ultrapure water as the resuspension medium, although not in *M. smegmatis*, the use of DNA-stabilizing and loss-reducing[16] TE buffer with carrier DNA solution was also tested. Nevertheless, the use of TE buffer with carrier DNA solution resulted in a significantly lower difference in Cq values between Pd-treated live and dead MAP cells (below 1 Cq) compared to the use of ultrapure water. Consequently, the TE buffer should not be used in a viability assay either as a dilution or as a resuspension medium as it impairs the ability of Pd compounds to suppress amplification of the target sequence in dead cells. Decreases in DNA amplification of 1.5 Cq and 2.3 Cq in heat-killed Pd-untreated *C. sakazakii* and *E. coli* cells, respectively, compared to live Pd-untreated cells both diluted in water were also recorded in the study by Soejima and Iwatsuki[10]. It is therefore necessary to find a more suitable medium that does not distort the viability assay utilizing Pd compounds.

The efficiency of the viability assay Pd-qPCR was compared with PMA-qPCR (Fig. 4), which has previously been applied in the viability determination of MAP cells[8]. Both bis(benzonitrile)dichloropalladium(II) and palladium(II)acetate (in the case of the use of ultrapure water as the resuspension medium) allowed higher differences in Cq values between Pd-treated live and dead MAP cells, by about 5 Cq and 4.3 Cq, respectively, than PMA dye. In addition, PMA showed higher permeability into live MAP cells than Pd compounds. Pd compounds thus proved to be a more suitable viability marker than PMA. Likewise, Soejima and Iwatsuki[10] evaluated Pd compounds as superior to the PMA agent for distinguishing between live and dead enterobacteria in water.

In summary, our study optimized a viability assay combining Pd compound treatment with qPCR to detect live MAP cells diluted in ultrapure water. Pd-qPCR proved to be more effective in distinguishing between live and dead MAP cells as compared to the previously established PMA-qPCR. In terms of practical use, further studies could focus on evaluating the ability of the optimized protocol to detect viable MAP cells in milk, tissue

or feces from infected animals or environmental samples. If efficacy is demonstrated in clinical samples, Pd-qPCR will facilitate the identification of MAP-infected cattle, thus ensuring effective monitoring of paratuberculosis.

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Author contributions

M.C. designed and performed all the experiments, analyzed and interpreted the data, and wrote the manuscript. M.B. participated in the initial experiments. V.B. performed the statistical analysis. P.K. designed the study and revised the manuscript. All authors approved the submitted version.

Competing interests

The authors declare no competing interests.

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REVIEW
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xMAP Technology: Applications in Detection of Pathogens

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xMAP technology is applicable for high-throughput, multiplex and simultaneous detection of different analytes within a single complex sample. xMAP multiplex assays are currently available in various nucleic acid and immunoassay formats, enabling simultaneous detection and typing of pathogenic viruses, bacteria, parasites and fungi and also antigen or antibody interception. As an open architecture platform, the xMAP technology is beneficial to end users and therefore it is used in various pharmaceutical, clinical and research laboratories. The main aim of this review is to summarize the latest findings and applications in the field of pathogen detection using microsphere-based multiplex assays.

Keywords: xMAP, magnetic microspheres, multiplex detection, nucleic acid detection, immunoassay, pathogen identification, diagnostics

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INTRODUCTION

High-throughput multiplex detection techniques are designed for the rapid, sensitive and specific testing of large numbers of analytes (nucleic acid assays, immunoassays, enzyme assays, or receptor-ligands) in a single biological sample. These techniques enable analysis of large numbers of samples. On the other hand, there are also classical single reaction detection methods based on determination of nucleic acids such as polymerase chain reaction (PCR) (Dunbar, 2006; Taylor et al., 2001), quantitative real-time PCR (qPCR) (Wuyts et al., 2015; Iannone et al., 2000), reverse transcription PCR (RT-PCR) (Weis et al., 1992) and reverse transcription quantitative PCR (RT-qPCR) (Bustin, 2000), or antibody-based tests like enzyme-linked immunosorbent assays (ELISA) (Engvall and Perlmann, 1971; Vanweeme and Schuurs, 1971) represent nowadays the "gold diagnostic standard" in many laboratories. Despite the previous implementation of these methods for routine rapid, sensitive, specific and cost-effective molecular diagnostics, their ability to simultaneously detect multiple analytes in a single reaction is limited and this limitation has yet to be overcome. The increasing amount of proteomic, transcriptomic and genomic sequence data from a large number of organisms accessible in public databases represents an exceptional opportunity for the development of new, multiplex detection technologies. The Luminex® xMAP technology (x = analyte, MAP = Multi-Analyte Profiling) that was invented in the late 1990s represents such a platform that can benefit from all the advances in DNA research (Angeloni et al., 2014). Although PCR allows multiplex amplification of several targets in a single run xMAP as a methodology represents a significant step forward, and was designed with the aim of creating a high-throughput bioassay platform, enabling rapid, cost-effective, and simultaneous analysis of multiple analytes within a single biological sample. As an open architecture platform, the xMAP system holds many benefits for the end user and therefore it is used in pharmaceutical, clinical and research laboratories (Dunbar and Li, 2010). The main aim of this review is to summarize the

state-of-the-art of xMAP technology applications in the detection of viral, bacterial, parasitical and fungal pathogens from different matrices.

xMAP TECHNOLOGY – IN THE BEGINNING WERE THE MICROSPHERES

The principle of xMAP technology is based on the concept of a liquid (suspension) array. In contrast to the conventional microarray technology where the identity of the analyte is characterized by its position on the glass slide, the xMAP technology uses different sets of microspheres in a liquid suspension as determiners of analyte specificity. Microsphere sets are internally dyed with two spectrally different fluorophores. The spectral signature is unique for each microsphere set and is determined by different concentrations of internal dyes, producing a 100-member array of spectrally distinct microsphere sets (**Figure 1**). Integration of a third internal dye has allowed the expansion of up to 500-member microsphere sets (Dunbar and Li, 2010). The surface of each microsphere set allows a simple chemical coupling of various reagents specific to a particular bioassay, such as nucleic acid assays, immunoassays, enzyme assays or receptor-ligand assays. A further fluorescent reporter (e.g., Streptavidin-R-phycoerythrin, Alexa 532, Cy3) is coupled to a target molecule, which allows its detection after specific capture on the microsphere surface.

There are different types of commercially available microspheres (**Table 1**), and their selection is generally determined by the type of instrumentation used for detection and the particular analyte of interest (Dunbar and Li, 2010; Houser, 2012). Basic microspheres are 5.6 μm polystyrene beads whose surface is covered by approximately 10^8 carboxyl groups (COOH) for covalent coupling of capture reagents (Tang and Stratton, 2006). Magnetic microspheres (**Figure 2**) differ in size and structure through the addition of a magnetite layer (Dunbar and Li, 2010; Houser, 2012). Usage of magnetic beads improves washing efficiency as the magnetic separation step enables the elimination of unwanted sample constituents. Moreover, MagPlex-TAG microspheres are covalently pre-coupled with unique 24 base pair-(bp)-long anti-TAG oligonucleotides that serve as an anchor for target sequences containing the complementary TAG sequence. This proprietary TAG system (xTAG technology) is optimized to have minimum cross-reactivity. An assay can be easily designed by adding a complementary TAG sequence into the sequence of the primer or detection probe of interest and hybridization to the anti-TAG sequence on the microsphere surface.

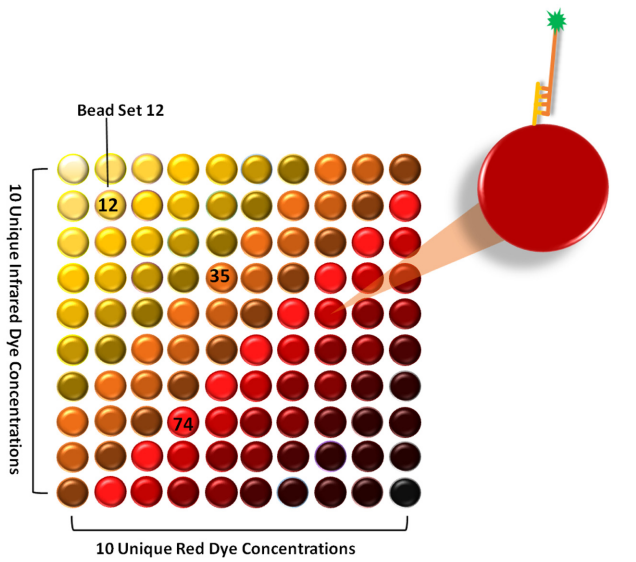

FIGURE 1 | The xMAP Technology based on internally dyed microspheres. Different concentrations of red and infrared fluorophores were used to create 100 distinct microsphere sets. Each set is able to conjugate to a specific target molecule (yellow and orange lines = nucleic acid; green star = fluorescent reporter).

Mechanism of Signal Detection and Overview of Available Instruments

The analysis of beads is in general performed by two lasers. The red classification laser/LED (635 nm) excites the inner fluorescent dyes of the microspheres, thus identifying a specific microsphere set according to its spectral signature. If the analyte of interest is present, the green reporter laser/LED (525–532 nm) recognizes the fluorescent reporter bound to the captured analyte on the microsphere surface.

There are approximately 10^4 microspheres from each set present in a single sample. This number represents the range in xMAP, in which it is possible to perform determination of quantity according to a calibration curve, similarly to qPCR. However, one must bear in mind that inclusion of a PCR amplification step prior to xMAP analysis does not reveal the real number of DNA molecules present in the original sample, but can only be used for the approximate estimation of DNA quantity. Therefore, xMAP can provide only semi-quantitative data.

The simultaneous reading of both spectra is performed in purpose-designed readers (**Table 2**). They differ by their mechanisms of fluorescence capture and by the maximum number of samples that can be analyzed.

The basic detection instrument, which is called MAGPIX, is compatible only with magnetic microspheres (MagPlex and MagPlex-TAG). The principle of microsphere analysis in the MAGPIX instrument is based on their immobilization in the monolayer on the magnetic surface (**Figure 3**). Unlike flow-based instruments, the fluorescent imager of the MAGPIX system reads all the microspheres at once, while generating data that is comparable with other methods. Reading a 96-well-plate takes about 60 min. The maximal reading capacity of MAGPIX instruments is limited to 50 bead sets.

Advanced detection instruments – the Luminex 100/200 (Bio-Plex 200) and FlexMAP (Bio-Plex) 3D – are based on flow cytometry principles. The microspheres with bound analyte are focused into a rapidly flowing fluid stream. Each microsphere

TABLE 1 | Commercially available microspheres.

Microsphere type	Size (μm)	Structure	Sets available	Instrument suitability	Analyte
MicroPlex®	5.6	Non-magnetic	100	Flow cytometry-based	All
MagPlex®	6.5	Magnetic	500	All xMAP	All
MagPlex-TAG™	6.5	Magnetic	150	All xMAP	Nucleic acid
LumAvidin®	5.6	Non-magnetic	100	Flow cytometry-based	Proteins
SeroMAP™	5.6	Non-magnetic	100	Flow cytometry-based	Proteins

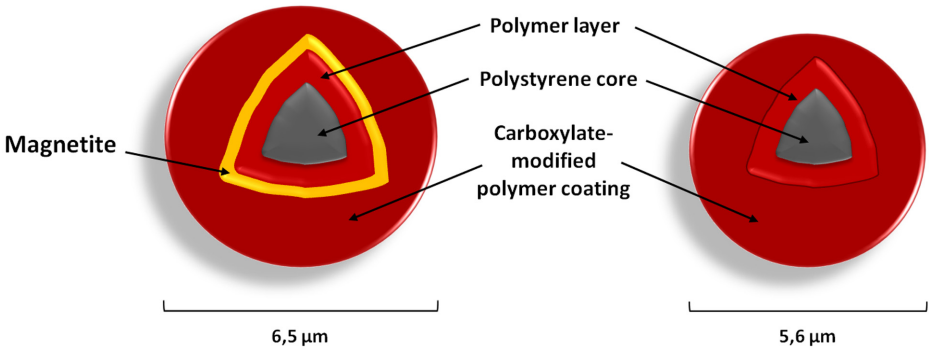

FIGURE 2 | Microsphere architecture. The polystyrene divinylbenzene core is surrounded by a polymer layer, which is formed by polystyrene methacrylic acid (infusion of dyes). The surface of each microsphere is irregular, porous and carboxylated. Magnetic microspheres have an additional layer of magnetite within the polymer layer and so differ also in size.

TABLE 2 | Detection instruments compatible with xMAP technology.

Instrument	Compatibility	Strategy	Analytes/reaction	Microplate type
Luminex MAGPIX®	Magnetic microspheres	Immobilization of microspheres in magnetic field	50	96-well plate
Luminex100®/200™	All types of microspheres	Flow cytometry-based	100 (80 with MagPlex)	96-well plate
FlexMAP 3D®	All types of microspheres	Flow cytometry-based	500	96 and 384-well plate

is then individually detected and digitally processed as the stream passes through the imaging cuvette. Flow cytometry-based platforms are convenient for applications with samples of limited size. The reading of a 96-well-plate is faster than in the MAGPIX system and takes 45 min or less. The capacity of the 3D platform is further increased by the possibility of analyzing 384-well plates.

MICROSPHERE-BASED MULTIPLEX ASSAY FORMATS

The microsphere-based technology can be applied in various assay formats, which can be divided, according to the type of analyte, into microsphere-based multiplex nucleic acid assay formats (MBMNA) and microsphere-based multiplex immunoassays (MBMI).

In general, xMAP-based assay formats are in comparison to other commonly used methods very open and flexible, ensuring the result data within few hours, while requiring only minimal amounts of sample.

Detection assays based on nucleic acids have a potential for high levels of multiplexing, approaching the levels of sensitivity achieved by target amplification methods like multiplex PCR or TaqMan chemistry assays, while using the same protocols of DNA/RNA extraction. Multiplex oligonucleotide ligation PCR assay format (MOL-PCR) is able to simultaneously perform detection and identification, strain typing, detect antibiotic resistance determination, virulence prediction, etc., thereby surpasses other methods like Multiplex Ligation-dependent Probe Amplification (MLPA) or qPCR. The disadvantage of technology is that it is not capable to perform quantitative analysis like qPCR, because providing only semi-quantitative data.

xMAP immunoassays surpass the common enzyme immunoassays in the ability of multiple simultaneous detection, while requiring smaller amount of sample and lower cost. Moreover, these assay formats produce superior dynamic range and sensitivity.

Nucleic Acid Assays (MBMNA)

xMAP technology is applicable in numerous nucleic acid assay formats such as, e.g., gene expression analysis, microRNA analysis, single nucleotide polymorphism (SNP) analysis or specific sequence detection. Basically, nucleic acid assays can be developed by coupling sequence-specific capture oligos to

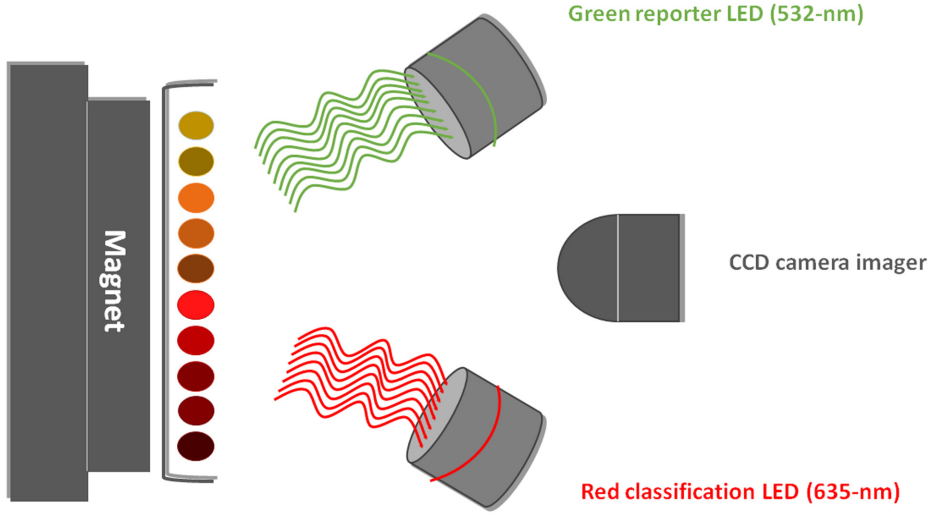

FIGURE 3 | Principle of analysis by the MAGPIX fluorescent imager. Magnetic microspheres immobilized on a magnet are recognized by LEDs and a CCD camera records the picture (LED, light-emitting diode; CCD, charge-coupled device).

magnetic microspheres or by use of xTAG technology (Angeloni et al., 2014).

When performing xMAP analysis of nucleic acids it is essential to include PCR amplification to enrich the number of targets in the sample to detectable levels. There are two general strategies for including a PCR step in the detection of pathogens using xMAP technology. The main difference between the two lies in which phase the PCR amplification is applied. In direct DNA hybridization (DDH), allele-specific primer extension (ASPE), single base chain extension (SBCE), and Oligonucleotide ligation assay (OLA) all the target DNA sequences are amplified in multiplex PCR prior to hybridization to microspheres. The disadvantage of these methods is that in assays containing large amounts of targets multiplex PCR leads to amplification bias, which is caused by the different lengths of the amplicons (Nolan et al., 2001). In contrast, in the multiplex oligonucleotide ligation PCR assay (MOL-PCR) sequence discrimination by detection probes occurs before the amplification step, which can subsequently be run just in singleplex PCR with universal primers.

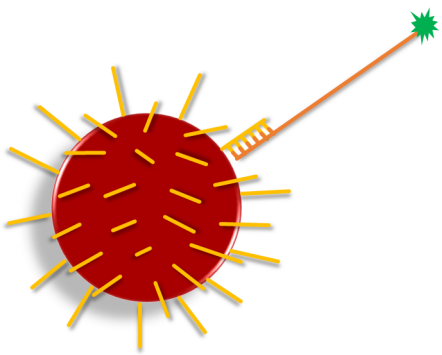

FIGURE 4 | Direct DNA hybridization (DDH, yellow lines = capture oligonucleotide; orange line = amplified target sequence; green star = fluorescent reporter). Target DNA sequence is amplified, while one of the primers is fluorescently labeled. Amplicons are then specifically hybridized (according to complementarity) to capture oligonucleotides on the microsphere surface.

Direct DNA Hybridization (DDH)

Direct DNA hybridization is one of the basic approaches used for the selective identification of sequences of interest from heterogeneous mixtures of DNAs (**Figure 4**). It is often used, e.g., for identification of species (Defoort et al., 2000; Page and Kurtzman, 2005; Righter et al., 2011; Liu Y. et al., 2012) or genotyping of pathogens (Letant et al., 2007; Zubach et al., 2012). In DDH, the amplification of target sequences is ensured by specific primer pairs, and one primer from each pair is fluorescently labeled at the 5′ end, permitting detection of the amplicon (Christopher-Hennings et al., 2013). The subsequent incubation of amplicon with microspheres leads to a direct and specific hybridization between matching capture and target sequences. Amplicon sequences should be 100–300 bp in length to minimize steric hindrance during hybridization and the capture sequence on microspheres should be 18–20 bp in size (Dunbar, 2006). The specificity of the capture sequences and stringency of hybridization conditions allow discrimination up to SNP. If the SNP or mutation discrimination is intended, the presumed mismatch should be located at the center of the capture sequence (Livshits and Mirzabekov, 1996). This assay format then requires a unique capture sequence coupled to a specific microsphere set to score each SNP allele (Kellar and Iannone, 2002).

Allele-Specific Primer Extension (ASPE)

Allele-specific primer extension (**Figure 5**) is an approach usually used for determination of allelic variants of pathogens (Page and

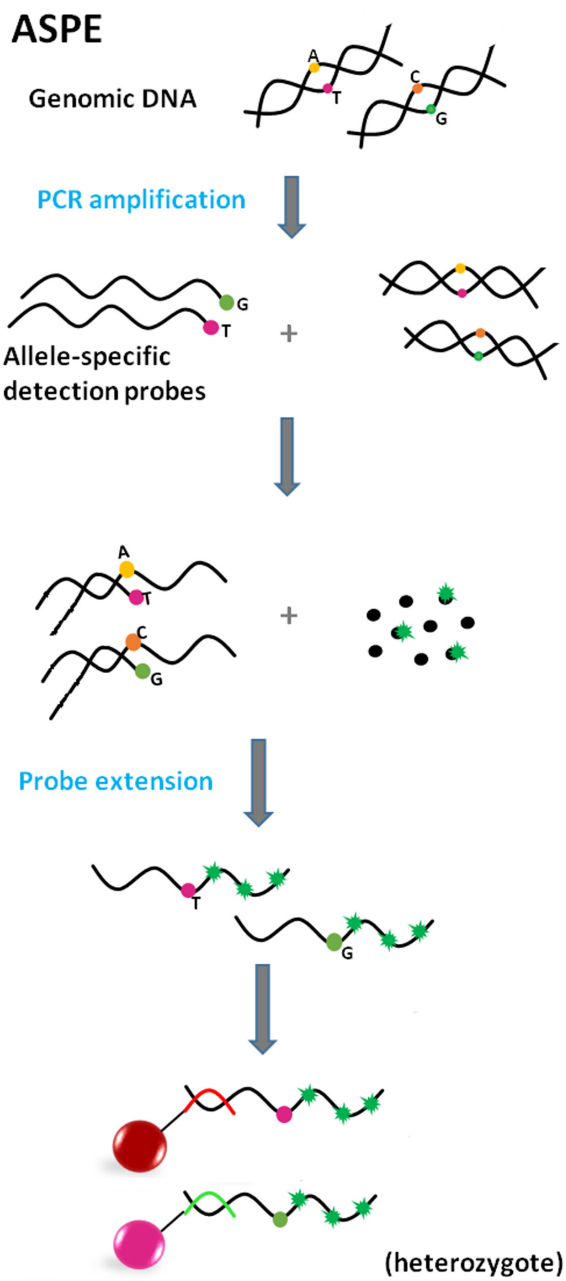

FIGURE 5 | Principle of Allele-specific primer extension (ASPE) (red and green line = anti-TAGs; green star = fluorescent reporter). Allele-specific detection probes, differing in one nucleotide on the polymorphic side, hybridize to amplified target sequence. After addition of DNA polymerase and dNTPs (one of which is fluorescently labeled), molecules are extended according to complementarity. Products are captured by anti-TAGs on the specific microsphere set.

Kurtzman, 2005; Lin et al., 2008). The defining characteristic of ASPE is the extension of two allele-specific detection probes, which contain a polymorphic site at the 3′ end, defining the particular allele variant. In this arrangement, DNA polymerase can extend detection probes by incorporation of dNTPs (one nucleotide is labeled, e.g., biotin-dCTP), if the allele is present in the sample. Just one probe is extended in the case of a homozygous target; conversely, in heterozygotes both probes are extended. The fluorescence signal is generated by a fluorophore bound to labeled dNTPs, incorporated within the extended probe.

Single Base Chain Extension (SBCE)

The use (Taylor et al., 2001) and assay format of SBCE is similar to the previously described ASPE. However, there are slight differences, mainly in the design of detection probes. In the case of SBCE (**Figure 6**), probe sequences are terminated one base before the polymorphic site (Chen et al., 2000; Ye et al., 2001). Due to this design the labeled dideoxyribonucleoside triphosphate (ddNTP) terminators serve as a "query" nucleotide and are used for single base probe extension at the same time; this assay requires the setting up of separate reactions for each of the four ddNTPs (ddC, ddG, ddA, and ddT). Moreover, PCR products from the previous step of PCR amplification of the target sequence need to be treated with exonuclease I and shrimp alkaline phosphatase (*ExoI*/SAP) before use as a template in the SBCE reaction (Ye et al., 2001; Dunbar, 2006) to get rid of unincorporated primers and dNTPs. Although SBCE has been proven to be highly specific and reliable (Chen et al., 1999; Syvanen, 1999), it is in the process of being replaced by less laborious methods.

Oligonucleotide Ligation Assay (OLA)

Oligonucleotide ligation-based formats include a ligation step of two oligonucleotide detection probes, which occurs in the presence of a target sequence of a specific pathogen. These assays are based on the ability of detection probes to hybridize next to each other on a complementary target DNA sequence (Landegren et al., 1988). If there are no mismatches near the junction site and there is a phosphate group at the 5′ end of a second probe (necessary for phosphodiester bond formation), annealing occurs; DNA ligase then recognizes the nick and forms a covalent bond between adjoining nucleotides while creating a single-stranded DNA molecule (Iannone et al., 2000). The most crucial step during the multiplexing of different ligation assays is the design of suitable probes with similar melting temperatures of between 51 and 56°C (Dunbar, 2006).

In the OLA assay format, the target DNA sequence is PCR-amplified prior to the ligation step of the annealed probes (**Figure 7**). OLA is suitable for SNP genotyping (Iannone et al., 2000; Taylor et al., 2001; Ye et al., 2001).

Multiplex Oligonucleotide Ligation PCR Assay (MOL-PCR)

The multiplex oligonucleotide ligation PCR assay represents an improved version of the previous OLA assay format. One advantage is that ligation is carried out prior to the PCR-amplification (**Figure 8**) (Nolan and White, 2004). Unlike in the OLA assay, one of the detection probes consists of a sequence complementary to the target sequence and an extension composed of the TAG sequence and primer binding site. The

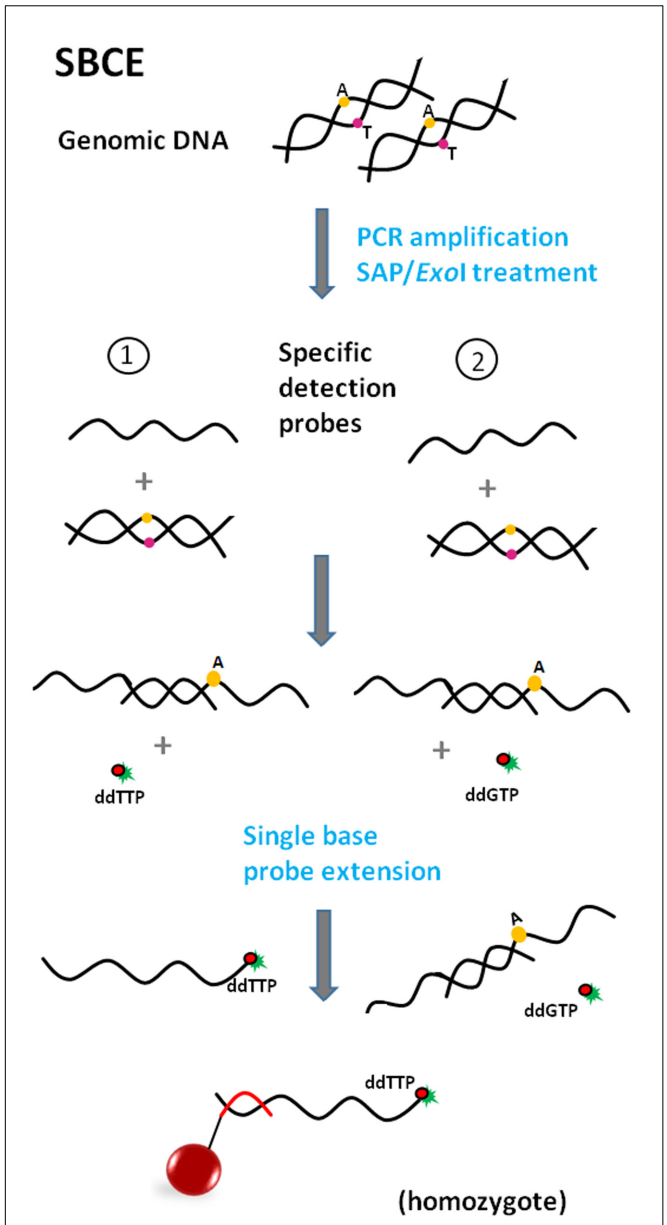

FIGURE 6 | Principle of Single base chain extension (SBCE) (red dot = dideoxynucleotide; green star = fluorescent reporter; red line = anti-TAG). Specific detection probes are terminated one base before the polymorphic site. Utilization of fluorescently labeled dideoxynucleotides necessitates a separate reaction for each nucleotide in focus (minimally two). Target DNA hybridizes with probes after amplification but only the mix with the proper ddNTP leads ultimately to the synthesis of a labeled product, which is captured by anti-TAG on the microsphere surface.

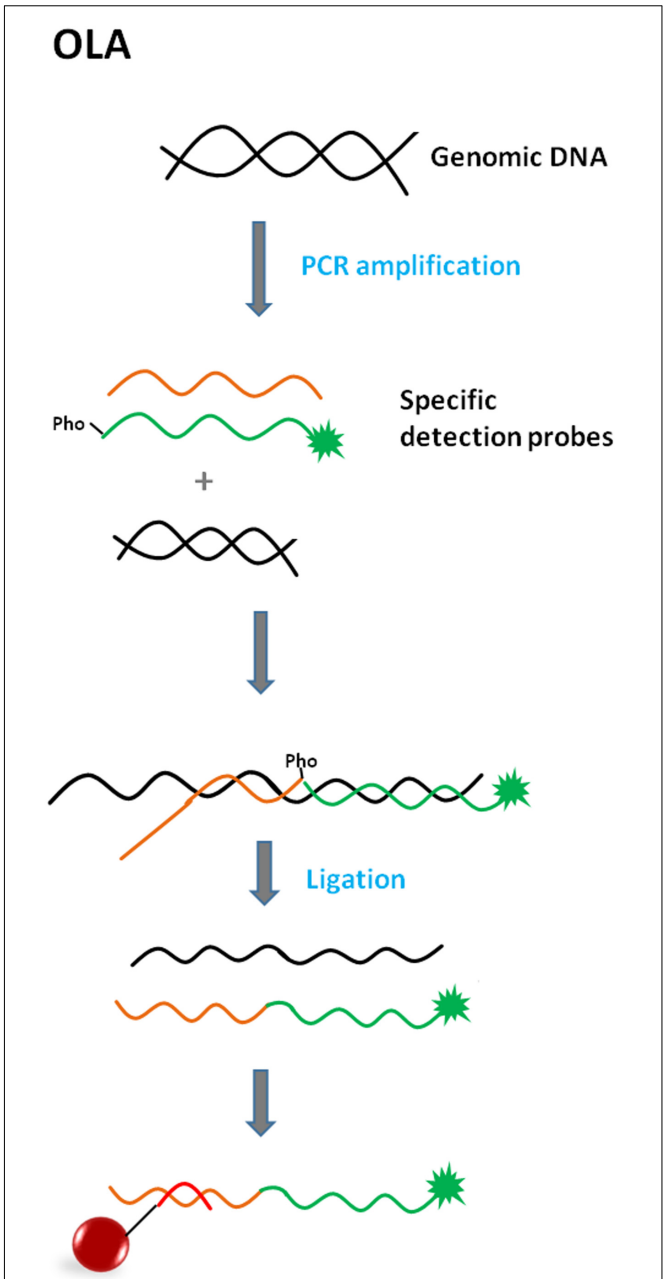

FIGURE 7 | Principle of Oligonucleotide ligation assay (OLA) (green star = fluorescent reporter; Pho = phosphate group; red line = anti-TAG). The target DNA sequence is PCR-amplified prior to the ligation step of the annealed probes. One of the detection probes consists of a sequence complementary to the target sequence (polymorphic site at the 3′ end if SNP identification is needed) and also an additional TAG tail sequence. The second detection probe is fully complementary to the target sequence and serves as a reporter due to its fluorescent label at the 3′ end. Detection probes bind next to each other, DNA ligase recognizes the nick and makes a bond. The product is captured by anti-TAG on the microsphere surface.

second probe is the same as the first except for the absence of the TAG sequence. Each probe pair is specific for a particular target sequence, but all pairs share the same primer sequence. Basically, these modular detection probes anneal to a target sequence, ligate into a complex single-stranded DNA molecule and only if this occurs does the molecule become a template for singleplex PCR using a universal pair of primers (one is fluorescently labeled). Additionally, all the ligation products are very similar in length (approximately 100 bp -120 bp), so the use of a universal primer pair during PCR makes the simultaneous amplification

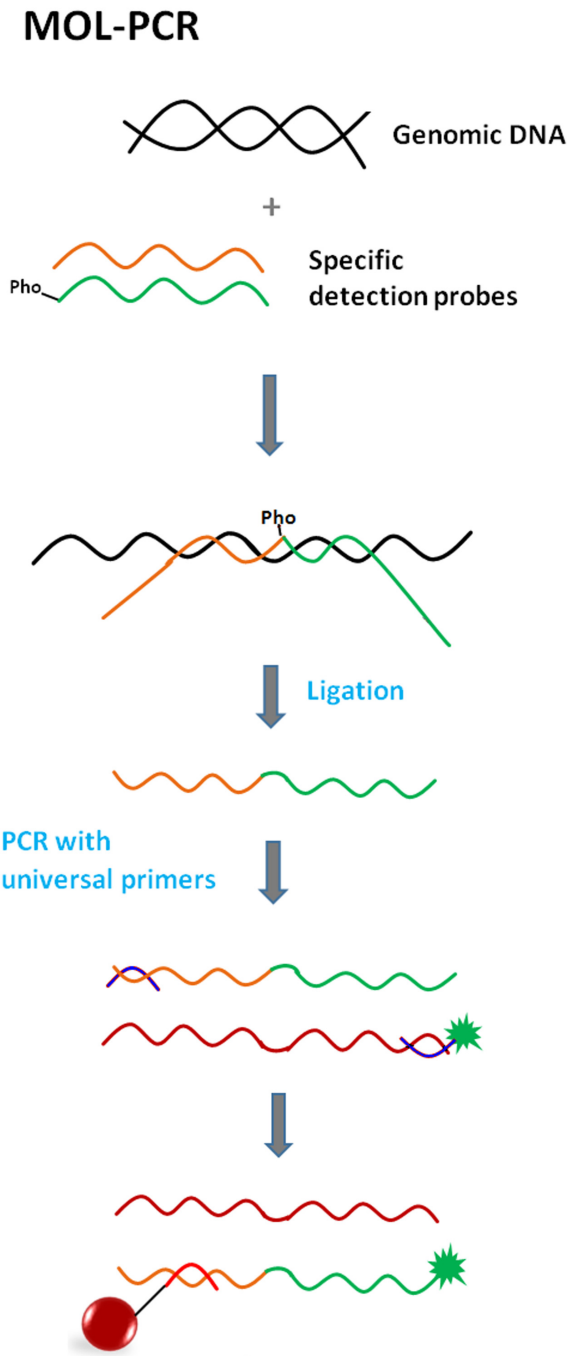

FIGURE 8 | Principle of Multiplex oligonucleotide ligation PCR assay (MOL-PCR) (orange line = detection probe 1; green line = detection probe 2; blue lines = universal PCR primers; burgundy line = amplified negative strand; green star = fluorescent reporter; Pho = phosphate group; red line = anti-TAG). Specific detection probes bind next to each other to target sequence via complementary parts, while the parts including the TAG sequence and binding sites for PCR primers form tails sticking out into space. DNA ligase recognizes the nick and makes a bond. The complex sequence of ligated probes becomes a template for singleplex PCR with universal primers; one of the primers is fluorescently labeled. Labeled amplicon hybridizes via its TAG sequence to capture anti-TAG on the microsphere.

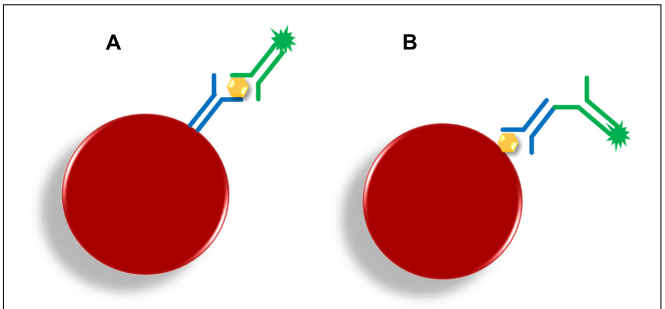

FIGURE 9 | Principle of microsphere-based multiplex immunoassays. **(A)** Capture sandwich (CS; yellow hexagon = target; blue Y = capture antibodies; green Y = detection antibody; green star = fluorescent reporter); **(B)** Indirect serological assay (ISA; yellow hexagon = capture antigen; blue Y = specific target antibody; green Y = detection anti-antibody; green star = fluorescent reporter).

of many short fragments highly feasible. All these facts ensure that MOL-PCR is not susceptible to the amplification bias that is characteristic of multiplex PCR or previously mentioned formats. Only a minimal amount of target/sample is required.

The MOL-PCR upgrade has the potential to have widespread impact on genomic assays, because not only is sequence detection and SNP identification possible, but the detection of indels (insertion/deletion), screening tests for pathogens (virus, bacteria, fungi) from various matrices or determination of antibiotic resistances is also feasible (Deshpande et al., 2010; Thierry et al., 2013; Wuyts et al., 2015). MOL-PCR could replace, e.g., MLPA or qPCR in certain applications in routine diagnostics (Deshpande et al., 2010).

Microsphere-Based Multiplex Immunoassay (MBMI)

Microsphere-based multiplex immunoassay (MBMIs) are typically biochemical tests that allow the detection or measuring of the concentration of an analyte (protein) in a solution through the use of an antibody or immunoglobulin (Angeloni et al., 2014). Single-analyte ELISA cannot support simultaneous detection of multiple specific antibody responses within a single serum sample (Bokken et al., 2012), and has further disadvantages, such as the requirement for a relatively large amount of sample, negligible non-specific binding or increased background. MBMIs represent an alternative for commonly used indirect tests like ELISA. The conversion of an ELISA assay to the MBMI format is uncomplicated, efficient, cost-saving and produces an assay with superior dynamic range and sensitivity (Baker et al., 2012). MBMIs are often used in the diagnostics of various pathogens including multicellular organisms, such as e.g., parasites, in tests where the current methods are not sensitive enough. The methods of choice are usually Capture Sandwich (CS) and Indirect Serological Assay (ISA) (**Figure 9**). However, the problems typical for methods based on serology remain: the need for periodical testing in order to avoid false negative results resulting from a wide and inevitable lag between infection and

development of a specific response against a parasite in the form of IgG antibodies (sero-positivity) (Nockler et al., 1995).

Capture Sandwich (CS)

The CS assay utilizes microspheres covalently coupled with a capture antibody (polyclonal antibodies should be purified and mono-specific) that takes up target molecules from the sample. This complex is recognized by a labeled detection antibody (Baker et al., 2012; Angeloni et al., 2014). The CS format can be used in cases where, for example, confirmation of pathogen identity within the inflammatory focus or altered tissue is needed.

Indirect Serological Assay (ISA)

In contrast to CS, in ISA a specific antibody against an antigen coupled with a microsphere is captured. If the binding of serum antibody to antigen occurs, a labeled secondary antibody (anti-antibody) then provides the visualization. ISA is typically used for serological screenings (monitoring and prevention purposes) that are carried out on serum samples (van der Wal et al., 2013).

APPLICATIONS OF xMAP TECHNOLOGY

The xMAP technology is used in many different applications. This chapter describes the use of this technology for multiplex detection of viral, bacterial, parasitical and fungal agents using the microsphere-based multiplex nucleic acid-assay formats (MBMNA) and microsphere-based multiplex immuno-assay formats (MBMI) described above.

Multiplex Detection and Typing of Viruses

Viruses are a very diverse group of infectious agents and are divided into groups according to a number of properties, e.g., type of nucleic acid, the presence of the viral envelope, antigenic structure, mode of transmission, pathogenicity, etc. They can be classified also according to the syndromes which they cause and mode of transmission, e.g., respiratory viruses, viruses causing gastroenteritis, tumors, hepatitis, rashes or neuroviruses.

To date, the majority of applications that enable multiplex viral detection and identification are based on the capture of viral nucleic acid by adoption of various DDH modifications.

Respiratory viruses are causative agents of the most common diseases of the human upper and lower respiratory tract, which are often associated with significant patient morbidity and mortality (Berry et al., 2015), e.g., H5N1 subtype of highly pathogenic influenza A virus (Neumann et al., 2010).

The MBMNA method for more effective detection and genotyping of H5N1 viral isolates from clinical samples comprising pharyngeal swabs and tracheal aspirates was developed and its efficiency was compared with RT-PCR and qPCR (Zou et al., 2007). The results using the MBMNA approach showed that this assay is vulnerable to viral mutations although the primers were designed according to conserved sequences. Therefore, there is a need to monitor viral mutations in order to reduce false-negative results and add new primers and probes to adapt to the mutations, which is a disadvantage of MBMNA. On the other hand, MBMNA holds a number of advantages compared to RT-qPCR and qPCR, e.g., allele-specific probes with TAG sequences can be recognized by a universal set of primers, thus potentially eliminating the problem with different primer sets (which may be incompatible) used in conventional methods. Moreover, amplification is carried out with a single set of universal primers where only one primer is labeled; therefore, the background is low and no post-PCR cleanup is required.

Another application of the MBMNA method was developed for the identification of human adenoviruses (HAdVs). Conventional serological identification of HAdVs serotypes is a time consuming process. Target-specific extension (TSE), which is a variant of ASPE was suggested to accelerate identification through the use of MBMNA for simultaneous identification of different serotypes; this is not possible using commercially available neutralization tests, antibody studies, or antigen detection by immunofluorescence or conventional PCR (Washington et al., 2010). Universal primers were used for nonspecific PCR amplification and serotype-specific probes coupled to tags were used for TSE. This MBMNA procedure is methodically simple, the cost is relatively low, and it enables diagnosis of up to five HAdV serotypes in a single reaction.

Besides the in-house assays described above, commercial kits have also been developed for the detection of respiratory viruses by xMAP, e.g., xTAG® Respiratory Viral Panel (xTAG RVP) (Krunic et al., 2011). xTAG RVP is multiplex nucleic acid test designed for detection of multiple respiratory virus nucleic acids in human nasopharyngeal swabs (Selvaraju and Selvarangan, 2012; Smith et al., 2012). Qualitative detection of a panel including respiratory syncytial virus (RSV), Influenza A virus (influenza A matrix, H1 subtype, H3 subtype, H5 subtype), Influenza B (Parainfluenza 1,2,3, and 4), Metapneumovirus (hMPV), HAdV, Entero-Rhinovirus, Corona NL63, Corona HKU1, Corona 229E, Corona OC43, and Bocavirus is possible. Bacteriophage MS2 and bacteriophage λ DNA were used as the internal controls. The detection of respiratory virus targets using the xTAG RVP, which detects 20 respiratory viral targets, was compared with individual qPCR nucleic acid amplification tests (NATs) (Pabbaraju et al., 2008). The xTAG RVP can detect all the respiratory viral targets included in the in-house NAT panel, which is used for detection of Influenza A, B viruses (IFVA, IFVB), parainfluenza virus types 1 to 4 (PIV 1–4), RSV, hMPV, and respiratory adenovirus types (ADV). Of the 1,530 samples tested, 532 were positive by xTAG RVP and 580 by in-house NATs for these targets. This gives the xTAG RVP a sensitivity of 91.2% and a specificity of 99.7%; in addition, xTAG RVP can detect picornaviruses (the in-house assays did not detect 88 picornaviruses) and coronaviruses and can subtype IFVA positives simultaneously. The xTAG RVP includes all the respiratory viral targets that are tested routinely for the diagnosis of acute respiratory tract infections; further, the technology is flexible and can easily allow for incorporation of other targets (e.g., human bocavirus) in the future.

The xTAG RVP assay was subsequently modified and was marketed as the xTAG RVP Fast assay, which has a simpler protocol; the results are obtained in a shorter time and handling of the amplified product is not required (amplified DNA is mixed

with TAG primers specific to each viral target), which could be a potential contamination risk (Pabbaraju et al., 2011). The respiratory samples were tested for a variety of respiratory viral targets by xTAG RVP and xTAG RVP Fast in parallel. The xTAG RVP was more sensitive than xTAG RVP Fast (88.6% versus 77.5%) for all the viral targets; in addition, some targets (influenza B virus, parainfluenza virus type 2, and human coronavirus 229E) were not detected using xTAG RVP Fast and, e.g., the sensitivity for detection of IFVB was very low (41.3%). Therefore, it is not suitable as the primary assay for the detection of IFVB.

In addition to respiratory viral diseases the MBMNA was successfully applied also for detection of viral pathogens causing acute viral gastroenteritis. Acute viral gastroenteritis is usually caused by four distinct families of viruses: rotaviruses, noroviruses, astroviruses, and adenoviruses (Liu Y. et al., 2012). The authors focused on simultaneous detection of rotavirus A (RVA), noroviruses (NoVs), sapoviruses (SaV), human astrovirus (HAstV), enteric adenoviruses (EAds) and human bocavirus 2 (HBoV2). Altogether 140 fecal samples were tested using the MBMNA and RT-PCR in parallel. The specificity of MBMNA was equal to the conventional RT-PCR (>90%), but MBMNA was faster in terms of detection of different viral pathogens in one tube (Liu Y. et al., 2012). The studies of (Hamza et al., 2014) were also directed to the detection of human enteric viruses (human adenovirus (HAdV), human polyomavirus (HPyV), enterovirus (EV), rotavirus (RoV), norovirus GI (NoVGI), and norovirus GII (NoVGII), but environmental water samples were tested (Hamza et al., 2014). MBMNA provided high specificity and no cross-reactivity, but was not as sensitive as qPCR for the identification of viral contamination in river water samples. In contrast, all wastewater samples that were positive in qPCR were also positive by the MBMNA and the detection limit was higher than qPCR; MBMNA was as sensitive as qPCR for viral detection in wastewater samples. Therefore, MBMNA could be a reliable method for the simultaneous detection of viral pathogens, but only in wastewater. For detection of gastrointestinal pathogens xTAG® Gastrointestinal Pathogen Panel - GPP is commercially available [multiplex detection of various viral, bacterial and parasitic nucleic acids in human stool samples (Beckmann et al., 2014; Perry et al., 2014; Wessels et al., 2014; Zboromyrska et al., 2014)]. In comparison to the two previous studies mentioned above only three enteric viruses (norovirus, rotavirus and adenovirus 40/41) can be identified by the GPP (See chapter 4.2).

Viruses such as human papillomaviruses (HPV) are also associated with oncogenesis. HPV belong to those viruses, which require simultaneous detection and typing to identify individual HPV types because the genotype determination is necessary for the investigation of epidemiology and behavior of individual HPV types. Therefore, DDH was designed for detection and genotyping of HPV using L1 consensus (primer systems, which can detect 10 to 100 molecules of HPV targets) resulting in the establishment of a method for simultaneous detection of 26 different HPV genotypes including 18 high-risk HPV and 8 low-risk HPV genotypes (Jiang et al., 2006). Subsequent analysis of the data showed that the 26-plex method precisely discriminated all 18 high-risk HPV targets and also 8 low-risk HPV targets. Another study focused on genotyping HPV also used specific probes targeting a region of the L1 gene (Zubach et al., 2012). DDH was optimized for the detection and genotyping of 46 mucosal HPV types, which are associated with infections of the genital, anal, and oropharyngeal mucosae and the method enables a more comprehensive coverage of HPV types compared with the previously mentioned study, where only 26 types of HPV were genotyped. The DDH was more sensitive than the Linear Array (a leading commercial genotyping method) in terms of distinguishing positive/negative HPV samples, but less sensitive for detection of multiple HPV types; another limitation was the inability of the PCR system to amplify certain variants of HPV68. HPV genotype detection was by combined whole genome amplification and xMAP technology showed that this method is highly specific and sensitive (Lowe et al., 2010). This approach is capable to identify all high risk HPV types with the analytical limit of detection 100 copies plasmid DNA.

Many viruses can cause infections with fatal consequences for human health, e.g., Hendra and Nipah viruses, which can infect cells of the central nervous system and may cause relapsing encephalitis (Clayton et al., 2013), Ebola virus, which causes lethal hemorrhagic disease in humans (Takada and Kawaoka, 2001) or Menangle virus, which causes an influenza-like illness with a rash in humans (Bowden et al., 2012); these zoonotic viruses are linked to bats. The surveillance of zoonotic viruses in wildlife populations is necessary in order to monitor the risk of emerging infectious disease outbreaks. For the complex detection and genotyping of paramyxoviruses in Australian bats two bat virus panel assays (BVPA) for detection of paramyxoviruses in Australian bats (BVPA-1) and for paramyxoviruses and filoviruses in non-Australian bats (BVPA-2) were introduced (Boyd et al., 2015). Examined RNA was extracted from the urine of bats and a total of 532 samples were tested in 11-plex BVPA-1 and 540 field 8-plex BVPA-2; both developed assays were proven to be reliable and accurate.

A number of pathogens, including viruses, are implicated in reproductive diseases of swine. (Chen et al., 2015) combined one-step asymmetric multiplex reverse transcription PCR (RT-PCR) with DDH for simultaneous detection of respiratory syndrome virus (PRRSV), porcine circovirus type 2 (PCV-2), porcine pseudorabies virus (PRV), classical swine fever virus (CSFV), and porcine parvovirus (PPV). All strains of these five viruses were accurately identified. The results showed that the combination of RT-PCR with the DDH assay is more accurate and specific than the other methods, e.g., conventional RT-PCR, and could be a useful tool in the diagnostics of swine diseases. MBMNAs could become very important for veterinary diagnostic testing and (Christopher-Hennings et al., 2013) reported the potential use of MBMNAs for detection of different pathogens in pigs using panels for the multiplex detection of swine pathogens (viruses and bacteria) in serum, lung, oral fluids, feces and spleen or liver.

Although direct diagnosis based on the detection of the nucleic acids of viral pathogens described above prevails, xMAP antibody-based tests for the detection and typing of viruses are also available. MBMI was used to develop a competitive immunoassay that measures HPV type 6, 11, 16, and 18 specific neutralizing antibodies (Opalka et al., 2003); this was later

validated for use in epidemiology studies and clinical vaccine trials (Opalka et al., 2003; Dias et al., 2005). MBMI was also compared with a Western blot assay for the detection of HIV-specific antibodies (Kong et al., 2016). The microspheres were coupled with anti-p24 monoclonal antibody and with HIV antigens: gp41, p17, p24, p31, and p66 recombinant protein. The results of both methods showed that MBMI sensitivity was 82.7% and Western blot assay sensitivity was 74.7%. The MBMI was more efficient and precise for screening several parameters and based on the acquired results it was better in HIV diagnostics than Western blots. For the determination of antibodies against HCV in patient serum samples MBMI based on the antigenic properties of four recombinant proteins was designed (Fonseca et al., 2011). Only a small number of samples was tested and that is why the specificity and sensitivity were 100%, but in spite of this the MBMI has the potential to become a viable alternative to standard tests due to its excellent specificity and it may be used for screening of HCV infection. Detection of antibodies against several Epstein-Barr virus (EBV) antigens in nasopharyngeal carcinoma patients (NPC) showed the possibility of simultaneous detection of multiple markers using MBMI, which is not possible with ELISA, and because of the distinct EBV serology spectrum in individual NPC patients, the multiplexed microsphere assay has powerful potential to allow serological diagnosis of NPC in the future (Gu et al., 2008). MBMI showed increased sensitivity and the possibility of quantifying antibodies, antigens, as well as other substances (e.g., hormones, cytokines, tumor markers, etc.), in contrast to conventional ELISA tests (duPont et al., 2005).

Multiplex Detection and Typing of Bacteria

The majority of applications for multiplex bacterial diagnostics are based on the detection of DNA. The most widely used approaches are based on the DDH, ligation assays or ASPE, but multiplex detection of bacteria may be performed as well using MBMI.

Direct DNA hybridization was used for the detection of pathogens causing foodborne diseases such as acute gastroenteritis and diarrhea, which are usually associated with ingestion of contaminated food. DDH was applied for the typing of 500 *Salmonella* isolates using the genes encoding the flagellar antigens H (*fliC* and *fljB*) (McQuiston et al., 2011). Allele-specific probes for fifteen H antigens, 5 complex major antigens and 16 complex secondary antigens according to the Kauffmann-White serotyping scheme were designed. Comparison of DDH with traditional serotyping methods revealed that the DDH cannot completely replace these methods because unfortunately not all flagellar antigen types were detected. A similar DDH assay for the typing of *Salmonella* focused only on the most common six serogroups of *Salmonella* in the United States (B, C_1, C_2, D, E, and O13), as well as serotype Paratyphi A, using the *rfb* genes required for O-antigen biosynthesis in *Salmonella* (Fitzgerald et al., 2007). In contrast with the previous study of McQuiston et al. (2011), the authors showed that the DDH was more specific than traditionally used methods for typing of *Salmonella*.

In the previous sections, it was described how DDH can be used for typing of pathogens; however, in most cases DDH is used only for the detection of pathogens, as described below. (Liu J. et al., 2012) attempted simultaneous detection of the enteric pathogens *Aeromonas*, *Campylobacter jejuni/coli*, *Shigella*, enteroinvasive *Escherichia coli* (EIEC), *Vibrio*, *Yersinia* and as well as *Salmonella* in fecal samples. However, there were some limitations to the method, which included the limited number of clinically significant pathogens or the inability to detect diarrheagenic *E. coli,* protozoa, or viruses. The full capacity of the DDH assay was utilized when the panel was expanded to include the most common bacterial/viral enteropathogens found in stool samples, such as *Salmonella*, *Shigella*, *Vibrio*, toxin B producer *Clostridium difficile*, *Campylobacter*, *Clostridium perfringens*, *Yersinia enterocolitica*, *Aeromonas*, *Escherichia coli* O157:H7, verocytotoxin-producing *Escherichia coli* and adenovirus, Group A rotavirus, norovirus GI and GII and astrovirus (Onori et al., 2014). The results showed that the assay is rapid, sensitive, specific, and reliable for screening and for exploring the etiology of gastrointestinal infections. The sensitivity of MBMNA was demonstrated to be greater than the routine methods (76.3% versus 66.5%), with the exception of *Salmonella* sp. and toxigenic *C. difficile* where the adoption of multiplex PCR did not always result in a significant improvement of specificity. The causative agents were not found in 44 of 245 (18%) of the presumed infectious gastroenteritis cases, but this could be due to the limitations of the detection panel, which did not include allele-specific probes for detection of parasitic enteric pathogens or emerging viruses related to gastroenteritis. Also, using DDH, detection of pathogenic bacteria occurring in environmental samples and causing acute and often fatal diseases (*Bacillus anthracis, Yersinia pestis*, *Francisella tularensis*, and *Brucella melitensis)* was optimized in a multiplexed format to allow the maximum sensitivity and specificity (Wilson et al., 2005). DNA was extracted robotically and in combination with DDH a rapid reliable screening approach was developed. Detection limits were from 100 fg to 10 pg starting DNA concentration when primer sets were multiplexed; in some cases the limits of detection were higher when primer sets were tested separately (range from 10 fg to 10 pg).

Besides the in-house assays developed for multiplex detection of bacteria described above, there are also commercial solutions based on xMAP technology for detection of the most common gastrointestinal pathogens and toxins. The xTAG® Gastrointestinal Pathogen Panel is a multiplex nucleic acid test designed for detection of various bacterial, viral and parasitic nucleic acids in human stool samples (Beckmann et al., 2014; Perry et al., 2014; Wessels et al., 2014; Zboromyrska et al., 2014). The panel allows qualitative detection of *Campylobacter* sp., *Clostridium difficile* (toxin A/B), *Escherichia coli* O157, Enterotoxigenic *E. coli* (ETEC) LT/ST, Shiga-like toxin producing *E. coli* (STEC) *stx1/stx2*, *Salmonella* sp., *Shigella* sp., *Vibrio cholerae*, *Yersinia enterocolitica,* HAdV serotypes 40 and 41, NoV GI and GII, Rotavirus A, *Giardia*, *Cryptosporidium* and *Entamoeba histolytica*. The xTAG GPP was tested and compared with routine tests, which are used in clinical diagnostic laboratories for screening of 17 kinds of enteropathogens, e.g.,

qRT-PCR kit for detection of viruses, culture methods for detection of bacteria or microscopic examination for detection of parasites (Deng et al., 2015). Samples with discordant results between the routine tests and xTAG GPP were tested by singleplex PCR and sequencing. The overall sensitivity of xTAG GPP was 96.3% and specificity was 99.8%. The sensitivity of xTAG GPP was 100% for all enteropathogens except *Salmonella* sp. (84.9%) and *C. difficile* toxin B (88.6%). The specificity was 100% for all targets except *Salmonella* sp. (99.2%), *Shigella* sp. (99.7%), *C. difficile* toxin B (99.2%), and norovirus GII (98.8%). xTAG GPP is also capable of detecting coinfections; 35 coinfections were detected using xTAG GPP, which is more than by the routine tests. However, the authors also reported some disadvantages as xTAG GPP failed to detect some important diarrheal pathogens (*Aeromonas*, *Plesiomonas shigelloides*) often detected by routine diagnostic tests; further, the detection of *Salmonella* exhibited low sensitivity (84.9%).

Ligation assays are also often used for multiplex detection of pathogenic bacteria. The main advantage over direct hybridization methods is the ability to simultaneously detect diverse signatures such as unique sequences, SNPs, indels and repeats (Song et al., 2010). MOL-PCR was initially optimized for the detection of the biothreat agents *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* (Deshpande et al., 2010). The pathogen-specific sets of MOLigo pair probes were designed and their specificity and sensitivity were tested using similar species of *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* and dilutions of isolated DNA, respectively. MOLigo pairs, which showed the highest specificity and sensitivity, were selected for compilation of a final probe panel, which was validated on extracted DNA from infected rodent liver and spleen, human blood or pleural fluid spiked with pathogen DNA. Nine from 10 unknown samples were successfully identified using the final probe panel. The results also showed the ability of this method to simultaneously detect multiple different signatures (SNPs, indels and repeats). The versatility of MOL-PCR was utilized when simultaneous detection of *Bacillus anthracis*, *Yersinia pestis*, and *Francisella tularensis* was supplemented by characterization of antibiotic resistance (ciprofloxacin and doxycycline) of these bacteria based on SNP analysis (Song et al., 2010). The allele-specific probes for detection and characterization of all the known resistance determinants performed well when tested individually, but multiplex use did not provide satisfactory results. Due to the ability to simultaneously detect diverse signatures such as unique sequences, SNPs, indels, and repeats, MOL-PCR can be used as a genotyping method as described below. A MOL-PCR-based 8-plex SNP typing method for *Mycobacterium tuberculosis* complex (MTBC) based on two phylogenetically equivalent sets of SNP markers that are specific for the six main human-associated lineages of MTBC was introduced (Stucki et al., 2012). MOL-PCR was compared with TaqMan qPCR and the obtained results showed that the sensitivity and specificity of both methods were similar (specificity 100%, sensitivity 98.6% MOL-PCR, 98.8% TaqMan) and that both methods were of comparative cost. MOL-PCR was ideal for classification of unknown isolates, while TaqMan qPCR was faster for confirmation of unknown isolates. MOL-PCR was also successfully used for genotyping of *Bacillus anthracis* in a 13-plex assay to score 13 phylogenetically lineage-specific canonical SNPs within the genome of *Bacillus anthracis* (Thierry et al., 2013).

Allele-specific primer extension was applied for identification of bacteria (Lin et al., 2008) even though it is more commonly used for SNP genotyping. ASPE was used for the identification of *Acinetobacter sp.* and antimicrobial susceptibilities of the clinical *Acinetobacter* species isolates were also determined (Lin et al., 2008). The 16S-23S rRNA gene intergenic spacer (ITS) regions of 13 distinct *Acinetobacter* species were amplified and then multiplex ASPE was performed. It was shown that this multiplex identification of *Acinetobacter* sp. is applicable also for determination of antibiotic resistance of the clinical *Acinetobacter* isolates. ASPE was compared with SBCE for identification of bacterial samples (Ye et al., 2001) and both methods provided similar results as they managed to correctly classify 17 bacterial species into 17 groups.

In addition to MBMNA also MBMI can be used for the direct multiplex detection of bacteria and their products (Dunbar et al., 2003). In MBMI direct fluorescence (detection antibody that incorporates a fluorescent label) is used for detection of reaction or of emerging product in contrast to ELISA and, in addition, MBMI enables measurement of multiple analytes simultaneously. For this reason, MBMI is preferred because time for detection is reduced and also test sensitivity is increased (Jun et al., 2012). Capture sandwich immunoassays (CS) were successfully applied for detection of organism-specific antibodies using microspheres coupled with antibodies for *Salmonella*, *Campylobacter*, *Escherichia coli,* and *Listeria* and it has been demonstrated that MBMI is a suitable method for multiplex detection of bacteria occurring in foodstuffs (Kim et al., 2010) or for detection of *Brucella* sp. from milk using capture-sensitive monoclonal antibodies for the lipopolysaccharide (LPS) O-antigen of *Brucella* sp. (Silbereisen et al., 2015). MBMI was also applied to test bacterial contamination of foods through the detection of staphylococcal enterotoxin B (SEB) (Kim et al., 2010), staphylococcal toxin A (SEA), and toxic shock syndrome toxin (TSST) produced by various strains of *Staphylococcus aureus* (Simonova et al., 2014) using sandwich immunoassays in which microspheres were conjugated with specific antibodies. A similar approach was used for the detection of pneumococcal serotype-specific polysaccharide and C-polysaccharide (C-Ps) antigens from urine samples (Sheppard et al., 2011). For the detection, MBMI was combined with the Binax NOW *Streptococcus pneumoniae* antigen detection kit. The specificity of MBMI was determined by testing 85 serotypes of *S. pneumoniae* and other strains of streptococci; 18 of the 26 non-pneumococcal serotypes gave C-P positive results, which showed that MBMI could be used for diagnosis of infection caused by *S. pneumoniae* only in combination with the Binax NOW assay.

Multiplex Detection of Parasitic Agents

Parasitic zoonoses are recorded worldwide and some of them have endemic character. Parasitic agents may pass from animals to humans in several ways, e.g., by direct contact, vector, consumption of raw or undercooked foodstuffs containing the infective stages or by infective stages released into environment

(Hubalek, 2003). In the context of animal health and human food consumption, a list of the top ten parasites has been defined by the UN's Food and Agriculture Organization (FAO) and World Health Organization (WHO) (**Table 3**). Although in the last decades a number of novel diagnostic methodological approaches has been developed, the current diagnosis of some parasitic diseases is still based only on a combination of clinical signs, anamnesis, and direct visual identification of parasitological objects (Anderson et al., 2015). The most common conventional diagnostic methods, such as microscopic examination, biochemical assays or ELISA, are available, but they are laborious, time-consuming and in many cases not reliable (Navidad et al., 2013). Improvements in this field are represented by molecular methods, including also routine PCR diagnostics, increasingly used for detection mainly of intestinal parasites, which are easy to recover from fecal specimens (Taniuchi et al., 2011) or potentially useful for other parasites found in secretions. With regard to the fact that parasites might exhibit very strictly confined localization within the host's body – intracellular/extracellular or tissue/organ, sampling can be very problematic and it often leads to a false negative results.

Outbreaks of diarrheal diseases are caused by a wide range of pathogens, including parasites. Stool microscopy (detection of eggs, parts of bodies etc.) is the gold standard in the diagnostics of intestinal parasites. However, the presence of parasites in stool may vary and could be naturally low, requiring multiple sampling. In fact, up to 80% of all cases of diarrhea remain without confirmed etiology (Vernacchio et al., 2006). Therefore, there is space for the development of more sensitive diagnostic assays (Taniuchi et al., 2011), which should provide more precise determination. Among the modern molecular diagnostic methods qPCR assays are most frequently used for determination of intestinal parasites. In areas where co-infections are common (up to 22% of cases are caused by two or more pathogens) (Jansen et al., 2008; Friesema et al., 2012), the application of multiplex assays is of great benefit. Several pioneering works have been published in relation to this topic. To date, in parasitology, improved multiplex qPCR assays were adapted to DDH, which enables parallel diagnosis of seven intestinal parasites (Taniuchi et al., 2011); separate reactions were optimized – 3-plex for protozoa (*Cryptosporidium* sp., *Giardia intestinalis*, and *Entamoeba histolytica*) and 4-plex for helminths (*Ancylostoma duodenale, Ascaris lumbricoides, Necator americanus*, and *Strongyloides stercoralis*). The final calculated sensitivity was 83% and specificity was 100%. The results of both DDH assays were equivalent or better in comparison to the parent multiplex qPCR. Moreover, this approach has been developed as a commercial diagnostic xTAG GPP tool– a 19-plex assay, which enables *inter alia* detection of the protozoa *G. intestinalis, E. histolytica* and *Cryptosporidium* sp. The overall performance of xTAG GPP compared with conventional methods (standard culture, microscopic examination, immunochromatographic tests, qPCR) showed a sensitivity of 94.5% (range 90 to 97%) and a specificity of 99% (range 98,5% to 99,9%) (Claas et al., 2013; Mengelle et al., 2013; Navidad et al., 2013). If multiplexing more than 20 targets, the limit of detection might be reduced for individual targets when compared to single-target detection (Navidad et al., 2013). However, the identification of multiple pathogens revealed that very often (in up to 65% of samples), the physicians do not request testing for the proper pathogen (Claas et al., 2013). Therefore, multiplexing refines the diagnosis and contributes to the selection of a suitable treatment.

It was mentioned above that microsphere-based assays can be arranged also as multiplex indirect immunoassays, although the conventional singleplex ELISA still represents the gold standard in serodiagnostics for screening of individual human/animal or higher numbers of samples at a population level (Ruitenberg et al., 1983; Nockler et al., 2000; Dubey et al., 2005). Recently, some studies have been done in order to improve the potential of this serological method and to upgrade it to the multiplex level. These studies are mostly focused on parasites with the ability to migrate through the tissues of the host's body – where PCR based detection would not be reliable. In this context, the most studied group of parasites are representatives from the phylum Nematoda, including also the important human pathogens, the *Trichinella* sp. The larvae may infect humans during the ingestion of raw or undercooked meat, mainly pork (domestic pig, wild boar) and can induce disease, whose consequences can be fatal (Dupouy-Camet, 2000; Pozio and Murrell, 2006). Inspection of meat for the most important species, *Trichinella spiralis*, is mandatory at slaughter (Anonymous, 2015), but currently used methods like artificial digestion and microscopic examination of pooled meat samples (Nockler et al., 2000) are archaic and usually do not properly reflect the real infection. Therefore, serodiagnostic methods are considered as a possible alternative and xMAP technology in the form of ISA, using excretory/secretory (E/S) products, was also developed and tested. The effectivity of ISA was tested with *T. spiralis*-positive pig meat samples. The system was developed as a duplex assay (with *Toxoplasma gondii*), using goat anti-swine secondary antibodies against specific antibodies. The results of this study corresponded to the infection status of the animals with an assay sensitivity of 68% and specificity of 100% (Bokken et al., 2012). When the immunoglobulin binding protein A/G (generic

TABLE 3 | Foodborne parasites with the greatest global impact (Anonymous, 2014).

Parasite	Type	Occurrence
Taenia solium	Tapeworm	Pork
Echinococcus granulosus	Hydatid worm or dog tapeworm	In fresh produce
Echinococcus multilocularis	Tapeworm	In fresh produce
Toxoplasma gondii	Protozoa	In meat from small ruminants, pork, beef, game meat (red meat and organs)
Cryptosporidium sp.	Protozoa	In fresh produce, fruit juice, milk
Entamoeba histolytica	Protozoa	In fresh produce
Trichinella spiralis	Worm	Pork
Opisthorchiidae	Flatworm	in fresh water fish
Ascaris sp.	Roundworm	In fresh produce
Trypanosoma cruzi	Protozoa	In fruit juice

Ig-binding protein), which can be used in multiple species in contrast with goat anti-swine secondary antibody, was included, the results showed a similar specificity of 95%, but an increase in sensitivity from 88% for anti-swine antibody to 94% with protein A/G. The xMAP technology-ISA exhibited 87% sensitivity and 95% specificity in comparison with the commercial Pourquier ELISA, and 98% sensitivity and 95% specificity in comparison with the Safepath ELISA (van der Wal et al., 2013).

With the rising popularity of MBMIs, ISA was also developed for other members of Nematodes, such as representatives from the genus *Toxocara* (Anderson et al., 2015). The infection by these parasites is typically peroral at areas contaminated by embryonated roundworm eggs, e.g., sand from childrens' playgrounds. The recombinant *T.canis* and *T. cati* E/S antigens Tc-CTL-1 and Tc-TES-26 were used to detect toxocara-specific antibodies in sera from humans pre-diagnosed as positive for visceral and ocular larval migrans (VLM, OLM). The specificity of ISA was 94% for both sets of samples, but there were differences in the sensitivity, which was 99% for VLM and 64% for OLM samples. It was recorded that a combination of recombinant antigens improves sensitivity in comparison with conventional immunoassays (e.g., Western Blot, ELISA), which employ native E/S antigens isolated from larvae (limited availability) that also exhibit cross-reactivity with antibodies from other helminthic infections so reducing its usefulness in regions with poly-parasitism.

Within the unicellular parasitic protozoa ISA was tested in representatives from the genus *Toxoplasma.* Unlike *T. spiralis*, no such regulations for meat control exist for *T. gondii,* even though its prevalence is higher and health complications can be very severe. Recombinant tachyzoite surface protein (SAG-1) was used for simultaneous serological detection in a set with *T. spiralis* E/S (Bokken et al., 2012). Similarly to *T. spiralis*, the results exactly reflected the load of infection; sensitivity was 86% and specificity was 96% for *T. gondii.* The obtained results repeatedly underline the potential of these assays for further implementation in routine diagnostic screening of a wide range of parasites.

As we have descibed, the ISA represents an improved methodological alternative to current serological diagnostics, enabling multiplex detection of pathogenic agents with higher sensitivity.

Multiplex Detection and Typing of Fungal Pathogens

Traditional diagnostic methods for the identifications of fungal pathogens are mostly based on phenotype analysis of fungal cultures or detection of antigens (polysaccharides), but these approaches are time-consuming and not very accurate (Diaz and Fell, 2004; Bovers et al., 2007; Landlinger et al., 2009; Babady et al., 2011). Rapid and correct identification methods are important for efficient therapy (Diaz and Fell, 2004), however, available qPCR assays have various levels of sensitivity and specificity and often have a limited range, targeting only a few yeasts or mold species (Landlinger et al., 2009; Babady et al., 2011).

The need for rapid and correct identifications of fungal pathogens was addressed by development of xMAP technology based detection methods (Diaz and Fell, 2004; Page and Kurtzman, 2005; Das et al., 2006; Bovers et al., 2007; Babady et al., 2011; Balada-Llasat et al., 2012; Farooqi et al., 2012; Landlinger et al., 2009). Majority of xMAP applications for the multiplex detection and identification of fungal pathogens are based on the capture of fungal nucleic acid by DDH assays.

To perform rapid and accurate identifications of fungal pathogens in immunocompromised individuals, the DDH was designed detect a wide range of the most commonly occurring clinically relevant fungal pathogens including species of the genera *Aspergillus* and *Candida* and other important pathogens such as *Cryptococcus*, *Fusarium*, *Trichosporon*, *Mucor*, *Rhizopus*, *Penicillium*, *Absidia*, and *Acremonium* (Landlinger et al., 2009). The DDH was used mainly for identifications of fungi due to its ability to detect coinfections with multiple fungal species in patients and may contribute to improved diagnosis of invasive fungal infections.

Studies employing xMAP technology were developed and successfully used to identify individual fungal species within *Candida* sp. (Page and Kurtzman, 2005; Farooqi et al., 2012), or *Trichosporon* sp. (Diaz and Fell, 2004). In these studies, DDH assays for fast and accurate detection and identification of important fungal pathogens were developed. In another study, the xMAP technology was used for genotyping of human pathogenic *Fusarium* sp. (O'Donnell et al., 2007). Fusaria were genotyped also by sequence analysis. The independent comparison of the results obtained via xMAP technology with results obtained via sequencing showed the xMAP incorrectly identified some of *Fusarium* isolates.

Besides the in-house assays described above, commercial kits have also been developed for the detection of fungal pathogens by xMAP, e.g., xTAG® Fungal Analyte-Specific Reagents (ASR) assay and the sensitivity and specificity of the assay were tested within identification of fungal isolates and positive blood culture bottles (Babady et al., 2011). The *Candida* 7-plex assay was tested within 43 of *Candida* strains and 16 bacterial strains with no-cross-reaction with any of the bacterial strains. The sensitivity and specificity were 100%. Using 11-plex assay were tested 51 mold species and the assay correctly identified all species of *Aspergillus*, with 100% specificity and sensitivity except *A. niger* (0/8 isolates). Other molds were identify also with 100% specificity and sensitivity except *Mucor* (0/6 isolates) and *Rhizopus* (1/6 isolates). Besides the testing of fungal isolates also positive blood culture bottles were tested for the presence of *Candida* species using *Candida* 7-plex assay. The sensitivity and specificity of the assay was 100% for each species. The mold 11-plex assay did not detect one *Rhizopus* species and the *A. niger* strains, so the results were similar as the previous mentioned results in the course of identification of fungal isolates.

In addition, ASR for identification of *Candida* species do not distinguish between members of *Candida* complexes, e.g., phenotypically indistinguishable groups II and III of *C. parapsilosis* (group I), which have been renamed *Candida orthopsilosis* and *C. metapsilosis*. Similarly, ASR for identification of *A. fumigatus* were unable to distinguish between members of the *A. fumigatus* complex. The results showed that xTAG® Fungal ASR assay could be used as an

adjunct to culture. The mold 11-plex assay has been developed specifically for the detection of specific species of mold, which may be reason why *Rhizopus, Mucor,* and *A. niger* have not been identified. Due to the equal treatment of infections caused by genera *Mucor* and *Rhizopus*, it would be better to design a panel to detect the most common genera of fungi, and not to focus on the detection of particular species.

The results showed that the xTAG® Fungal ASR assay is an attractive alternative to reference methods, due to its speed and ability to simultaneously identify multiple fungal species (Balada-Llasat et al., 2012).

DDH assay is able to not only identify the fungal pathogens, but it can be used for a genotyping of fungal pathogens. It was applied for identification of closely related pathogenic yeasts *Cryptococcus neoformans* and *Cryptococcus gattii* that may cause meningoencephalitis in immunocompromised individuals (Bovers et al., 2007). Six haploid genotypic groups within these pathogens can be distinguished by several molecular methods e.g. PCR fingerprinting or intergenic spacer genotyping. Besides these haploid groups, hybrids have been described as well. AD hybrids are hybrids between the two varieties of *C. neoformans* and also hybrids between *C. neoformans* var. *neoformans* and *C. gattii* have been described. The DDH has been adapted for the detection of the genotypes within *Cryptococcus neoformans* and *Cryptococcus gattii*. The detection limit was calculated from 4×10^1 to 2×10^3 cells for the various specific probes for each of the six haploid genotypic groups. The results showed that DDH is highly specific method and it is possible not only identify cryptococcal isolates at the species and genotype levels but also allows identification of hybrid isolates that have two alleles of the specific probes region and also able to identify cryptococci in cerebrospinal fluid. However, the optimization of DNA extraction methods is needed before routine use in clinical laboratories.

CONCLUSION

Detection and identification of pathogens, as well as an understanding of pathogen variation, the pathogenesis of the diseases they cause, and timelines of infection and antimicrobial resistance, are all required in order to obtain the full picture of disease progression and to select effective cures for infected individuals or populations. As the amount of input data required for such decisions increases, so too does the number of tests that are required during laboratory examinations. The multiplex assays for the detection and typing of pathogens using xMAP technology are tools of choice as they are capable of providing all of the important information within a reasonable timeframe, and without excessive labor or costs. The major improvement of xMAP assays is that they add another dimension to the simple detection, which is represented by the simultaneous analysis of many targets within a single sample, and they therefore represent complementary tools to procedures for the detection and quantification of pathogens such as qPCR, culture, or ELISA assays. The significance of such a complex approach for the multiplex detection has grown in recent years, which is documented by the increase in published data and of application of the commercial assays in routine diagnostics.

AUTHOR CONTRIBUTIONS

Conception of the review: PK; Design of the work: VM, NR, PK; Writing this review: NR and VM (These authors contributed to this work equally); Revision of the manuscript: MK, PM, PK; All authors approved the version to be published in *Frontiers in Microbiology* and agreed to be accountable for all aspects of the work.

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A novel perspective on MOL-PCR optimization and MAGPIX analysis of in-house multiplex foodborne pathogens detection assay

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Multiplex oligonucleotide ligation-PCR (MOL-PCR) is a rapid method for simultaneous detection of multiple molecular markers within a single reaction. MOL-PCR is increasingly employed in microbial detection assays, where its ability to facilitate identification and further characterization via simple analysis is of great benefit and significantly simplifies routine diagnostics. When adapted to microsphere suspension arrays on a MAGPIX reader, MOL-PCR has the potential to outperform standard nucleic acid-based diagnostic assays. This study represents the guideline towards in-house MOL-PCR assay optimization using the example of foodborne pathogens (bacteria and parasites) with an emphasis on the appropriate choice of crucial parameters. The optimized protocol focused on specific sequence detection utilizes the fluorescent reporter BODIPY-TMRX and self-coupled magnetic microspheres and allows for a smooth and brisk workflow which should serve as a guide for the development of MOL-PCR assays intended for pathogen detection.

Foodborne pathogens (FBP) represent one of the major threats to public health, especially in developing countries[1], and thus, food safety remains one of the most important global health issues. Foodborne illnesses are usually related to pathogenic viruses, bacteria, and parasites as well as toxic chemical substances entering the body through contaminated food or water[2]. Similar clinical manifestations may have at their root various causes, and, therefore, a complex and effective system of prevention is required; in this process, the timely detection and further characterization of pathogens plays a critical role[3]. Nucleic acid-based assays are ideal for such purposes due to their high specificity, ability to be modified to detect almost any target, minimal sample amounts required (high sensitivity), the short time required for analysis, and the capability for automation[4]. Such assays should also enable simultaneous screening of numerous molecular markers, which would be time- and cost-effective in comparison to gold standard methods, such as, e.g. cultivation, immunoassays, biochemical tests, and PCR/qPCR that require multiple serial or parallel assays and have limitations in multiplexing ability[3,5].

The multiplex oligonucleotide ligation-PCR (MOL-PCR) first described by Deshpande *et al.*[4] combines analysis of multiple types of molecular markers in a single multiplex reaction[4,6,7] and therefore is capable of specific sequence detection in parallel with SNP identification or detection of indels (insertion/deletion). Owing to these features, MOL-PCR represents a sensitive tool for complex screening of pathogens (virus, bacteria, fungi) in various matrices or to establish the presence of genes responsible for antibiotic resistance. The visualization of MOL-PCR products can be achieved using several detection platforms, e.g. fixed microarrays, gel-based formats, or microsphere suspension arrays[4]. The latter way of visualization using a Luminex MAGPIX (Luminex Corp., Texas, USA) or Bio-Plex MAGPIX Multiplex Reader (Bio-Rad, California, USA), hereafter referred to as MAGPIX, allows for simple workflow and reproducible results. The MAGPIX is a simple instrument based on a suspension array using magnetic microspheres. The instrument consists of a magnet for immobilization of magnetic microspheres and two diodes that read the fluorescent spectra of dyes within the microspheres and reporter molecules captured on their surface[8]. Moreover, MAGPIX has the ability to analyze up to 50 markers (limited by the capacity of regions for magnetic microspheres) in a single sample.

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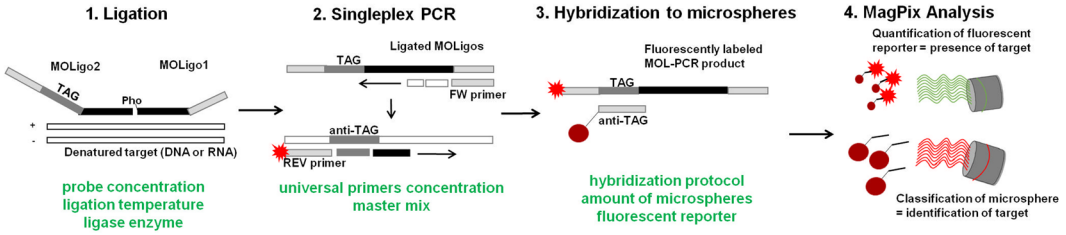

Figure 1. Overview of MOL-PCR assay workflow. The workflow is illustrated for one target. Green headings indicate several parameters of the MOL-PCR reaction, which must be optimized in order to achieve the best results. White = target DNA. Black = complementary region of specific detection probes. Gray = universal primer sites. Red stain = fluorescent reporter.

Carrying out of a typical MOL-PCR assay involves three crucial steps: multiplex oligonucleotide ligation of specific probes for detection of molecular markers, PCR for signal amplification, and the hybridization of PCR products to magnetic microspheres followed by signal detection on MAGPIX (Fig. 1). The advantages of MOL-PCR lie in the multiplex ligation step, which is carried out prior to the PCR amplification and in the fact that specific detection probes are modular structures bearing binding sequences for universal primers enabling singleplex PCR[9]. Compared to other ligation-based methodologies such as multiplex ligation-dependent probe amplification (MLPA) or the oligonucleotide ligation assay (OLA), where multiplexed PCR is the limiting factor of high multiplexing (due to different lengths of the amplicons), in MOL-PCR the ligation step represents the actual detection event and the subsequent PCR does not serve for amplification of template DNA[10]. This ensures that MOL-PCR is not susceptible to amplification bias or cross-hybridization, which are characteristic not only of other ligation-based assays but also of multiplex PCR[8,11].

Modular detection probes used in MOL-PCR, in particular MOLigo1, consist of two segments: a target-complementary sequence at the 5′-end followed by a reverse-complement of the universal FW primer sequence; importantly, the 5′-end of MOLigo1 is phosphorylated to enable ligation to MOLigo2. The longer MOLigo2 contains three segments: a universal REV primer sequence at the 5′-end linked to an inner specific 24-bp xTAG sequence (synthetic sequences based on just three bases T, A, and G; provided by Luminex Corp., Texas, USA) and target-complementary sequence at the 3′-end. If hybridization of probes to their target sequence occurs, DNA ligase links them covalently into one complex sequence (100–120 nucleotides long). Ligated sequence serves as a template for singleplex PCR, where the REV primer carries a fluorescent reporter (depicted as red stain in Fig. 1) marking the product. MOL-PCR products are hybridized to the anti-TAG sequences on the surface of magnetic microspheres and detected in MAGPIX.

Although the popularity of MOL-PCR is increasing, only little is found in the literature regarding the optimization steps which must be implemented before finalization of the working protocol. However, home-made assays intended for routine use need to be optimized in terms of differentiation between positive and negative results, cost, and effort. To date, relevant papers have focused mainly on detection of single nucleotide polymorphisms (SNPs)[6,10,12], have utilized indirect labeling by the fluorescent dye streptavidin-R-phycoerythrin (SAPE)[5–7,13], and were adapted for measurement on the Luminex 100/200 (Luminex Corp., Texas, USA) flow cytometer device[4,6,10,12]. Further, in all cases commercially pre-coupled MagPlex-TAG Microspheres (Luminex Corp., Texas, USA) were used. While these papers did describe the reaction conditions in detail, they nevertheless failed to provide a comprehensive description of the optimization procedure. Wuyts *et al.*[7] created guide of optimization focused on MagPlex-TAG Microspheres and following a slightly adapted workflow of the commercially available manufacturer's Cookbook protocol[14]. However, a precise and detailed description of optimization experiments together with troubleshooting is crucial and essential for the development of any in-house MOL-PCR assay for pathogen detection. Therefore, the present study was meant to be a guideline through such MOL-PCR optimization and was aimed at identifying and analyzing in detail individual parameters of the working protocol on two model organisms. These parameters, may, if inappropriately chosen, negatively affect the workflow and performance of the whole analysis. The final optimized conditions were tested in an 11-plex model assay targeting a set of bacterial and parasitic pathogens transmitted via food matrices. In addition, in this study, we tried as much as possible to fulfill criteria with respect to the availability of reagents and equipment, affordability, and the skills required to perform the method, with the aim of making this approach feasible for a wide range of users who carry out detection and identification of pathogens in food.

Materials and Methods

Target organisms and DNA template. The most important parameters in the development of a MOL-PCR assay designed for detection of pathogens intended for routine application were optimized in two model organisms, the foodborne pathogens *Yersinia enterocolitica* (*YE*) and *Toxoplasma gondii* (*TG*).

The *YE* CAPM 6154 bacterial isolate (serotype O2,3; biotype 3) was obtained from the Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute (Brno, Czech Republic). A single colony from a culture was resuspended in dH_2O and genomic DNA (gDNA) was released by application of heat lysis at 100 °C for 15 min. After centrifugation, 50 µl of supernatant were diluted in 450 µl of dH_2O (10X dilution).

TG oocysts obtained from the National Reference Laboratory for Parasites at the University of Veterinary and Pharmaceutical Sciences (Brno, Czech Republic) were used for gDNA isolation using an extraction protocol previously described in Reslova *et al.*[15]. Briefly, 10 µl of oocysts in H_2O (approximately 2×10^6 oocysts) were

Oligo type	xTAG	Sequence 5′-3′
TG_MOLigo1		Pho-CGGAAACATCTTCTCCCTCTCC**TCTCACTTCTTACTACCGCG**
TG_MOLigo2	A018	*ACTCGTAGGGAATAAACCGT*Gtaattgaattgaaagataagtgt TCCAGGAAAAGCAGCCAAGC
YE_MOLigo1		Pho-GGAGTAATAGGTTCGTTTG**TCTCACTTCTTACTACCGCG**
YE_ MOLigo2	A019	*ACTCGTAGGGAATAAACCGT*gtgtgttatttgtttgtaaagtat GAACTCGATGATAACTGG
Universal FW primer		**CGCGGTAGTAAGAAGTGAGA**
Universal REV primer		**ACTCGTAGGGAATAAACCGT*

Table 1. Sequences for primers and MOLigo pairs. Target-hybridizing complementary sequence is underlined, universal forward primer in bold, universal reverse primer in italics, and xTAG sequence is differentiated by lowercase. Pho = phosphorylation, *Fluorescent label.

incubated in 200 µl of extraction buffer (100 mM Tris-Cl from Sigma-Aldrich, Missouri, USA; 10 mM EDTA from Amresco, Ohio, USA; 100 mM NaCl from Carl Roth, Germany; 1% SDS from Sigma-Aldrich, Missouri, USA; 1.5 mM dithiothreitol from Roche, Switzerland and 0.06 mg proteinase K from Qiagen, Germany) overnight at 55 °C. Proteins were precipitated using 3 M sodium acetate and gDNA-containing supernatant was precipitated by ice-cold 99.5% isopropanol. After incubation at −70 °C for 30 min and centrifugation the pellet was washed using 200 µl of 70% ethanol. Finally, pelleted gDNA was dissolved in 50 µl of dH_2O and diluted to a concentration of 1 ng/µl. Both gDNAs were stored at −20 °C until further use.

Probe design. Specific MOLigos were targeted to the one-copy *ail* gene, which encodes an outer membrane protein and a multi-copy 529-bp repeat element, for the detection of *YE* and *TG*, respectively. These targets were chosen with regard to their frequent and validated use in detection methods and the availability of their sequences in the NCBI GenBank database. Then, an approximately 100-bp long region was chosen manually and tested in OligoAnalyzer 3.1 (https://eu.idtdna.com/calc/analyzer) with the aim of finding the most suitable region for probe hybridization. The complementary region of each probe should meet certain parameters, as described in detail in Deshpande *et al.*[4]. The melting temperature of target complementary regions was set to be at least 47 °C, which is high enough to ensure detection specificity[4]. The optimal melting temperatures of designed MOLigos has been suggested to be in the range 47–58 °C with an average of 53 °C in most probes. Moreover, the difference in melting temperatures between the two complementary regions of one MOLigo pair did not exceed 1.5 °C. Sequences with hairpin melting temperatures higher than 30 °C and sequences for which potential dimer formation ($dG < -7$ kcal/mol) was identified were excluded. After setting the parameters, complementary sequences were checked in Nucleotide BLAST to guarantee their uniqueness for the desired target and to ensure that probe efficiency would not be reduced by off-target interactions. MOLigo probes were synthesized using standard desalting purification (Generi-Biotech, Czech Republic; Table 1).

Coating of microspheres. MagPlex Microspheres (12.5×10^6 microspheres/ml; Luminex Corp., Texas, USA) were coupled with anti-TAG sequences according to an adapted Bio-Plex Bead Coupling protocol by Bio-Rad, California, USA.

Magnetic microspheres of regions 19 and 34 were tempered to room temperature, vortexed on max speed, and sonicated for 30 s in an Ultrasonic Cleaning Bath (BioTech, Czech Republic). Then, 400 µl of each set were transferred into separate DNA LoBind Tubes (Eppendorf, Germany) and placed in the DynaMag-2 magnetic separator (Thermo Fisher Scientific, Massachusetts, USA) for 1 min in order to remove the supernatant. The pellet was resuspended in 45 µl of 0.1 M MES buffer, pH 4.5 (Sigma-Aldrich, Missouri, USA) and then vortexed and sonicated. Coupling reactions were started by the addition of 2 µl of 100 µM (stock in dH_2O) A018 and A019 anti-TAGs (complementary to xTAG sequences in MOLigo probes in Table 1) with C6-amino modifications at the 5′-end; each anti-TAG was transferred into a separate tube with different microspheres and vortexed. Then, 2.5 µl of a 10 mg/ml freshly prepared solution of EDAC powder (Thermo Fisher Scientific, Massachusetts, USA) were added and the mixture was vortexed immediately. Tubes were incubated for 30 min in the dark at room temperature with vortexing every 10 min. Then, addition of 2.5 µl freshly prepared EDAC was repeated together with incubation and vortexing. After the coupling reaction, microspheres were washed in 1 ml 0.02% Tween 20 (Alpha Diagnostic, Texas, USA) by vortexing and the supernatant was removed using the magnetic separator. The pellet was resuspended in 1 ml 0.1% SDS, vortexed, and the supernatant was removed on the separator. Finally, the pellet was resuspended in 80 µl of 1X TE buffer (pH 8.0; SERVA, Germany), vortexed, sonicated, and the coated microspheres were stored in the dark at 4 °C where they remain stable and ready to use for more than one year[14].

A hemocytometer was used to enumerate coupled microspheres (microspheres/µl). Microspheres were vortexed, diluted 1:100 in dH_2O, and 10 µl were transferred to the hemocytometer, where four large corners of the grid were counted. The following simple formula was used for enumeration: microsphere concentration (microspheres/µl) = (sum of microspheres in four large corners) × 2.5 × 100 (dilution factor). The average concentration for both regions was 50,000 microspheres/µl (should be at least 40,000 microspheres/µl), which is sufficient for at least 1,600 reactions.

The quality of microsphere coating was verified by direct hybridization to xTAG oligonucleotides (A018 and A019, the same as the xTAG sequence in MOLigo2) fluorescently labeled at the 5′-end, which were then used in a concentration gradient of 0–100 femtomoles. These oligos simulated MOL-PCR products and the

	Condition	Reference	Tested parameters [optimized highlighted]								
Multiplex oligonucleotide ligation	Ligase enzyme	Ampligase; *Taq*	Ampligase	Hifi *Taq*	*Taq*						
	MOLigo concentration [nM]	10000; 5; 4; 2; 1	10000 (nlp)	1000 (nlp)	50	10	5	1			
	Ligation temperature [°C]	60; 58; 50	69.7	67.7	63.9	63.3	62	61.2	59.8	59.2	56.3	55
	Ligation profile	30x; 10x cycling	20x cycling	incubation							
Singleplex PCR	Master Mix	AmpliTaq Gold; HotStarTaq	AmpliTaq Gold	EliZyme	OneTaq	Accustart II	Platinum	HotStarTaq			
	Primer concentration [µM]	0.125; 0.1	0.25	0.125	0.0625	0.05					
	Labeled-primer concentration [µM]	5; 0.5	2.5	1	0.5	0.25					
Hybridization and MAGPIX analysis	Microsphere type	pre-coupled MagPlex-TAG; MicroPlex	self-coupled Mag-Plex	pre-coupled MagPlex-TAG							
	Microspheres per reaction	5000; 2500; 1250; 1000; 750	5000	3500	2500	1500	1000	750	350		
	Reporter dyes	SAPE; Alexa532	HEX	TAMRA	BODIPY-TMRX	DY480	Cy3	Alexa532	SAPE	biotin-dCTP	

Figure 2. Table showing individual parameters of MOL-PCR assay which should be taken into account during optimizations. Reference values represent data reported in up-to-date literature (Deshpande *et al.*[4]; Thierry *et al.*[6]; Stucki *et al.*[10]; Wuyts *et al.*[5]; Woods *et al.*[13]) and optimized values are based on protocol described in present study. In green = significant values fullfilling interpretation criteria. (nlp) = no ligation product.

validation reaction was prepared in the same way as proper analysis according to the protocol described below (Hybridization to microspheres and MAGPIX analysis).

Multiplex oligonucleotide ligation. The multiplex ligation step was separated from PCR amplification[4] in order to avoid the high background signal levels when combining both steps in one reaction[10]. For the optimizations, each ligation reaction was prepared in a singleplex manner and contained only one specific MOLigo pair and one gDNA template. In this set-up, various parameters of the ligation reaction were tested (Fig. 2: Multiplex oligonucleotide ligation) and all experiments were run in quadruplicates. The optimized ligation reaction mix combined 5 nM of each MOLigo probe (Table 1) with 2.5 µl of 10X Hifi *Taq* DNA ligase reaction buffer, 0.5 µl of Hifi *Taq* DNA ligase (New England BioLabs, Massachusetts, USA), and 2.5 µl of template DNA (corresponds to ~2.5 ng of isolated DNA). The reaction was brought to a final volume of 25 µl with PCR H_2O (Top-Bio, Czech Republic). The thermal cycling program (DNA Engine Dyad, Bio-Rad, California, USA) included 10 min of denaturation at 95 °C followed by 20 cycles of 30 s at 95 °C and 1 min at 60 °C. Reactions were then cooled to 10 °C and used immediately in the PCR amplification step. Each experiment consisted of a positive sample containing template DNA and a no-template-control (NTC) with PCR H_2O instead of template to monitor cross-reactivity and contamination.

Singleplex PCR. Different master mixes and concentrations of universal primers were tested (Fig. 2: Singleplex PCR).

The optimized singleplex PCR reaction was performed in a final volume of 24 µl, which included 12 µl of 2X EliZyme HS Robust MIX (Elisabeth Pharmacon, Czech Republic), 0.0625 µM of universal FW primer, 0.25 µM of BODIPY-TMRX-labeled REV primer (Table 1), and 6 µl of ligation product. The thermal cycling program consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Reactions were cooled to 10 °C and either used immediately in the microsphere hybridization step or stored at 4 °C pending hybridization.

Several master mixes were tested in the optimization experiments, namely AccuStart II PCR ToughMix (QuantaBio, Massachusetts, USA), AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Massachusetts, USA), One*Taq* Hot Start 2X Master Mix with GC Buffer (New England BioLabs, Massachusetts, USA), Platinum Hot Start PCR Master Mix (Invitrogen, California, USA), and HotStarTaq DNA Polymerase (Qiagen, Germany). Each singleplex PCR reaction and thermal cycling protocol was run according to the relevant instructions from the manufacturer. In order to increase SAPE signal, we tested addition of Biotin-16-dCTP (Jena Bioscience, Germany) to the PCR reaction mix in a ratio of 1:1 to the dCTPs included within the original EliZyme master mix.

Hybridization to microspheres and MAGPIX analysis. The MOL-PCR products were hybridized to a particular magnetic microsphere set using xTAG sequences complementary to anti-TAG sequences covalently linked to the microspheres' surface. All microspheres present in the reaction were then analyzed by a MAGPIX reader, whose red diode identified a specific microsphere set according to its spectral signature and whose green diode measured fluorescent intensity emitted by the fluorescent reporter bound to the captured analyte on the microsphere surface.

Altogether, three parameters of the hybridization step were tested (Fig. 2: Hybridization and MAGPIX analysis). An optimized microsphere mix consisting of self-coupled MagPlex microspheres of regions 19 and 34 specific to *YE* and *TG* MOLigo pairs contained 2,500 microspheres of each set, 800 mM NaCl, and 50 mM MES buffer; the volume of each reaction was adjusted to 5 µl with 1X TE buffer (pH 8.0). Ten microliters of MOL-PCR product were mixed with 5 µl of microsphere mix in 0.2 ml Tear-off Strips which were then closed with strip caps (Bioplastics, Netherlands). The hybridization reaction (based on an adjusted protocol by Deshpande *et al.*[4]) was performed in a thermal cycler and consisted of denaturation at 94 °C for 1 min, followed by a slow ramp down in temperature to 25 °C at a rate of 0.1 °C per second. Before analysis on a MAGPIX reader, the reaction volume was increased to 60 µl by adding 45 µl of analysis buffer containing 10 mM Tris-Cl (pH 8.0), 0.1 mM EDTA, 90 mM NaCl and 0.02% Tween 20. Strips were placed into a 0.2 ml Multo Rack bench (Bioplastics, Netherlands) and

analyzed in MAGPIX device (Bio-Plex MAGPIX from Bio-Rad) using Bio-Plex Manager MP Software (Bio-Rad, California, USA).

When biotinylated REV primers or biotin-dCTPs were included during the optimization of the PCR step, the analysis buffer was enriched with SAPE (Thermo Fisher Scientific, Massachusetts, USA) in a final concentration of 3 μg/ml and with bovine serum albumin (BSA) in a final concentration of 0.1%. Samples were additionally incubated at 37 °C for 15 min. MAGPIX analysis was then carried out in the same manner but with heating at 37 °C.

Median fluorescence intensity (MFI) values were calculated from the analysis of at least 50 microspheres of each region per sample. After the analysis, the MFI values were exported from Bio-Plex Manager 6.1 Software (Bio-Rad, California, USA) into an Excel file to calculate signal-to-noise ratios.

Multiplex assay. The functionality of the optimized protocol described above, including the probe design, coating of microspheres, multiplex oligonucleotide ligation, singleplex PCR, hybridization to microspheres and MAGPIX analysis, was demonstrated on a model multiplex assay developed in-house.

For the 11-plex panel, common representatives of foodborne pathogens were selected and five bacterial systems (*Y.enterocolitica; Escherischia coli* CAPM 5358, serotype O26, gene *wzy*; *Listeria monocytogenes* CAPM 5879, serotype 1/2b, gene *rnc*; *Campylobacter jejuni* CAPM 6316, serotype 10, gene *hipO;* and *Salmonella enterica* CAPM 5445, serotype Typhimurium, gene *ttr*C), five parasite systems (*T. gondii; Trichinella spiralis*, target *ITS1; Taenia saginata*, gene *cox1*; *Giardia intestinalis* generic for all assemblages, gene *ef1α;* and *Giardia intestinalis* assemblage A, gene *tpi*), and an internal amplification control (IAC) were designed. The IAC was designed as a non-competitive synthetic sequence based on two ancient DNA sequences[16]; specifically the mitochondrial DNA of two extinct species – the Tasmanian tiger (*Thylacinus cynocephalus*) and the giant moa (*Dinornis struthoides*). This synthetic sequence was cloned into a plasmid and served for the differentiation of truly negative and false negative (inhibited) samples[17]. Sequences of MOLigo probes included within this model multiplex assay are available upon request.

Genomic DNAs were extracted using the same procedure as described above with respect to bacterial or parasitic affiliation. All bacterial isolates were obtained from the Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute and single colony was used for heat lysis resulting in 10X diluted lysates. As an input into parasitic DNA isolation using extraction buffer was used muscle larvae of *T. spiralis* (provided by the International Trichinella Reference Center, Rome, Italy) and a part of a *T. saginata* body segment (originating from a natural infection). Genomic DNA of *G. intestinalis* was ordered commercially from ATCC, 30888D (Virginia, USA). DNA of each species was diluted to concentration of 1 ng/μl.

A single ligation reaction mix was prepared for all samples, which thus contained 22 specific MOLigo probes, each in final concentration 5 nM and 10^3 of plasmid DNA as the template for the IAC. Four NTCs were prepared and the experiment was run in quadruplicates. For each sample one template DNA was used (~2.5 ng) and microsphere mix consisted of 11 regions specific to individual pathogens.

Data analysis and interpretation. For each experiment, two controls were used for the calculation of the signal-to-noise ratio: (1) A microsphere-only control called "blank" to report background fluorescence (contained analysis buffer, microsphere mix, fluorescent reporter, and dH_2O instead of MOL-PCR product) and (2) a NTC to report cross-reactivity of MOLigos and contamination level in the absence of any template.

In an Excel file, all measured MFI values were corrected by subtraction of the blank MFI and then an average value for each sample/NTC was calculated from the quadruplicates. The signal-to-noise ratio was calculated by dividing the MFI of the sample by the corresponding MFI of the NTC. A signal-to-noise ratio of at least 4 and MFI of at least 200 were the two criteria used to determine "positive samples"[4]. Regarding the resulting data, it should be noted that even a slight increase in MFI values of NTCs as divisors can cause profound differences in the signal-to-noise ratios between samples.

Three criteria were considered for the comprehensive numerical evaluation of individual tested conditions for both targets *YE* and *TG*: MFI of the positive samples (MFI+), MFI of the NTCs (MFI-) and height of the signal-to-noise ratio (SNR). Each criterion has been given a different relevance (weight), corresponding to its importance during the data interpretation. In all parameters of the optimization, each tested condition was given a rating as a weighted mean of six ranks in individual criteria achieved in comparison test with other conditions. For each criteria, the following scale (expressing the importance) was chosen: MFI + 3 (high fluorescence intensity of positive samples is desirable, because a MFI decrease in the case of low DNA concentrations or high level of multiplex is expected), MFI- 2 (low fluorescence intensity of no-template controls is desirable but not so significant beyond a limit of 200 MFI) and SNR 1 (height of signal-to-noise ratios serve for differentiation of positive samples in disputed cases). Taking into account all monitored criteria, the most suitable condition for each parameter was chosen based on its achievement of the best ratings (see Supplementary Table S1) and further utilized within the optimized protocol.

Results and Discussion

Different conditions were tested to evaluate whether deviations from the reference values (Fig. 2) had a significant impact on the MFI values and signal-to-noise ratios. If no impact was observed, no further conditions were tested. Optimized conditions for individual parameters were further used in the subsequent experiments.

In the case of bacteria (*YE*), a simple method for DNA isolation, such as heat lysis of a single colony, is preferable for routine purposes and was previously described for preparation of a DNA template for a MOL-PCR assay[6,7,10]. For eukaryotes (*TG*) and multicellular organisms, DNA isolation using extraction buffer was previously described and enables acquisition of very pure DNA[15,18]. In the present study, no differences were observed in the MOL-PCR optimization analyses when utilizing gDNA templates isolated by two distinct methods. The use of

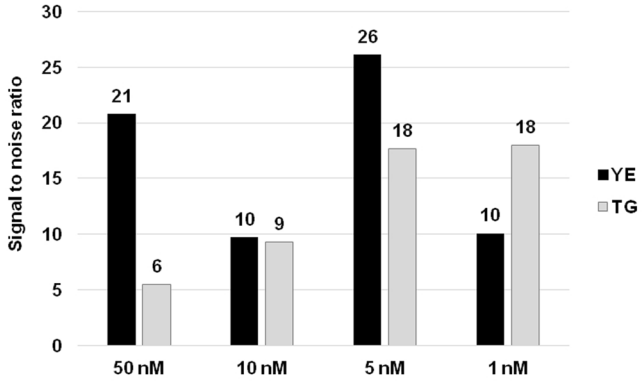

Figure 3. Determination of optimal concentration of MOLigo probes. The optimal concentration of each probe for both targets was found to be 5 nM. Other concentrations resulted in an increased background of NTCs, which led to a decrease in the signal-to-noise ratio. YE = *Y. enterocolitica;* TG = *T. gondii*.

2.5 µl of 10X diluted lysate and 2.5 ng of isolated DNA resulted in very high signals in comparison to NTCs (based on the results in Figs 3–8). In general, MOL-PCR is sensitive enough to allow usage of low concentrations of DNA (in the order of 1–0.1 ng of input material[6,10]); however, more concentrated DNA gives better results[7] as there is a decrease of signal intensities with decreasing amounts of DNA[10].

Song *et al.*[12] developed the MOLigoDesigner web-based design tool (http://moligodesigner.lanl.gov) especially for the purposes of MOLigo probe design and probe quality check[6,10,12]; however, this helpful online tool is no longer available. Another possibility is the download of ThermoNucleotideBlast (http://public.lanl.gov/jgans/tnt-blast/)[4,13,19], which is useful for melting temperature calculations or the purchase of Visual OMP software (DNA Software)[5,7]. Nevertheless, for an individual experienced with primer or probe design, common online tools such as Nucleotide BLAST and OligoAnalyzer 3.1 are sufficient. In some cases, it is complicated to find a suitable complementary sequence, which strictly conforms to all criteria, especially when the usable sequence region for the design of MOLigos is too short. The most problematic criterion during probe design appears to be the melting temperature. During probe design, it is recommended to design more variants of specific detection MOLigo pairs combining positive and negative (reverse complement) strands of the target complementary region as well as to switch TAG sequences between MOLigo1 and MOLigo2. Computational analysis indicated the absence of non-specific interactions; however, different results were obtained for each variant (see Supplementary Fig. S1 and Table S1). The utilization of a variant carrying the TAG sequence on MOLigo2 seems to be ideal, although no pattern in targeting a particular strand of target sequence was found. In initial testing, half of each assay product was visualized on an agarose gel stained with ethidium bromide and the second half hybridized to magnetic microspheres. Probe variants showing no ligation product or high NTC values were eliminated or redesigned.

Optimization of the multiplex oligonucleotide ligation step. In the very first paper dealing with MOL-PCR by Deshpande *et al.*[4], ligation and PCR were conducted in a single reaction using Ampligase (Epicentre, Wisconsin, USA) and Amplitaq Gold DNA polymerase (Roche, Switzerland). Polymerase was activated during the ligation step through a slow release mechanism and was able to subsequently perform the amplification of ligated MOLigos. However, follow-up studies by Thierry *et al.*[6] and Stucki *et al.*[10] were unsuccessful with such a workflow which resulted in high background noise. Therefore, the ligation step was performed separate from the PCR step optimization experiments and high signals compared to the background noise were obtained after optimization.

The ligation step which constitutes the actual detection event includes a specific MOLigo pair intended for the detection of a particular marker. The total concentration of probes in a MOL-PCR reaction seems to be a crucial factor. In the literature, the concentration ranges from 1 nM[7] up to 10 µM[6] (Fig. 2). For evaluation of the probe concentration effect on the signal-to-noise ratio, the concentration of each probe in the ligation mix was tested at 1 nM, 5 nM[4], 10 nM, 50 nM, and 1 and 10 µM. Based on numerical data evaluation (see Supplementary Table S1), the optimal concentration of MOLigo probes was established at the very low level of 5 nM of each probe as other concentrations resulted in increased MFI values of NTCs and thus decreases in signal-to-noise ratios (Fig. 3). If higher concentrations of 1 or 10 µM were used, no ligation product was detected on an agarose gel. Low concentrations are important especially in assays with high levels of multiplexing, e.g. the same concentration of 5 nM was used in a biothreat panel combining more than 10-plex assays[4], while an even lower concentration of 2 nM was utilized in an 8-plex assay for SNP-typing (three probes per SNP) of the *Mycobacterium tuberculosis* Complex[10] and in three MOL-PCR assays for subtyping of *Salmonella* Typhimurium by 52 molecular markers[5].

The ligation temperature should be considered in order to ensure high specificity and efficiency of the detection event, since the usage of higher temperatures (near the melting temperatures of the used MOLigos) increases the annealing stringency[20]. To determine the temperature that most efficiently ensured the formation of a covalent bond between the two MOLigos after hybridization to the target sequence, and its impact on the signal-to-noise ratio, a temperature gradient from 55 to 70 °C was tested. While maximum MFIs varied only very little, temperature had an impact on NTCs; based on numerical data evaluation (see Supplementary Table S1) ligation temperature between 59–60 °C was considered as the most suitable. This value is higher than in the majority of

previously reported data, where a ligation temperature of only 50 °C was used in a 10-plex biothreat panel[4] and an 8-plex assay for *M. tuberculosis*[10]. This might reflect different requirements for optimal performance of each thermostable ligation enzyme; in the case of Hifi *Taq* DNA ligase, typical nick-ligation procedures should be performed at 60 °C. Therefore, a temperature range of about 59–60 °C fulfills the prerequisites for the best activity and fidelity of the enzyme based on oligo-template properties and ensures the reduction of errors caused by mismatched base pairs.

According to the manufacturers' recommendation, a novel thermostable Hifi *Taq* DNA ligase may efficiently seal nicks in DNA when incubated at the optimal ligation temperature for 15 minutes or for repeat cycles of the denaturation and annealing steps. In an attempt to shorten the working protocol, the longer cycling protocol including initial denaturation for 10 min at 95 °C followed by 20 cycles of 30 s at 95 °C and 1 min at 60 °C was compared with a shorter incubation protocol consisting of initial denaturation for 5 min at 95 °C followed by 15 min incubation at 60 °C. This comparison showed (see Supplementary Fig. S2) that linear amplification occurring during cycling does not have any significant effect on the fluorescent intensity of positive samples; however, such amplification suppresses non-specificity in NTCs and thus increases signal-to-noise ratios. Based on numerical data evaluation (see Supplementary Table S1), the ligation protocol based on cycling was further utilized.

The best efficiency and lowest background noise was obtained with the Hifi *Taq* DNA Ligase (see Supplementary Table S1), of which it has been claimed that it is the NAD^+-dependent DNA ligase with the highest fidelity currently available commercially[21]. There is the possibility to use Ampligase Thermostable DNA Ligase (Epicentre) or *Taq* DNA Ligase (New England BioLabs, Massachusetts, USA) instead, although the fidelity is greatly reduced[21]. Also, the usage of Ampligase or *Taq* ligase may result in the reduced effectiveness of some previously functioning MOLigo pairs (in the case of *YE;* see Supplementary Fig. S3) caused by a significant increase in NTCs; such pairs would thus become unusable and would have to be redesigned.

To ensure optimal performance of the ligation reaction and to suppress cross-reactivity in problematic MOLigo pairs, fish sperm DNA can be added to the ligation reaction mix. The addition of 50 ng of DNA from fish sperm (SERVA, Germany) in 1X TE buffer markedly reduced NTCs in a problematic detection system (see Supplementary Fig. S4) and increased the signal-to-noise ratio from less than 4 to up to 39, thus allowing data interpretation and further use of such a system in future assays. The exact mechanism of action is unknown, but one can assume that carrier DNA prevents the non-specific binding of MOLigos to other DNA sequences or attachment to tube surfaces[22], which is a common reason for carrier DNA addition to mastermixes. Utlization of carrier DNA in MOL-PCR was previously described by Woods *et al.*[13], who included carrier DNA at a concentration of 0.15 mg/ml in the ligation mix of their 11-plex assay for Shiga toxin-producing *E. coli*. The positive properties of carrier DNA have also been described with respect to DNA isolation, where the addition increased the yield of DNA[23].

Optimization of singleplex PCR. Subsequent singleplex PCR amplification was performed using the successfully ligated MOLigos as a template and the universal primer pair, with the REV primer carrying a fluorescent reporter. Most ligase reaction buffers usually contain white precipitate particles, which need to be fully dissolved by warming in hand and/or vigorous shaking. Buffers should not also be subject to repeated freeze-thawing, which may distort results leading to high MFI values of NTCs. These particles are likely to consist of dithiothreitol (DTT) used to preserve enzyme stability during *in vitro* reactions. DTT was recently described as a possible cause of markedly increased background, and thereby reduced signal-to-noise ratios[24].

The significant drop in signal when incorporating higher loads of PCR products in the microsphere hybridization reaction was described previously[6]. To limit possible bias during the hybridization of labeled MOL-PCR product to microspheres, the concentration of universal primers was altered with the aim of producing predominantly fluorescently-labeled single-stranded products. Universal primers (Table 1) were adopted from Thierry *et al.*[13] and four-fold less FW primer than BODIPY-TMRX labeled REV primer at concentrations of 0.125/0.5 μM, 0.25/1 μM, 0.0625/0.25 μM, and 0.05/2.5 μM were used in comparison to a uniform concentration of both primers of 0.25 μM. This asymmetric PCR amplification strategy improved hybridization efficiency and increased maximal MFI values for positive samples (see Supplementary Fig. S5). Fifty-fold less[6] FW primer (0.05/2.5 μM) led to the highest MFI values; however, a noticeable increase of MFI values of NTCs was also observed. Based on numerical data evaluation (see Supplementary Table S1), final concentrations of 0.0625 μM for FW primer and 0.25 μM for labeled REV primer were considered as the most efficient despite the fact that the uniform concentration of 0.25 μM for both primers resulted in higher signal-to-noise ratios (Fig. 4) but considerably lower MFI values.

Another important factor influencing the effectiveness and cost of the MOL-PCR reaction is the choice of the master mix. In order to find a balance, the performance of six different master mixes was tested (Fig. 5), including the previously utilized HotStarTaq DNA Polymerase[5–7] and the upgraded AmpliTaq Gold 360 Master Mix[4,13]. The MOL-PCR reaction using OneTaq HotStart was not successful and no products were detected, since the results did not meet even one of the interpretative criteria. HotStarTaq DNA Polymerase, on the other hand, reached the highest MFI values. However, the increase of NTC noise was significant in *YE* and, with a signal-to-noise ratio of 4, was on the border of positivity. Based on numerical data evaluation (see Supplementary Table S1), the highest efficacy was achieved with the EliZyme HS Robust mix, which in addition, offers time savings through the shorter cycling profile and compound complexity of stock solution.

Optimization of hybridization step and MAGPIX analysis. In the last step, the specific hybridization of fluorescently labeled MOL-PCR product to the magnetic microspheres enables detection in a MAGPIX reader. This final step requires several parameters to be considered such as the type and number of magnetic microspheres, choice of fluorescent reporter or working protocol. As presented in Fig. 2, all previously developed

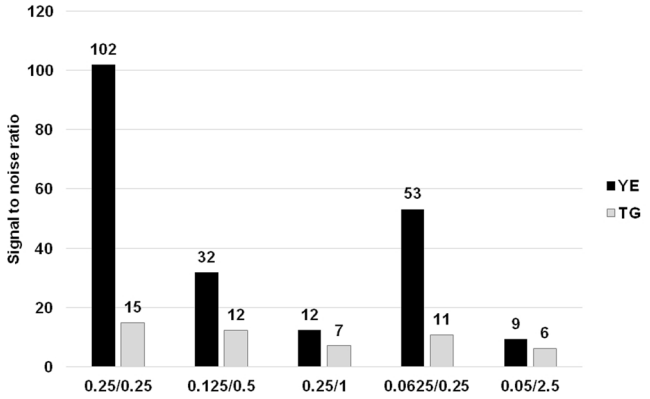

Figure 4. Optimization of the concentration of universal primers utilized in the singleplex PCR. The values on the x-axis represent FW primer/labeled REV primer concentration [μM]. The most suitable final concentration of FW primer was found to be 0.0625 μM and that of labeled REV primer 0.25 μM. YE = *Y. enterocolitica;* TG = *T. gondii*.

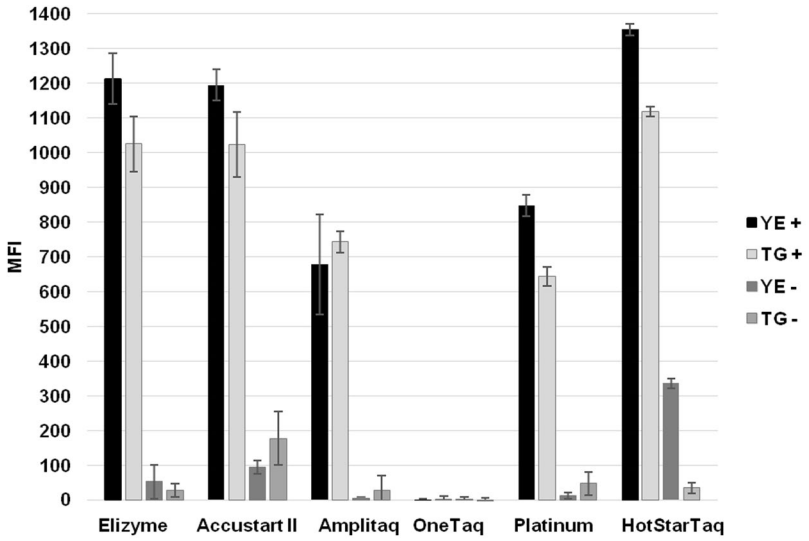

Figure 5. Impact evaluation of Master Mix used in singleplex PCR. In graph: Elizyme = 2X EliZyme HS Robust MIX (Elisabeth Pharmacon, Czech Republic); Accustart II = AccuStart II PCR ToughMix (QuantaBio, Massachusetts, USA); Amplitaq = AmpliTaq Gold 360 Master Mix (Thermo Fisher Scientific, Massachusetts, USA); OneTaq = OneTaq Hot Start 2X Master Mix with GC Buffer (New England BioLabs, Massachusetts, USA); Platinum = Platinum Hot Start PCR 2X Master Mix (Invitrogen, California, USA); HotStarTaq = HotStarTaq DNA Polymerase (Qiagen, Germany). YE = *Y. enterocolitica;* TG = *T. gondii*; plus sign in legend = positive sample; minus sign = NTC.

MOL-PCR assays utilized commercially available pre-coupled MagPlex-TAG Microspheres or MicroPlex Microspheres (Luminex Corp., Texas, USA). However, the fluorescent intensity of pre-coupled microspheres (Fig. 6) was less than half that achieved by self-coupled MagPlex microspheres used in other experiments and, with a signal-to-noise ratio of less than 3 in the case of *TG*, did not fulfil the stipulated criteria. Based on numerical data evaluation (see Supplementary Table S1), self-coupled MagPlex Microspheres were considered as more efficient and for this reason were utilized within all optimization experiments.

Findings concerning microsphere number per reaction in ligation assays are based on analyses on flow cytometer devices. The general recommendation for obtaining accurate results is to use up to 2,500 input microspheres[25] per reaction and to count 100 events[26]. Bruse *et al.*[27] concluded that an input of 200 microspheres and a minimum of 20 events provide the same results as higher amounts that have been recommended[27] and Jacobson *et al.*[28] went even further and declared that as few as 10 microspheres are sufficient under appropriate conditions[28]. However, flow cytometer devices differ from MAGPIX readers in their mechanisms of measurement and little data are available for this basic instrument. Woods *et al.*[13] screened Shiga toxin-producing *E. coli* using 1,000 microspheres per reaction and counted a minimum of 100 events in their 11-plex assay. Wuyts *et al.*[5,7] used 750 input microspheres for subtyping of *Salmonella* Typhimurium and counted a minimum of 50 events. Before setting this parameter, they tested 375, 750, and 2,500 microspheres per reaction and obtained the best results

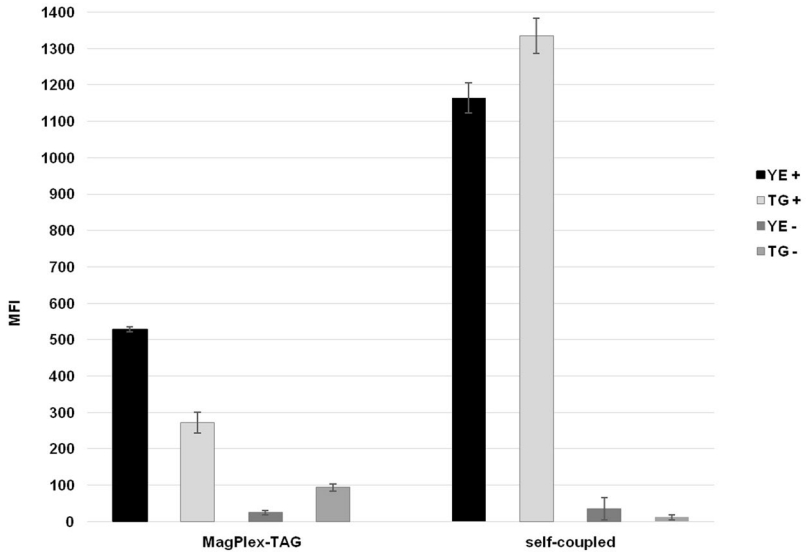

Figure 6. The efficiency of commercially pre-coupled MagPlex-TAG Microspheres in comparison to self-coupled MagPlex Microspheres. In *TG*, the obtained signal-to-noise ratio of 2.89 with the usage of pre-coupled microspheres did not fulfil the set criteria. YE = *Y. enterocolitica;* TG = *T. gondii*; plus sign in legend = positive sample; minus sign = NTC.

with 750 microspheres, since the lower number resulted in counts of below 50. For deeper exploration, we tested 350, 750, 1,000, 1,500, 2,500, 3,500, and 5,000[4] microspheres per reaction with a minimum count set to 50 events (see Supplementary Fig. S6). Regarding high MFI, low NTCs (high signal-to-noise ratios), and minimal deviations between measurements, 2,500 microspheres per reaction showed slightly better efficiency. Moreover, lower numbers resulted in counts of less than 50 (see Supplementary Table S2); specifically, 350 microspheres were on average below the minimum event count in 97% of samples, 750 microspheres in 64%, 1,000 microspheres in 76%, and 1,500 in 38% of samples. Meanwhile, the use of 2,500 and more microspheres resulted in event counts that were below the minimum in less than 3% of samples. Based on numerical data evaluation (see Supplementary Table S3), where also the criterion of microsphere count below 50 was included, we conclude that the reference number of 2,500 microspheres per reaction generates the most reliable data.

The choice of fluorescent reporter dye may also significantly affect the resulting data. SAPE is recommended by Luminex as the most efficient reporter (100% relative fluorescence intensity) for read-outs on a MAGPIX[29]. The "Relative reporter intensities"[29] graph shows that other relevant reporters lag far behind, with Alexa Fluor532 (28%) coming a distant second, Cy3 (19%) third and BODIPY-TMRX (7%) determined to be the sixth most suitable dye. We re-evaluated this finding and tested other available dyes in an experiment including universal REV primers labeled at the 5′-end with seven different molecules whose spectra are relevant to MAGPIX diodes: HEX, TAMRA, BODIPY-TMRX, DY480, Cy3, Alexa Fluor532, and biotin binding with SAPE (Fig. 7). The highest MFI values and signal-to-noise ratios (see Supplementary Fig. S7) were obtained with biotin-SAPE usage as previously stated. However, SAPE utilization also showed the greatest deviations between measurements reaching a difference in MFI of up to 1,000. Based on numerical data evaluation (see Supplementary Table S1) the performance of Alexa Fluor532 and Cy3, previously reported as another suitable dyes for MOL-PCR assay[4,10], were greatly overcome by BODIPY-TMRX. On the other hand, DY480 did not meet either the minimum MFI of at least 200 or a signal-to-noise ratio of at least 4 and was evaluated as inappropriate for analysis on a MAGPIX reader. It is worth noting that the use of a direct label on a universal REV primer holds an advantage over indirect biotin-SAPE labeling in that only one labeled primer that marks all successfully ligated probes during a PCR reaction is needed; with biotinylation usage, meanwhile, a further incubation step of SAPE binding is required. This provision ensures time savings, reduces the financial burden, and diminishes the risk of cross contamination. For this reason and also in order to minimize deviations in measurements, we have found BODIPY-TMRX to be the most suitable and efficient fluorescent reporter for our MOL-PCR assay, and we utilized this dye for all optimization experiments presented within the study.

An alternative strategy aimed at increasing the fluorescence intensity based on incorporation of labeled nucleotides into target DNA sequence during the PCR step, was tested using biotin-16-dCTPs. Biotin-dNTPs are commonly used for allele-specific primer extension assays[30,31] on MAGPIX and its potential for the MOL-PCR assay was tested. The results showed significantly increased MFI values reaching tens of thousands in positive samples (see Supplementary Fig. S8). However, with NTCs of about 1,000 MFI, the signal-to-noise ratios were below the efficiency of SAPE (biotinylated REV primer) or BODIPY and with the regard to the higher price of biotinylated nucleotides and requirement for a subsequent incubation step, were not rated as the most appropriate labeling variant for MOL-PCR products. To conclude, fluorescent reporters other than SAPE showed lower absolute fluorescence; nevertheless, the performance is stable during the course of measurements, interpretative criteria are clear, and the working protocol is short.

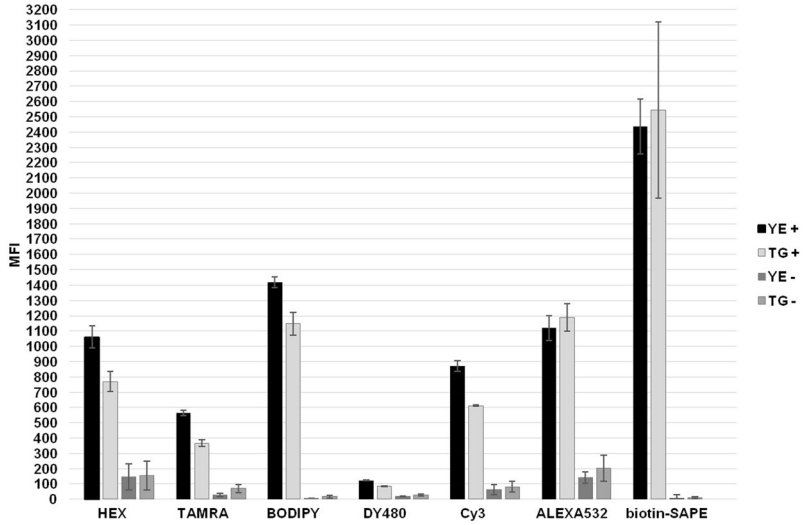

Figure 7. Comparison of efficiency of fluorescent reporters. 5′-end fluorophore modifications in graph: HEX = HEX (Eurofins Genomics, Luxembourg); TAMRA = TAMRA (Eurofins Genomics, Luxembourg); BODIPY = BODIPY-TMRX (Eurofins Genomics, Luxembourg); DY480 = DY480 (Eurofins Genomics, Luxembourg); Cy3 = Cy3 (Generi-Biotech, Czech Republic); ALEXA532 = Alexa Fluor532 (Invitrogen, California, USA); biotin-SAPE = biotin (Generi-Biotech, Czech Republic) and SAPE (Thermo Fisher Scientific, Massachusetts, USA). YE = *Y. enterocolitica;* TG = *T. gondii*; plus sign in legend = positive sample; minus sign = NTC.

Optimized conditions in the multiplex assay. To demonstrate the efficiency of the parameters optimized in the singleplex MOL-PCR systems and to prove the functionality of the described working protocol in a multiplex format, a model 11-plex FBP panel was created (Fig. 8). It is important to mention that this multiplex assay does not have a practical meaning as the sample with such pathogen composition is unlikely to be found and systems will be reorganized for future use.

IAC proved itself to be a reliable indicator of the accuracy of the results and all bacterial and parasitic systems fulfilled the set criteria (see Supplementary Fig. S9), proving their functionality for future experiments. Nevertheless, some systems showed a sharp decline in signal-to-noise ratios manifested by both a decrease of MFI values in positive samples and an increase of NTCs in comparison to their performance in singleplex assays, e.g. *TG* had a ratio of 6 in multiplex but reached a ratio of 69 in a singleplex test with fluorescent dyes (see Supplementary Figs S7 and S9); for similar issues might be a solution a division of the multiplex panel into separate groups, where individual systems would be combined on the basis of species relatedness or possibility of their common occurrence in particular matrix[32], where the systems included would be tested for mutual interactions. At higher levels of multiplexing (more than 20 targets), the limit of detection (0.1 ng of input DNA as described above) might be reduced for individual targets when compared to their values in singleplex[33].

Within this manuscript we do not include the limits of detection or specificity tests for particular systems of our model multiplex assay, these evaluations must be performed after optimization of MOL-PCR and MAGPIX analysis as whole, before implementation of the specific multiplex panel into routine practice.

Accessibility criteria. Microsphere-based assays based on xMAP technology (Luminex Corp., Texas, USA) require the purchase of a Luminex instrument. The most basic and also cheapest instrumentation suitable for such assays is a MAGPIX reader utilizing magnetic microspheres. According to the analyte of interest the MAGPIX is adjustable for a wide range of applications, e.g. nucleic acid assays and protein or immuno-assays. Moreover, many laboratories already have access to such instrumentation.

The most significant expenses of a typical Luminex-based assay are the ligation enzyme, PCR master mix, and microspheres. Regarding the MAGPIX reader, MagPlex-TAG Microspheres (2.5×10^6 microspheres/ml) are commercially available. With a 1 ml volume of microsphere stock solution and when using a microsphere mix with 2,500 microspheres/reaction, these commercial microspheres are sufficient for 1,000 reactions. In comparison, self-coupled MagPlex Microspheres are sufficient for at least 4,000 reactions; the necessary number of microspheres can be prepared in a single coupling procedure. Even with the inclusion of costs for reagents required for coating and labor, the self-coupled beads are still financially preferable (4% of the cost per reaction) over commercial microspheres.

Using the optimized MOL-PCR method described in the present study, the cost per reaction for detection of one marker is approximately €2. The costs drop even lower when multiplexing. The highest fidelity was proven when using Hifi *Taq* DNA Ligase, which represents the state-of-the-art in the field of thermostable ligases; however, this novel ligase is a factor that significantly increases the cost of analysis. If the price is the significant criterion for laboratory purposes, Ampligase or *Taq* DNA Ligase might be used as an alternative. Nevertheless, the reduced efficiency described above has to be taken into account. When using Ampligase, the price for one sample

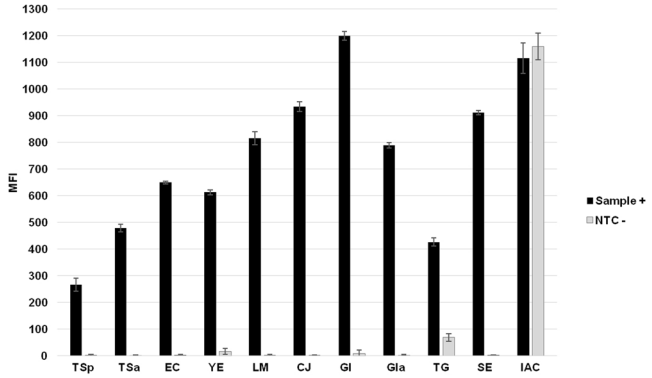

Figure 8. 11-plex foodborne pathogens panel run with optimized protocol utilizing BODIPY-REV primer. In graph: TSp = *Trichinella spiralis;* TSa = *Taenia saginata*; EC = *Escherischia coli*; YE = *Y. enterocolitica;* LM = *Listeria monocytogenes;* CJ = *Campylobacter jejuni*; GI = *Giardia intestinalis* generic; GIa = *Giardia intestinalis* assemblage A; TG = *T. gondii*; SE = *Salmonella enterica;* IAC = internal amplification control.

reaches €0.68, which roughly corresponds to €0.8 per assay, and similar to what was calculated by Stucki *et al.*[10]. However, the price of maintenance reagents necessary for proper operation of the MAGPIX reader should be also taken into account. It is in particular a Calibration kit, which is used approximately once a week and a Verification kit, which has to be used after each instrument startup; therefore an amount of approximately 20 cents per sample (considering a 96-well plate) should be included.

Another important factor is the time needed to perform the assay. In comparison to the closely related MLPA, which requires about 20 hours to complete, the MOL-PCR assay can be performed very rapidly[4]. MLPA remains, even in the version adapted to the microsphere array and when using the same detection platform as MOL-PCR, tedious due to the number of steps or necessity for overnight hybridization[34]. The MOL-PCR assay is designed to be performed in 96-well plates, while simultaneously allowing each well to be screened for up to 50 markers, and provides high-confidence results within 6 h[6,12]. Hands-on-time might be further shortened to less than 4 hours by usage of directly labeled universal primers and with the assay workflow described above (see overview in Fig. 2).

Conclusion

Modern diagnostics should meet current demands with respect to accuracy, rapidity, and complexity, in order to combine all necessary data within a simple methodology. Although the multiplexing of PCR diagnostic assays is desirable, there are technological limits to multiplexing in various derivatives of PCR. MOL-PCR adapted to Luminex instruments has the potential to overcome these limits and has proven to be suitable for a number of diagnostic approaches relying on a complex and multiplex analysis of the sample. Further, there is no necessity to change technology as the PCR cycler is nowadays present in each laboratory and the technique can be adapted to current instrumentation. However, even MOL-PCRs whose suitability has been proven in previous studies must be precisely optimized in order to serve as a robust and functional tool in routine diagnostics. To the best of our knowledge there is no complex study available which would comprehensibly identify all parameters needed to design a reliable in-house multiplex MOL-PCR assay and which would characterize the impact of each parameter on the performance of the assay. Through the present study as a guideline to the optimization process, we, therefore, intend to bring this approach closer to the wider body of professionals and to facilitate the spread of the MOL-PCR technology to complex routine microbial analysis of food.

Data Availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information file).

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Author Contributions

N.R. performed molecular work and analysis, created data outputs and wrote the manuscript. V.H. contributed to molecular work and analysis. J.H. contributed to molecular work and analysis. M.K. provided parasites, consulted on data interpretation and revised the manuscript. P.K. designed the study, secured the biological material, provided financial support, consulted on data interpretation, and revised the manuscript. All authors contributed to the final version of the manuscript.

Additional Information

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microorganisms

MDPI

Article

Development and Inter-Laboratory Validation of Diagnostics Panel for Detection of Biothreat Bacteria Based on MOL-PCR Assay

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Abstract: Early detection of biohazardous bacteria that can be misused as biological weapons is one of the most important measures to prevent the spread and outbreak of biological warfare. For this reason, many instrument platforms need to be introduced into operation in the field of biological warfare detection. Therefore the purpose of this study is to establish a new detection panel for biothreat bacteria (*Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Brucella* spp.) and confirm it by collaborative validation by using a multiplex oligonucleotide ligation followed by polymerase chain reaction and hybridization to microspheres by MagPix detection platform (MOL-PCR). Appropriate specific sequences in bacterial DNA were selected and tested to assemble the detection panel, and MOLigo probes (short specific oligonucleotides) were designed to show no cross-reactivity when tested between bacteria and to decrease the background signal measurement on the MagPix platform. During testing, sensitivity was assessed for all target bacteria using serially diluted DNA and was determined to be at least 0.5 ng/µL. For use as a diagnostic kit and easier handling, the storage stability of ligation premixes (MOLigo probe mixes) was tested. This highly multiplex method can be used for rapid screening to prevent outbreaks arising from the use of bacterial strains for bioterrorism, because time of analysis take under 4 h.

Keywords: MOL-PCR; biothreat bacteria; magnetic bead; bioterrorism; detection panel

1. Introduction

Bioterrorism refers to the deliberate abuse of pathogenic microorganisms (bacteria, viruses or their toxins) to spread life threatening diseases on a large scale and thus devastating the population of the area. Bacterial strains can be misused as biological weapons not only as a threat to human health but also in terms of agricultural abuse and ecotoxicological risks [1]. The use of biological agents in comparison with conventional weapons is very attractive to terrorists because of their relatively low cost and relative availability [2]. The most efficient way for delivery of biological agents is in the form of aerosols. However,

there are a number of other ways in which biological agents can be disseminated, e.g., via food, feed, or water contamination [3]. *Bacillus anthracis*—anthrax, *Yersinia pestis*—plague, *Francisella tularensis*—tularemia or *Brucella* spp.—brucellosis are among the most abused bacteria in biological weapons [4]. The Centre for Disease Control and Prevention (CDC) classifies biological agents into categories A–C, where the category A represents the most dangerous pathogens. All the biothreat bacteria listed above belong to group A, except for *Brucella* spp., which is classified in category B [5].

B. anthracis is a spore-forming bacterium and is classified as one of the most important pathogens for abuse as a bioterrorist weapon [6]. The respiratory route of infection caused by inhalation of spores is a minor issue in the context of global human anthrax cases but a serious issue when associated with bioterrorism [7]. Anthrax containing letters from October 2001 confirmed that only a small amount of *B. anthracis* spores are sufficient for an outbreak due to a terrorist action [8]. The infectious dose ranges from less than 10 to over 10,000 spores depending on factors like the route of the infection or the health status of the exposed individual [7–10]. Another destructive disease is the plague caused by *Y. pestis*. Person to person transmission of *Y. pestis*, i.e., primary pneumonic plague, is possible by way of airborne droplets or aerosols and if untreated, it is 100% fatal [11,12]. A short incubation period, very low infectious dose requirement of only 1 to 10 organisms, and the progressive nature of the infection classifies this bacterium into group A of pathogens at risk of bioterrorism abuse [13,14]. *F. tularensis* is able to cause a highly infectious disease called tularemia by as little as a few microbes aspirated from the surrounding air. Tularemia is a disease of wild animals (rodents, hares, and rabbits) that can be transmitted to humans [15]. *F. tularensis* is classified into four subspecies, but only two of them are the causative agents of disease in humans. Type A is subsp. *tularensis* (predominantly found in North America), and type B is subsp. *holartica* (predominantly found in Eurasia) [16]. *Brucella* spp. causes a serious contagious disease transmissible to humans, which results in reproductive failure of infected animals [17]. Genus *Brucella* includes several species exhibiting host adaptations. *B. abortus* (cattle), *B. melitensis* (sheep and goats), and *B. suis* (pigs) belong to the most common and virulent types not only for livestock but also for wildlife and humans [18,19]. Transmission of 100 to 1000 cells are sufficient for the development of the disease [20]. Although brucellosis has been eradicated in most developed countries, it is still found in many developing countries [21].

In order to prevent the spread of the agent after a bioterrorist attack and to apply efficient preventive measures to stop the spread of infectious diseases, it is important to quickly identify and specify bacterial species [22]. The method of choice for fast and reliable identification of pathogens in various matrices is polymerase chain reaction (PCR) [23]. However, analysis of numerous samples for the presence of multiple infectious agents by PCR requires modifications of the PCR workflow. One of the possibilities is to use PCR as a suspension arrays in which the fluorescently labelled PCR product is visualized by its attachment to a specific bead. A various modifications of suspension arrays exists [24]. The latter approach, multiple oligonucleotide ligation PCR (MOL-PCR), allows the use of only a single pair of universal primers which makes optimization of the whole assay easier. The first step is then accomplished by ligation of specific targets sequence which will create a template with specific complementary primer sequences for annealing of the universal primers. The PCR products can be labelled either by the post-PCR conjugation of streptavidin-phycoerythrin (SAPE) complex with e.g., biotinylated primer or by direct labelling of a PCR primer with fluorescent dyes (Alexa Fluor532, BODIPY-TMRX). The last step is the hybridization of the PCR product using a specific 24 base DNA sequence, which is already part of the probe and its complementary sequence located on the selected set of magnetic microspheres. Precise optimization of the MOL-PCR assay is crucial and key points in the optimization process were experimentally identified [25]. Since that time, MOL-PCR has been successfully adopted for parallel detection of many human, animal, or even insect pathogens [25–31]. All proposed detection panels show high specificity and sensitivity and are useful for screening a wide spectrum of samples in general.

To address the need of security bodies to be capable of simultaneously detecting the four major biothreat bacteria (*B. anthracis*, *Brucella* spp., *Y. pestis* and *F. tularensis*), a comprehensive and specific protocol for the MOL-PCR suspension array was developed in this study. The scheme of the whole MOL-PCR reaction is figuratively described in the our previous study [25]. The main aim of the study was to construct a panel of detection markers for all listed pathogens using at least two DNA markers (chromosomal markers and virulence genes for each pathogen). The whole multiplex detection panel comprised an internal control (IC) to exclude inhibition of the PCR by impurities potentially present in the sample. All the diagnostic parameters including analytical specificity (inclusivity and exclusivity) and sensitivity (limit of detection (LoD)) were determined and the multiplex panel was validated according to the FDA Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods and Feeds [32] by a ring trial among four laboratories to prove its diagnostic potential in routine practice.

2. Materials and Methods

2.1. Bacterial Strains and DNA Extraction

Bacillus anthracis, *Francisella tularensis* subsp. *tularensis*, *Francisella tularensis* subsp. *holartica*, *Brucella abortus*, *Brucella suis* and *Brucella melitensis* obtained from the Collection of Animal Pathogenic Microorganisms (CAPM) at the Veterinary Research Institute (Brno, Czech Republic) were used for testing inclusivity. *Yersinia pestis* was purchased from the National Collection of Type Cultures—NCTC (Public Health England, Salisbury, United Kingdom) and was also used for inclusivity testing. Other bacterial DNA from *Staphylococcus aureus*, *Listeria monocytogenes*, *Bacillus cereus*, *Yersinia enterocolitica*, *Campylobacter jejuni*, *Salmonella enterica*, *Escherichia coli* O26, *Escherichia coli* O157, *Enterococcus faecalis*, *Enterococcus faecium*, *Vibrio parahaemoliticus*, *Yersinia pseudotuberculosis*, *Clostridium tetani*, and *Clostridium botulinum* were also obtained from the CAPM and other collections and were used for testing exclusivity (Table 1). Genomic DNA (gDNA) was purified using the DNeasy® Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's protocol with a few modifications as described previously [33]. DNA concentrations were determined spectrophotometrically using a NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and diluted in sterile distilled water to the required concentrations, if appropriate. To test exclusivity and long-term storage effect of the ligation mix, DNA was diluted to a concentration of 10 ng/µL.

Table 1. List of bacteria used in this study.

Species	Accession No.
Bacillus anthracis	CAPM 5001
Yersinia pestis	NCTC 5923 ^{T}
Francisella tularensis	CAPM 5600
Brucella abortus	CAPM 5520
Brucella suis	CAPM 6073 ^{T}
Brucella melitensis	CAPM 5659 ^{T}
Staphylococcus aureus	CCM 885 ^{T}
	CAPM 5736
	CAPM 5756
	CAPM 5755
Methicillin-resistant *Staphylococcus aureus*	CAPM 6565
Listeria monocytogenes	CAPM 5580
	CAPM 5879
Bacillus cereus	CAPM 5631
Yersinia enterocolitica	DSM 9499
Yersinia enterocolitica	DSM 13030 ^{T}

Table 1. *Cont.*

Species	Accession No.
Campylobacter jejuni	CAPM 6316
	CAPM 6341
Salmonella enterica	CAPM 5439
Salmonella enterica	CAPM 5445
Salmonella enterica	CAPM 5456
Salmonella enterica	CAPM 6324 ^{T}
Escherichia coli O26	CAPM 5358
Escherichia coli O157	CAPM 6557
Enterococcus faecalis	CAPM 6575
	CAPM 6577
Enterococcus faecium	CAPM 6563
	CAPM 6590
Vibrio parahaemoliticus	CAPM 5939
Yersinia pseudotuberculosis	CAPM 6495
Clostridium tetani	NCTC 5409
Clostridium botulinum	NCTC 3815

Abbreviations: CAPM = Collection of Animal Pathogenic Microorganisms; CCM = Czech Collection of Microorganisms; NCTC = National Collection of Type Cultures; ^{T} = type strain, DSM = DSMZ-German Collection of Microorganisms and Cell Cultures.

2.2. Internal Control

An internal control (IC) was added to each reaction to differentiate a false negative from truly negative results because of the inhibition of the MOL-PCR reaction. The IC was designed as a synthetic sequence based on the joined mitochondrial DNA sequences of two extinct species, Thylacine (*Thylacinus cynocephalus*, GenBank Acc. No. FJ515781.1) and Moa bird (*Dinornis struthoides*, GenBank Acc. No. AY326187.1). This 150 bp control synthetic sequence was synthesized de novo and cloned into a plasmid [34]. The essence of internal control is to be positive in all samples as well as in negative controls without a template—no template controls (NTC). It confirms that all reaction steps have taken place correctly.

2.3. Design of MOLigo Probes (Short Specific Oligonucleotides)

Pathogen-specific sequences (specific genes for detection of bacteria) were selected according to previously published data. The References are listened in Table 2. Most previous work, of which there were specific targets chosen, were based on qPCR method. Sequences of each species were extracted from the National Centre for Biotechnology Information (NCBI) database. For detection of a specific target sequence are designed MOLigo probes. Each pair of MOLigo probes is specific for a particular target sequence, but all MOLigo pairs contain the same sequence for annealing the universal primers (Reverse—Rw and Forward—Fw). One of the MOLigo probes also contains the unique 24 base DNA sequence called an xTAG (from Luminex corporation; https://www.luminexcorp.com/magplex-tag-microspheres/), by which PCR products hybridize to a magnetic microsphere with a covalently linked anti-TAG sequence. The specific parts of MOLigo probe sequences (specific sequence for a given target—part 1 and part 2) were tested with OligoAnalyzer 3.1 tool (https://eu.idtdna.com/calc/analyzer) to identify properties such as melting temperature, hairpins, dimer formation etc., of the individual target sequences used for probe design. Optimal probe sequences were finally checked by NCBI BLAST for possible non-target interactions. A more detailed scheme for designing probes for the MOL-PCR reaction is shown in Figure 1. MOLigo probe synthesis was performed by standard desalination purification (Generi-Biotech, Czech Republic). The final size of the ligation product ranged from 102 to 113 base pairs. All other parameters for MOLigo probe design were used according to the previous study [25].

Table 2. Specific MOLigo pair sequence used for detection with microspheres code and xTAG assignation (bead and xTAG numbers of unique sequences are available in the Luminex catalog).

Pathogen	Marker	Reference	Sequence 5′-3′	Size of LP	xTAG	Bead
Bacillus anthracis	*BA5345*	[35]	PHO-AATTACAAGTATTATTCAGAGAACGT**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTtattagagtttgagaataagtagt**GGTATTTTTTGCTTCAATGGTG	112	A033	033
	pagA	[36]	PHO-CTGTATCAGCGGTATTTAAAC**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTtgagtaagtttgtatgtttaagta**ATCTAATATCGGCATTTAATCTTG	109	A065	034
Yersinia pestis	*pla*	[37]	PHO-TTCTGTTGTTTTGCCTTGACATTCTCC**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTgtgttatagaagttaaatgttaag**CATAATGACGGGGCGCTCA	110	A030	030
	caf1	[38]	PHO-AGGAACCACTAGCACATCT**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTgtaagattagaagttaatgaagaa**CTTACTCTTGGCGGCTATAAAAC	106	A051	051
Francisella tularensis	*23kDA*	[39]	PHO-TGAGATGATAACAAGACAACAG**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTgtaagagtattgaaattagtaaga**AACTAAAAAAAGGAGAATGATTATGAG	113	A066	020
	fopA	[40]	PHO-ACTATCTAGAAATGTTCAAGCAAGTGT**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTagtaagtgttagatagtattgaat**GGGTGGTGGTCTTAAGTTTGA	112	A038	038
Brucella spp.	*omp2a*	[41]	PHO-CAGGCTACGAATCCAGAAA**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTatttgttatgataaatgtgtagtg**CGCACTGAATCTCTGTTTTTC	104	A042	012
	bcsp31	[42]	PHO-TATGCCATTCGCCGCCTGA**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTgtgattgaatagtagattgtttaa**CATTCTTCACATCCAGGAAACCCGAC	109	A046	046
Internal control	aDNA	[34]	Pho-ATTAGCACAATGAATAATCATCG**TCTCACTTCTTACTACCGCG ACTCGTAGGGAATAAACCGTattgtgaaagaaagagaagaaatt**TATACACACGCAATCACCAC	107	A014	036
Forward primer		[43]	**CGCGGTAGTAAGAAGTGAGA**			
Reverse primer			BODIPY-TMRX-**ACTCGTAGGGAATAAACCGT**			

Abbreviations: Pho = phosphorylation; underlined = specific sequence for target gene; bold = universal forward and reverse primer; lowercase = xTAG sequence; LP = ligation product.

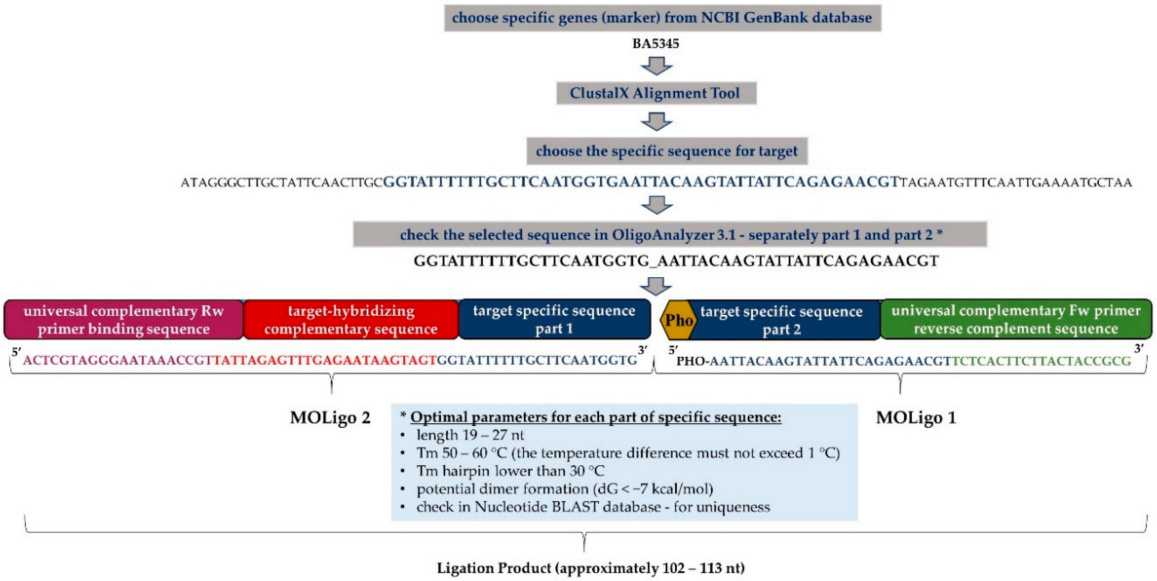

Figure 1. The structure of MOLigo probes demonstrated on a specific marker BA5345 for *Bacillus anthracis*: ClustalX Alignment Tool—part of a software program BioEdit, designed to perform mulTable 3. 1—analytical tool for oligonucleotides identifying their properties; MOLigo1 consists of a specific sequence for selected target (part 2) and a universal complementary forward primer (Fw)-binding sequence, MOLigo1-specific sequence is phosphorylated at its 5′end; MOLigo2 includes a specific sequence for selected target (part 1), target-hybridizing complementary sequence, which is specific for each microsphere and universal complementary reverse primer (Rw)-binding sequence. The final size of the ligation product ranges from 102 to 113 nucleotides (nt). * Optimal parameters for target specific sequences defined according to Deshpande et al. [26].

2.4. MOL-PCR Assay

2.4.1. Coating the Magnetic Microspheres with Anti-xTAG Oligonucleotides

Magplex®—C Microspheres sets (6.5 µm magnetic, carboxylated and internally labeled with two fluorescent dyes for bead identification) were purchased from Luminex Corporation. Each set of microspheres (12.5×10^6 microspheres/mL; Luminex Corp., Texas, USA) was vortexed and 200 µL was collected into a protein low bind DNA tube (Eppendorf, Germany). The tubes were placed on a DynaMag-2 magnetic separator (Thermo Fisher Scientific, Waltham, MA, USA) for approximately 1–2 min and then the supernatant was aspirated. The magnetic bead pellet was suspended in 22 µL of 0.1M MES buffer pH 4.5 (Sigma-Aldrich, St. Louis, MO, USA) and further vortexed and sonicated for 30 s in an ultrasonic cleaning bath (Desen Precision Instruments, Fuzhou, China). Subsequently, 2 µL of 100 µM antiTAG (C6-amino modifications at the 5′-end, specific modification to form a covalent bond with the surface of the microspheres) was added into the appropriate tube with beads and vortexed. After that, 1.25 µL of a freshly prepared 10 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide solution (solved in ultrapure H_2O) (Thermo Fisher Scientific) was added and tubes were incubated in the dark at room temperature for 30 min. This step was performed twice with a fresh preparation of the 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide solution. The tubes were vortexed every 10 min during this time. Afterwards, the beads were washed with 1 mL of 0.02% Tween 20 (Alpha Diagnostic, Texas, USA) and 1 mL of 0.1% SDS (Sigma-Aldrich) and resuspended in 40 µL of TE buffer (pH 8.0; SERVA, Heidelberg, Germany) with vortexing and removal of the supernatant using a magnetic separator after each step. The coated beads were then stored in the dark at 4 °C. To validate bead coating, direct hybridization using xTAG oligonucleotide sequences fluorescently labelled with Bodipy dye (validation TAGs) was performed using

the hybridization protocol described below. The signal intensity using validation tags and coated beads was around 1000–1200 median fluorescent intensity (MFI). Prior to any downstream reaction, the real number of beads was determined by enumeration on a hemocytometer and adjusted to 40,000 beads/µL.

2.4.2. Multiplex Oligonucleotide Ligation

Each multiplex ligation mix included 5 nM individual MOLigo pair probes (Table 2) with 2.5 µL10X Hifi Taq DNA ligase reaction buffer, 0.5 µL Hifi Taq DNA ligase (New England BioLabs, Massachusetts, USA), 0.1 µL of 0.05 ng/µL IC cloned into the plasmid and 2.5 µL template DNA. The reaction was brought to a final volume of 25 µL with H2O. Ligation protocol in the thermocycler (DNA Engine Dyad, Bio-Rad, Foster City, CA, USA) was set to: 10 min of denaturation at 95 °C followed by 20 cycles of 30 s at 95 °C and 1 min at 59 °C. Ligation products were stored at 10 °C until the next step of the MOL-PCR reaction.

2.4.3. Singleplex PCR

Amplification of the ligation products was performed in a final volume of 24 µL which consisted of 12 µL 2X EliZyme HS Robust MIX (Elisabeth Pharmacon, Brno-Židenice, Czech Republic), 0.0625 µM universal FW primer and 0.25 µM BODIPY-TMRX-labelled REV primer (Table 2). Ligation products were added to the already prepared tubes with PCR mix at a ratio of 1:3, i.e., a volume of 6 µL. PCR consisted of initial denaturation at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s.

2.4.4. Hybridization and MAGPIX Analysis

Hybridization of amplified PCR products to microspheres mediated by an xTAG sequence inside the amplified DNA and a complementary antixTAG sequence covalently bound to the surface of the magnetic beads, was performed in a bead mix formed by 1250 beads for each target (Table 2) per 1 sample; 2.5 µL of 800 mM NaCl (VWR, Stříbrná Skalice, Czech Republic) and 0.8 µL of 50 mM β-(N-Morpholino)ethanesulphonic acid (MES) monohydrate free acid ≥99%, ultrapure buffer (VWR) and adjusted to a final volume of 5 µL with 1xTE buffer at pH 8.0 (VWR). About 5 µL of the bead mix was pipetted into 0.2 mL tear-off strips (BIOplastics, Landgraaf, The Netherlands) followed by the addition of 10 µL of the PCR products from the previous step. The prepared strips with bead mix and PCR products were run on the thermocycler with the following protocol: denaturation 96 °C for 90 s, followed by 37 °C for 30 min and hold at 37 °C until further processing. After hybridization was completed, 45 µL of analysis buffer was added (10 mM Tris-Cl (pH 8.0); 0.1 mM EDTA; 90 mM NaCl and 0.2% Tween 20). Strips with hybridization products and analysis buffer were placed on a 0.2 mL Multi Rack bench (Bioplastics) and analyzed in a MAGPIX instrument (Bio-Plex MAGPIX from Bio-Rad) using xPONENT 4.2. ® SOFTWARE (Luminex, Austin, TX, USA).

2.5. Specificity and Sensitivity of MOL-PCR Assay

The specificity of the developed MOL-PCR panel was tested using selected pathogens (Table 1). A concentration gradient (5×; 10×; 40×) of extracted DNA from bacterial strains (*B. anthracis*, *Y. pestis*, *F. tularensis* and *Brucella* spp.) was performed to confirm inclusivity. Concentrations of stock bacterial DNA ranged from 20 ng/µL (*B. anthracis* DNA) to approx. 220 ng/µL (*Y. pestis*, *F. tularensis* and *B. suis* DNA). A ten-fold serial dilution was made from the lowest concentrations (40×) and used to determine sensitivity of the liquid array detection system. The diluted DNA was analyzed in quadruplicate for reproducibility testing. The LoD was determined as the lowest concentration of sample measured at 100 median fluorescence intensity (MFI). The detection limit was determined for each pathogen as well as for the two detection markers. In the evaluation, the appropriate no template control (NTC) was subtracted for the appropriate marker.

2.6. Variability in Preparation of Ligation Mix and Stability during Long-Term Storage

The effect of the ligation mix composition (various MOLigo probes) and the stability during long-term storage was investigated with two independent batches of 100 premixed ligation reactions. In the first batch, ligation buffer and only MOLigo probes 1 were mixed in separate tubes and stored separately (named Ligation premix 1 = LP1); the same was done with all MOLigo probes 2 (LP2). Ligation premix 1 and 2 were mixed together just before the ligation step. Performance of this protocol was compared with all the MOLigo probes mixed and stored together in ligation buffer. Ligase was always kept separated and added to the ligation premixes just before the ligation step. *B. anthracis*, *Y. pestis*, *F. tularensis*, and *B. melitensis* DNA at 10 ng/µL were used as template in the testing and the entire MOL-PCR protocol was performed on days 0, 3, 5, 8, and 10 to investigate the freeze-thawing effect of ligation premixes. The difference in storage during freezing was compared for each of the genes by using a paired t-test, i.e., the MOLigo probes separately (LP1 and LP2) and/or the ligation mix (MIX) prepared together. The analysis was performed using Statistica 13.2 software (StatSoft Inc., Tulsa, OK, USA) and differences with P values < 0.05 were considered as statistically significant.

2.7. Inter-Laboratory Validation of the Biothreat Detection Panel

Master stocks of all chemicals were prepared, aliquoted, and frozen at the Veterinary Research Institute, Brno (VRI). Thereafter, 16 designed MOLigo pairs of probes with 1 pair of IC probes were divided into two tubes, one with MOLigo probes 1 and the second with MOLigo probes 2. Ligase was supplied separately. Pure bacterial DNA was isolated at VRI and mixed with DNA isolated from soil to mimic a natural background. Each laboratory analyzed the concentration gradient of reference DNA—*B. anthracis*, *Y. pestis*, *F. tularensis*, *B. melitensis*, *B. suis*, *B. abortus* (4 samples for each pathogen—stock solution; 5×; 10×; 40×), non-target DNA (Excess) from *S. aureus* at 20 ng/uL concentrations and 4 NTC– water only. The prepared aliquots were shipped on dry ice to partner laboratories at University of Veterinary and Pharmaceutical Sciences, Brno, Military Veterinary Institute, Hlucin and Department of Biological Protection, Techonin. The raw data from testing in all laboratories were exported as Excel files. Each sample was analyzed in biological duplicate and technical duplicate.

2.8. Data Analysis and Interpretation

MFI values obtained from analysis of at least 50 microspheres of each target sequence per sample were used for test evaluations. NTC with no target DNA was used to determine the background MFI value for each microsphere region. The respective NTC MFI value for the microspheres region was subtracted from the measured MFI values. Samples with MFI values higher than 100 compared to background MFI were considered as positive.

3. Results and Discussion

3.1. Biothreat Bacteria Panel Optimization

Specific genes have been selected for four bacteria that can be primarily abused as biological weapons, namely *pagA*, *BA5345* (both *B. anthracis*), *pla*, *caf1* (*Y. pestis*), *23kDA*, *fopA* (*F. tularensis*), and *omp2a* and *bcsp31* (*Brucella* spp.). These specific sequences in bacterial pathogens have been selected according to previous studies based on PCR methods, in particular real-time PCR. Relative references are listed in Table 2. Some of these are chromosomal markers, and other genes are placed on plasmids and cause a virulence of selected pathogen. Because of the gene deletion on the plasmid, two markers were preferably used for detection to confirm more identity. The assembled multiplex MOL-PCR containing all the MOLigos freshly prepared each time were tested with pure reference DNA of *B. anthracis*, *Y. pestis*, *F. tularensis*, and *Brucella* species (Figure 2) and unrelated bacterial species (Figure 3). The difference between positive and NTC samples was demonstrated when the threshold 100 MFI was applied. At the same time, the IC of MOL-PCR reaction was between 600 and 1000 MFI which shows low level of cross-reaction in amplifi-

cation between target and IC. Specificity, particularly inclusivity (Figure 2) and exclusivity (Figure 3), did not show any cross-reactions or false positive or false negative interactions among all four bacterial species (Table 1). A similar detection panel for biothreat bacteria has already been assembled by Deshpande et al. 2010 [26]. Compared to Deshpande et al. study [26], *Brucella* species detection MOLigo probes and IC were added to reveal the reaction inhibition. The inclusion of IC is necessary in multiplexing for detection in diagnostic PCR assays to detect false negative results [44]. Frequently as prevention of false positive results is the most commonly used uracil DNA glycosylase [45]. A huge advantage of this method is its multiplexity and detection of a large number of pathogens in one reaction and therefore it is possible to extend the panel with other pathogens if necessary. Additionally, each bacterium was detected by two independent targets. Based on the previous findings, BODIPY-TMRX fluorescent dye was used for labelling the hybridization product instead of SAPE (time consuming) or Alexa Fluor 532 [25]. This approach enabled the omission of the separate step of PCR product labelling by phycoerythrin since BODIPY-TMRX dye successfully survives PCR amplification. As in most studies, the ligation step was separated from the PCR step to reduce the background signal and to allow better control over each step [27,43]. Compared to our previous study [25], the number of microspheres per sample was reduced to 1250 beads since this amount was sufficient to determine background and sample MFI but at reduced cost per reaction. Time generally plays a huge role in preventing the spread of the disease, epidemic or a biological warfare, so this method seems to be very suitable, as it is able to obtain results within 4 h and detect up to 50 targets and test a large number of samples at one time. All these things are important for the rapid implementation of measures to protect the population. Of course, the qPCR method (reaction time approximately 1.5 h) is most often used as the "gold standard" for the detection of bacteria, but unfortunately this method does not offer such a huge possibility of multiplexity in the same time.

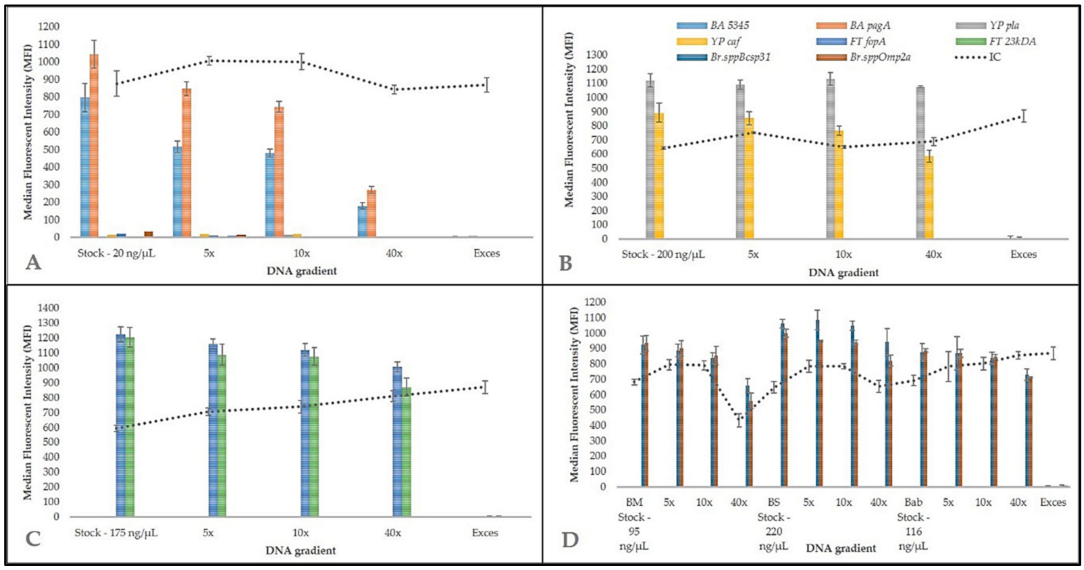

Figure 2. Biothreat detection panel. MOL-PCR biothreat assay for detection of (**A**) B. *anthracis*, (**B**) Y. *pestis*, (**C**) F. *tularensis*, and (**D**) *Brucella* spp. The biothreat panel was verified using DNA gra-dients from the collection of pathogenic microorganisms (CAPM) and evaluated by MFI after sub-tracting the NTC (H2O only) to the appropriate target. IC confirms that the individual reaction steps have taken place correctly. Exces (DNA from S. *aureus*) was used for confirmation as another negative control.

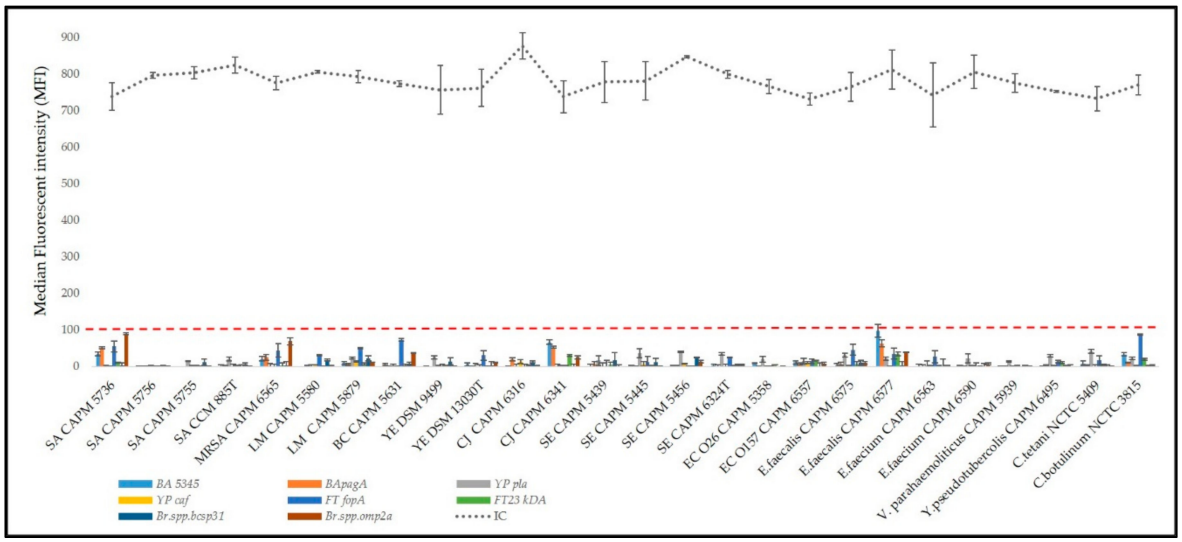

Figure 3. Exclusivity of biothreat detection panel based on MOL-PCR method. The exclusivity test was performed on various bacterial strains (Table 1) and evaluated as MFI. The specificity of the probes used in the biothreat panel was confirmed by the values obtained being below 100 MFI when using unrelated DNA. An IC confirmed the correct course of the MOL-PCR reaction.

3.2. Sensitivity Test of the MOL-PCR Reaction

MOL-PCR sensitivity was determined using ten-fold serial dilution of *B. anthracis*, *Y. pestis*, *F. tularensis*, and *B. melitensis* DNA (Figure 4). LoD for *B. anthracis* was 0.5 ng/µL for *BA5345* or 0.25–0.05 ng/µL for *pagA*. In the case of *Y. pestis*, *pla* target sequence could be detected at 0.0005 ng/µL and detection limit for *caf* ranged between 0.5 and 0.05 ng/µL. In the case of *F. tularensis*, *fopA* could be detected at 0.44–0.044 ng/µL and 23*kDA* had a detection limit of 2.2 to 0.44 ng/µL. In the case of the *Brucella melitensis*, the LoD was determined to be 0.29–0.0029 ng/µL for both target sequences (Table 3). In other studies, the LoD was determined for the Mycobacterial complex based on MOL-PCR and the detection limit was around 0.1 ng/µL. In another study, using a 13-plex for *B. anthracis* SNP typing, the LoD was 2 ng genomic DNA [27–30]. In conclusion, the biothreat panel reached a detection limit of at least 0.5 ng/uL for all pathogens and targets which corresponds with similar studies based on MOL-PCR methods. The results obtained after the evaluation of the limit of detection are that MOL-PCR method has a lower sensitivity than detection methods based on the use of DNA, thus qPCR achieves very low sensitivity. However, the MOL-PCR method is mainly a screening and semi-quantitative method, where it is very important to detect different types of pathogenic bacteria during one laboratory examination.

Table 3. Measured limit of detection (LoD) values for the MOL-PCR method using established detection panel for the biothreat bacteria.

Pathogen	Gene (Target)	LoD (ng/µL)
B. anthracis	*5345*	>0.5
	pagA	0.25–0.05
Y. pestis	*pla*	<0.005
	caf	0.5–0.05
F. tularensis	*fopA*	0.44–0.044
	23kDA	2.2–0.44
B. melitensis	*bcsp31*	0.24–0.024
	omp2a	0.24–0.024

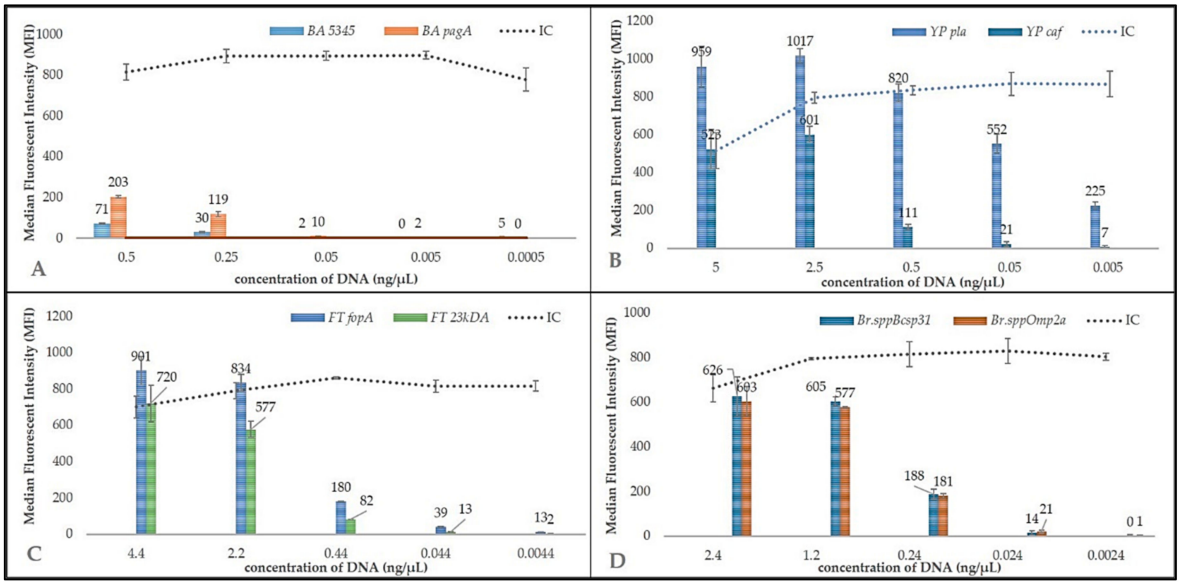

Figure 4. Sensitivity test.—The detection limit of the MOL-PCR system was performed in the form of a concentration gradient of isolated DNA of all bacteria and for two targets, namely: (**A**) *B. anthracis*, (**B**) *Y. pestis*, (**C**) *F. tularensis*, (**D**) *B. melitensis.*

3.3. Long-Term Storage Effect of Ligation Mix

In a previous study, the effect of mixing and storing the ligation premix (MOLigo probes) was described [46]. There were up to 40 probe pairs divided into three premixes of which each premix contained 8, 5, and 3 pairs and the remaining 8 pairs were added prior to ligation mixture preparation. Based on these results, the impact of storage on background signals (MFI measured in the NTC) was confirmed [46]. For this reason, we performed two experiments (Figure 5) to compare the freezing effect of different MOLigo mix variants. In the first experiment, all MOLigo pair probes were frozen together with the ligation buffer. In the second experiment, MOLigo 1 probe and MOLigo 2 probe premixes were frozen separately. Immediately before use, premixes 1 and 2 were mixed together and ligase was added. From the point of view of stability, the separation of MOLigo probes (LP1 + LP2) resulted in better performance because none of the genes showed an increasing or decreasing trend in NTC MFI values during storage (Stability over time—trend). In contrast, the MOLigo mix method showed an increasing trend ($p < 0.05$; significance of slope of regression line) of NTC MFI values during storage of the three genes (BA *pagA*, YP *caf*, and BMspp *omp2a*). For seven of the eight genes, the MFI values for the mixed probes protocol were higher than for the LP1 + LP2 method, except for the FT *23kDA* gene. The average differences between the two protocols were statistically significant in six cases ($p < 0.05$ at least). Only the BA *pagA* and BMspp *bcsp31* genes did not show a statistically significant difference between LP1 + LP2 and mixed probes protocols (Figure 5). Our suggestion is that some undesirable cross-interactions between individual probes may occur. We therefore recommend to freeze the probe mix MOLigo 1 and MOLigo 2 separately.

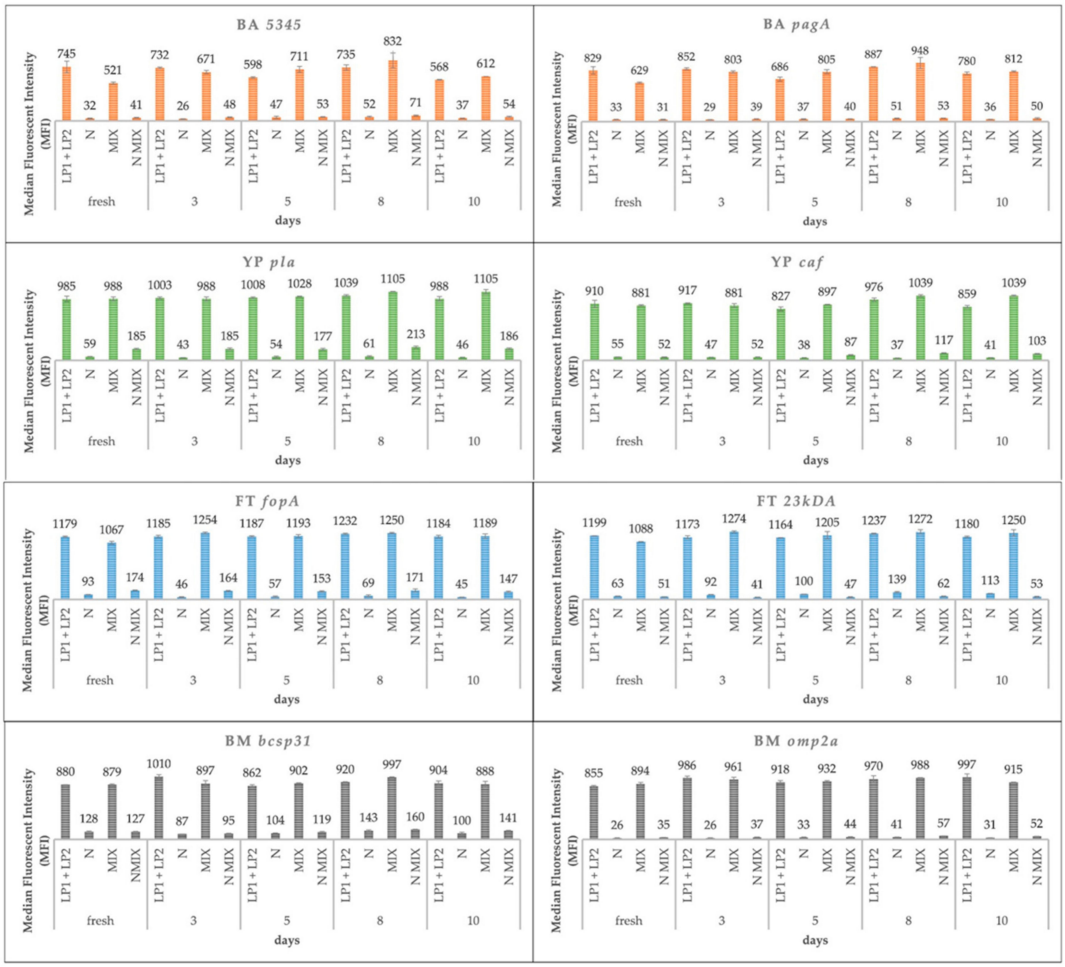

Figure 5. Long-term storage effect of ligation mix.—Experiment based on evaluating the stability of different types of ligation premix storage over time (MOLigo probes separately LP1 + LP2 or all MOLigo probes together—MIX). Better protocol performance was recorded when the two probes premix were stored separately. Abbreviations: LP = ligation premix; N = Negative control; MIX = mix of all probes in the ligation buffer.

3.4. Inter-Laboratory Validation of Biothreat Detection Panel

Inter-laboratory tests were performed to evaluate the reproducibility and robustness of the MOL-PCR biothreat panel in routine settings. Although reproducibility is defined as a quantitative parameter, it is possible to use it as a qualitative measure in validation of qPCR assays [44]. The results among all four laboratories showed agreement with one exception of one negative technical replicate of 40 × diluted sample analyzed at Military Veterinary Institute (Figure 2; Supplementary Figures S1–S3), however, the final interpretation of the results showed 100% agreement. Internal control (IC) was correct in all reactions and across all departments. The same applies to no template control (NTC) and the use of unrelated DNA (Exces) to evaluate the specificity of the probes. It was shown that the validation criteria postulated for qPCR assays by Food and Drug Administration (FDA) are applicable on MOL-PCR as well [47].

3.5. On-Site Usability of the Panel

To simplify the feasibility of the detection tool described here, its future conversion into a microfluidic chip or disposable cassette format is possible. A similar approach has already been introduced by Luminex corporation—ARIES®. This modification could not only simplify the analysis for point of care testing, but also significantly reduce the material costs and the possibility of technical error [48,49].

In conclusion, the presented protocol for the detection of four biothreat bacteria was developed and validated in this study. It connects the versatility of the MOL-PCR principle, single-tube detection of all bacteria, and increased sensitivity of detection/identification of each bacterium by at least two DNA targets. In addition, the IC of the process was included to exclude false negative results. The performance of the MOL-PCR assay was validated by a ring trial in four independent laboratories and the results showed that this assay can be implemented as a routine diagnostic for the most dangerous bacteria that could be misused as biological weapons. Of course, it is possible to enlarge the panel with detection targets for various other pathogens that could be misused as bioterrorist weapons. This method can be finished under 4 h, mainly due to its multiplexity and thus the detection (screening) of multiple amounts of pathogens in one reaction at the same time, which is indispensable in preventing bioterrorist action.

Supplementary Materials: The following are available online at https://www.mdpi.com/2076-2607/9/1/38/s1. Figure S1: Biothreat panel validated in University of Veterinary and Pharmaceutical Sciences Brno—collaborative validation of established biothreat detection panel based on MOL-PCR reaction. A concentration gradient of DNA isolated from biothreat bacteria (**A**) *B. anthracis*, (**B**) *Y. pestis*, (**C**) *F. tularensis*, **(D)** *B. melitensis*, *B. suis* and *B. abortus* was used to validate, verify, and to test the robustness of the method. IC confirmed the correct course of the whole reaction. The exclusivity of the detection probes was confirmed by unrelated DNA (*S. aureus*). Panel was evaluated by MFI after subtracting the NTC (H_2O only) to the appropriate target. Figure S2: Biothreat panel validated in Centre of Biological Defence Techonin—collaborative validation of established biothreat detection panel based on MOL-PCR reaction. A concentration gradient of DNA isolated from biothreat bacteria (**A**) *B. anthracis*, (**B**) *Y. pestis*, (**C**) *F. tularensis*, (**D**) *B. melitensis*, *B. suis* and *B. abortus* was used to validate, verify, and to test the robustness of the method. IC confirmed the correct course of the whole reaction. The exclusivity of the detection probes was confirmed by unrelated DNA (*S. aureus*). Panel was evaluated by MFI after subtracting the NTC (H_2O only) to the appropriate target. Figure S3: Biothreat panel validated in Military Veterinary Institute Hlucin—collaborative validation of established biothreat detection panel based on MOL-PCR reaction. A concentration gradient of DNA isolated from biothreat bacteria (**A**) *B. anthracis*, (**B**) *Y. pestis*, (**C**) *F. tularensis*, (**D**) *B. melitensis*, *B. suis* and *B. abortus* was used to validate, verify and to test the robustness of the method. IC confirmed the correct course of the whole reaction. The exclusivity of the detection probes was confirmed by unrelated DNA (*S. aureus*). Panel was evaluated by MFI after subtracting the NTC (H_2O only) to the appropriate target.

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MOL-PCR and xMAP Technology

A Multiplex System for Fast Detection of Food- and Waterborne Viruses

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Viruses are common causes of food- and waterborne diseases worldwide. Conventional identification of these agents is based on cultivation, antigen detection, electron microscopy, or real-time PCR. Because recent technological advancements in detection methods are focused on fast and robust analysis, a rapid multiplexing technology, which can detect a broad spectrum of pathogenic viruses connected to food or water contamination, was utilized. A new semiquantitative magnetic bead–based multiplex system has been designed for simultaneous detection of several targets in one reaction. The system includes adenoviruses 40/41 (AdV), rotavirus A (RVA), norovirus (NoV), hepatitis E virus (HEV), hepatitis A virus (HAV), and a target for external control of the system. To evaluate the detection system, interlaboratory ring tests were performed in four independent laboratories. Analytical specificity of the tool was tested on a cohort of pathogenic agents and biological samples with quantitative PCR as a reference method. Limit of detection (analytical sensitivity) of 5×10^0 (AdV, HEV, and RVA) and 5×10^1 (HAV and NoV) genome equivalents per reaction was reached. This robust, senstivie, and rapid multiplexing technology may be used to routinely monitor and manage viruses in food and water to prevent food and waterborne diseases. *(J Mol Diagn 2021, 23: 765–776; https://doi.org/10.1016/j.jmoldx.2021.03.005)*

Gastrointestinal diseases of various origin are still among the leading causes of morbidity and mortality worldwide with almost 5 billion episodes and more than 1.6 million deaths a year.[1,2] The greatest impact of these diseases can be observed in developing countries.[3–5] As for developed countries, the mortality level is significantly lower, but the economic impacts associated with morbidity are substantial. Significant costs are also associated with safety measures taken to mitigate the impact of possible infection on the general population. The causative agents can be viruses or nonviral agents (bacteria, parasites). In the case of viruses, a more serious impact on public health is seen.[6–8] Enteric and hepatic viruses can be transmitted through contaminated food and water, and cause disease outbreaks. The potential for these diseases to spread in today's globalized world is measureless.[9,10] Nevertheless, the microbiological monitoring of safety of food and water resources is currently still focused primarily on bacterial contamination rather than on viruses. Therefore, despite increasing attention to food safety, food- or waterborne viruses cause hundreds of thousands deaths every year. The most vulnerable populations are the most severely hit, namely small children, the elderly, and immunocompromised individuals.[11] Determining the causative agent of an outbreak can be quite demanding due to similar clinical signs and symptoms between affected individuals. In addition, standard

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diagnostic tests, which are selected and sequentially performed according to presumed etiology, can take several days. Individual methods also have their limitations, such as different sensitivity, specificity, and processing times.[8,12] All of these can negatively affect the further spread of the causative agent and possible patient treatment options. Appropriate and timely therapy can shorten illness and reduce morbidity, and in some cases, can even be life-saving.[12] Furthermore, performing individual analyses for the detection of given pathogens can be very costly.

The most commonly mentioned pathogenic viruses in food and water contamination include norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), adenoviruses 40/41 (AdV), and rotaviruses (RV).[13] The detection of these viruses can be quite complicated. Cell cultures have significant limitations (time- and work-intensive, noncultivable viruses), as well as insufficiently sensitive electron microscopy or immunological methods.[9] Antibody tests can be time consuming. The gold standard in molecular diagnostics for detection of food- or waterborne viruses is quantitative PCR (qPCR) or RT-qPCR.[14–16] Regularly updated International Organization for Standardization methods for the detection and quantification of HAV and NoV from various food sources have been released.[17,18] qPCR and methods derived from them are able to specifically and sensitively detect the viral genomes of interest. Nevertheless, these methods also have their disadvantages, namely the limited possibility of multiplexing. There are not enough fluorescent acquisition channels/fluorescent reporter dyes to create sufficiently robust systems. Typically, only three to four targets (sequences) are detected at a time in a single reaction. In addition, the sensitivity of the method usually decreases when creating multiplex qPCR systems (interference between individual sets of primers, off-target interactions, reagent depletion). This may be a problem in diagnosis of some low–infectious-dose pathogens.[9,10,19]

Based on this knowledge, our aim was to overcome these limitations and improve the detection potential of food- and waterborne viruses by nucleic acid–based molecular techniques. A combination of multiplex oligonucleotide ligation (MOL) with PCR and xMAP technology (Luminex Corporation, Austin, TX) was employed to create a detection system comprising the most common viral agents responsible for gastrointestinal problems in humans. The system was designed to be used in routine diagnostics (analysis of human, animal, environmental, and food samples); therefore, basic optimization and validation parameters were determined as well.

Principle of Combination of MOL-PCR with xMAP Technology

The MOL-PCR technique combines ligation of multiple pairs of molecular probes (MOLigos) in the presence of target DNA molecules with amplification of ligated products by singleplex PCR using universal primers.[20–24] MOL-PCR is able to work only with DNA templates, therefore, in the case of RNA viruses, a preceding step of reverse transcription is required. Because it is usually not possible to determine in advance whether DNA or RNA viruses are present in the analyzed sample, all samples undergo reverse transcription before MOL-PCR (RT-MOL-PCR). Ligation experiments were also performed directly with the RNA templates and appropriate ligation enzymes. However, these tests proved to be unsuccessful (unpublished data).

xMAP technology (Luminex Corporation) uses advanced fluidics, optics, and digital signal processing in combination with color-coded microsphere technology to provide new solutions for multiplexed assays, reducing time, labor, and unnecessary costs.

The key step of the protocol presented in this study (Figure 1) is the ligation reaction. It is the phase of actual detection of the potentially present template (DNA/cDNA). Specific pairs of probes are linked together only in the presence of detected target. One probe (MOLigo-1) contains a section complementary to the target sequence and the reverse-complement sequence of the universal forward primer sequence. The 5′-end of this MOLigo-1 is also phosphorylated to enable ligation. The second probe (MOLigo-2) has three sections: a sequence complementary to the target, a specific TAG sequence, and a sequence of a universal reverse primer. The TAG sequence (xTAG; Luminex Corporation) is a 24-nucleotide–long synthetic sequence containing a combination of only three bases (T, A, and G). When ligation occurs, the MOLigo-1 and MOLigo-2 are covalently linked together to form a complete sequence with a length of about 100 to 120 nucleotides. This sequence can serve as a template for the following singleplex PCR, through which the ligation product is amplified. One of the universal primers used for this PCR is labeled by fluorescent dye; amplicons thus carry a fluorescent signal, which is detected in the final step of the analysis by hybridization to magnetic microspheres and using a MAGPIX instrument (Luminex Corporation). Each set of these microspheres is composed of a unique content of two red dyes (red and infrared), thus giving them a specific spectral profile. Their surface is covered with short, specific 24-mer oligonucleotides, the anti-TAGs (Luminex Corporation). Binding of the amplicons to the beads is mediated by the above-mentioned TAG sequences, which are complementary to the anti-TAG sequences. Using the MAGPIX device, the beads with bound amplicons are immobilized on a magnet, and two diodes (two lasers with different wavelengths) read the signals to identify a particular set of microspheres and read the fluorescent intensity of bound reporter molecules. The open architecture of this system allows the creation of detection tools with the ability to specify up to 50 different targets (MAGPIX limitation of region numbers for magnetic microspheres) in one sample/reaction.[23–25]

The amplified products are similar in length due to the use of universal primers. Therefore, there is no inteference due to template preference within the reaction.[26,27] This method

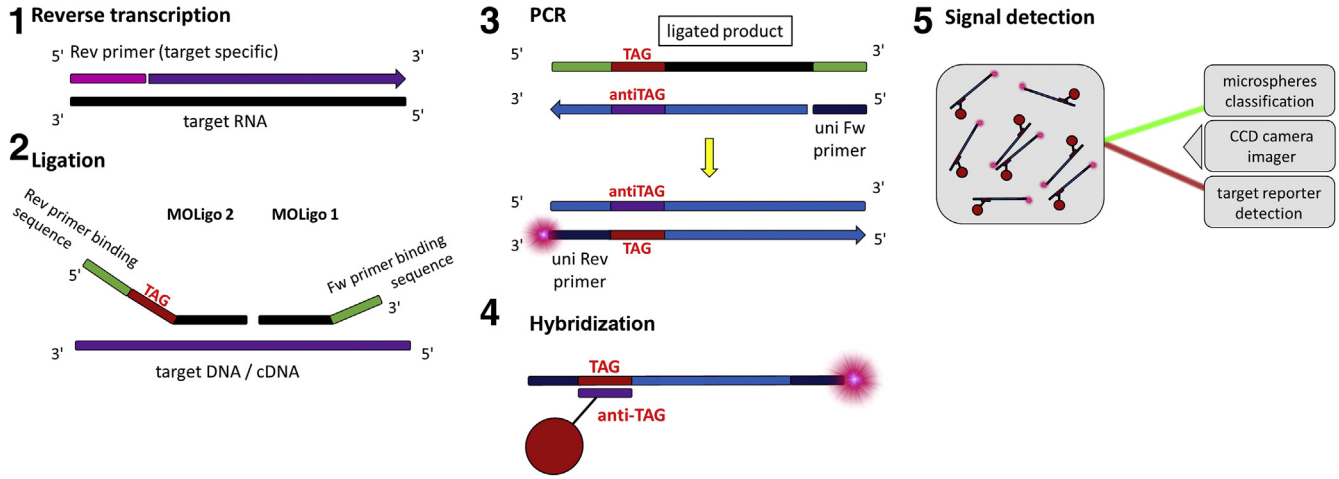

Figure 1 Flow chart of the analysis. 1. Reverse transcription: conversion of RNA targets into cDNA using specific primers. 2. Ligation: specific probes (MOLigo 1 and MOLigo 2) are linked together in the presence of target DNA/cDNA. 3. PCR: amplification of the ligation products by universal primers. 4. Hybridization: hybridization of the amplified products to magnetic microspheres. 5. Signal detection: classification of the magnetic microspheres and reading the signal of bound fluorescent products. CCD, charge-coupled device; Rev primer, target-specific reverse primer; uni Fw primer, universal forward primer; uni Rev primer, fluorescently labeled universal reverse primer.

is superior to other xMAP methods that rely on detection during the PCR step. These PCR-based methods include oligo ligation assays,[28,29] primer extension techniques,[30,31] target-specific PCR sequence detection assays,[32,33] and direct hybridization methods.[34,35] Though each of these approaches uses slightly different molecular mechanisms, initial template amplification by PCR is a shared step in all methods, which limits thier utility.

Materials and Methods

Viruses Targeted and Origin of the Samples

The multiplex MOL-PCR system for detection of viruses connected to contaminated food and water was developed for the simultaneous detection of adenoviruses 40/41 (AdV), rotavirus A (RVA), noroviruses [genogroup I (NoV GI); genogroup II (NoV GII)], hepatitis E virus [genotype 1 (HEV-1); genotype 3 (HEV-3)], and hepatitis A virus (HAV). The viruses used in this study for optimization and verification of the system were obtained either as cell culture products from the Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute (CAPM, Czech Republic) or as reference materials provided by Public Health England and by Paul-Ehrlich-Institut (a WHO Collaborating Center, Germany) (Table 1). The detection reliability for each target was further verified on real biological samples (human, animal, and environmental origin). These were human stool and serum samples of patients with hepatitis A, hepatitis E, or gastrointestinal diseases, and informed consent was obtained from all patients. Animal feces, livers, sera, and bile originated from wild boar (*Sus scrofa*) from hunting grounds situated in different locations of the Czech Republic. Samples of drinking and service water were associated with possible waterborne outbreaks of hepatitis or gastrointestinal diseases. All material was delivered shortly after sampling and processed without delay. All samples were provided through collaboration with hospitals and institutions in the Czech Republic.

The nucleic acid of viruses from CAPM (cell cultures) and reference materials was isolated using the NucliSENS miniMAG system (BioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions. All reference materials were stored and processed in compliance with the provider's instructions. Fecal, serum, liver, or bile samples were prepared according to Vasickova et al.[36] Briefly, 250 mg of stool/fecal content was suspended in 2.25 mL of phosphate-buffered saline, homogenized by vortexing, and then clarified by centrifugation at 3,000 × *g* and 4°C for 15 minutes. Nondiluted bile samples were clarified by centrifugation at 1,500 × *g* and 4°C for 10 minutes. Subsequently, 140 μL of supernatant (feces/bile samples) and nondiluted serum samples were taken and used for nucleic acid isolation with the QIAamp Viral RNA kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. Nucleic acid from liver tissue was extracted using the RNeasy mini kit (Qiagen). Fifty milligrams of liver tissue was homogenized mechanically with 1.2 mL of RLT buffer containing β-mercaptoethanol and 1-mm zirconia beads (BioSpec Products, Bartlesville, OK) at 6400 rpm for 40 seconds in a MagnaLyser instrument (Roche Diagnostics, Basel, Switzerland). Homogenized samples were then centrifuged at 13,100 × g for 3 minutes, and the supernatant was mixed with an adequate amount of 70% ethanol (1:1). Subsequently, 1200 μL of the mixture was applied to the spin column, and the manufacturer's protocol followed. Water samples were processed as previously described.[37] Briefly, water samples (10 L) with pH lowered to 3.5 were passed through negatively charged filters (cellulose nitrate HA Millipore membrane, 0.45-μm pore size;

Table 1 Viruses in This Study

Classification	Viruses and abbreviations	Samples origin (collection)
+ssRNA viruses > *Picornavirales* > *Picornaviridae* > *Hepatovirus*	Hepatitis A virus, HAV	Hepatitis A virus, ATCC VR-1402, strain HM175/18f (CAPM) Hepatitis A virus RM000HAV (PHE)
+ssRNA viruses > *Hepeviridae* > *Orthohepevirus*	Hepatitis E virus genotype 1, HEV-1	1st WHO International Reference Panel for HEV Genotypes for Nucleic Acid Amplification Technique (NAT)-Based Assays (PEI)
+ssRNA viruses > *Hepeviridae* > *Orthohepevirus*	Hepatitis E virus genotype 3, HEV-3	
+ssRNA viruses > *Caliciviridae* > *Norovirus*	Norovirus genogroup GI, NoV GI	Norovirus GI RMNOROGI (PHE)
+ssRNA viruses > *Caliciviridae* > *Norovirus*	Norovirus genogroup GII, NoV GII	Norovirus GII RMNOROG2 (PHE)
dsDNA viruses > *Adenoviridae* > *Mastadenovirus*	Human adenovirus F type 40, AdV40	Human adenovirus 40, CAPM V-662 (CAPM)
dsDNA viruses > *Adenoviridae* > *Mastadenovirus*	Human adenovirus F type 41, AdV41	Human adenovirus 41, CAPM V-661 (CAPM)
dsRNA viruses > *Reoviridae* > *Sedoreovirinae* > *Rotavirus* > *Rotavirus A*	Rotavirus A, RVA	Rotavirus A, CAPM V-177, strain Lincoln (CAPM) Rotavirus A, CAPM V-334, strain OSU 6 (CAPM)

CAPM, Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute (Czech Republic); dsRNA, double-stranded RNA; dsDNA, double-stranded DNA; PEI, Paul-Ehrlich-Institut (Germany); PHE, Public Health England (UK); +ssRNA, positive-sense, single-stranded RNA.

Merck, Darmstadt, Germany). Elution of viral particles by beef extract solution (3% w/v; pH 9.5) in glycine buffer (0.05 mol/L) followed. The eluate was then flocculated by lowering the pH to 3.5 and centrifuged at 7,500 × *g* and 4°C for 30 minutes. The pellet was finally dissolved in phosphate-buffered saline, and nucleic acid was isolated using the NucliSENS miniMAG system (BioMerieux).

Viral Load Quantification by RT-qPCR/qPCR

Because qPCR was considered the reference method, the number of genome equivalents of targeted viruses in isolated nucleic acid used for optimization, verification, and validation was determined by standardized RT-qPCR/qPCR systems. HAV, NoV GI, and NoV GII RNA was detected using a one-step duplex RT-qPCR assay according to the International Organization for Standardization report ISO/TS 15216-2.[38] Adenovirus was detected as previously described by Wong et al.[39] HEV RNA was detected using triplex RT-qPCR with two sets of oligonucleotides targeting two different loci of the HEV genome as previously described.[36] RVA was detected using the commercially available Rotavirus A genesig Detection Kit (PrimerDesign, Chandler's Ford, UK). To ensure the quality, validity, and reliability of the results, all RT-qPCR/qPCR samples were always performed in duplicates, and an internal amplification control according to the instructions of the respective above-mentioned systems was incorporated.

All primers for noncommercial RT-qPCR/qPCR systems were synthesized with standard desalting purification by Generi-Biotech (Hradec Králové, Czech Republic). TaqMan probes were provided by Eurofins Genomics (Luxembourg City, Luxembourg). Amplification and fluorescence was analyzed on a LightCycler 480 (Roche Diagnostics); the results were analyzed using the Fitpoint analysis option of the LightCycler 480 software version 1.5.0.39. The targeted viruses were absolutely quantified on the basis of a calibration curve derived from a 10-fold diluted *in vitro* transcript (RNA: NoV, HAV, HEV, and RVA) or plasmid DNA (AdV).[36]

External Control of the Analysis

The presented detection assay uses an external control to verify the correct course of the analysis. Artificially prepared virus-like particles based on the bacteriophage MS2 served as a control.[40] The virus-like particles contain unique mitochondrial sequences of two extinct organisms: thylacine (*Thylacinus cynocephalus*) and giant moa (*Dinornis struthoides*) in RNA form. All samples in this study were artificially contaminated with the virus-like particle suspension prior to processing (5×10^6 virus-like particle genome equivalents).

MOLigos and Primers Design

MOLigos and primers for RT used in this study (Table 2) were designed from conserved regions of viral genomes to meet optimal conditions in accordance with Reslova et al.[20] Parameters (melting temperature, GC content, hairpin and dimer formation) for these were calculated using the OligoAnalyzer Tool (Integrated DNA Technologies, Coralville, IA). After setting the parameters, BLAST (Basic Local Alignment Search Tool) analyses were performed using the National Center for Biotechnology Information database to confirm the specificity and homology of the MOLigos and primers. All of them were thus *in silico* tested against all available records in the National Center for Biotechnology

Table 2 MOLigos and Primers

Oligo	xTAG	Sequence	Length
HAV_RP		5′-GTCCCCAATTTAGACTCCTACAGCT-3′	25 nt
HEV_G1_RP		5′-CACAAGCAAATAAACTATAACTCCCG-3′	26 nt
HEV_G3_RP		5′-AAGGGGTTGGTTGGATGAAT-3′	20 nt
NoV_GI_RP		5′-GTCYTTAGACGCCATCRTC-3′	19 nt
NoV_GII_RP		5′-TCAYTCGACGCCATCTTCAC-3′	20 nt
RVA_RP		5′-AACTGCAGCTTCAAAAGAAGAG-3′	22 nt
EAC_RP		5′-GGTTTAGAATGTTTTCTCCCGT-3′	22 nt
HAV_MOLigo_1		PHO-5′-ATGGAGCTGTAGGAGTCTAATCTCACTTCTTACTACCGCG-3′	40 nt
HAV_MOLigo_2	A033	5′-ACTCGTAGGGAATAAACCGTtattagagtttgagaataagtagtGGGTCAACTC CATGATTAGC-3′	64 nt
HEV_G1_MOLigo_1		PHO-5′-GTGGGTAAAACTCGGGATCTCACTTCTTACTACCGCG-3′	37 nt
HEV_G1_MOLigo_2	A037	5′-ACTCGTAGGGAATAAACCGTtgtatatgttaatgagatgttgtaCAGCGCCTTA AGATGAAG-3′	62 nt
HEV_G3_MOLigo_1		PHO-5′-ATTCATCCAACCAACCCCTTTCTCACTTCTTACTACCGCG-3′	40 nt
HEV_G3_MOLigo_2	A036	5′-ACTCGTAGGGAATAAACCGTttgtgtagttaagagttgtttaatCTTCGCCCT CCCCTAT-3′	60 nt
NoV_GI_MOLigo_1		PHO-5′-GAYGATGGCGTCTAARGACTCTCACTTCTTACTACCGCG-3′	21 nt
NoV_GI_MOLigo_2	A038	5′-ACTCGTAGGGAATAAACCGTagtaagtgttagatagtattgaatGCRATCTYY TSCCCGAWTWYGTRAAT-3′	70 nt
NoV_GII_MOLigo_1		PHO-5′-ATCGCAATCTKGCYCCCRTCTCACTTCTTACTACCGCG-3′	38 nt
NoV_GII_MOLigo_2	A048	5′-ACTCGTAGGGAATAAACCGTtatgaatgttattgtgtgttgattTVAGCACDT GGGAGGGCG-3′	62 nt
AdV_40/41_MOLigo_1		PHO-5′-GCCAGCACBTWCTTTGACATTCTCACTTCTTACTACCGCG-3′	40 nt
AdV_40/41_MOLigo_2	A043	5′-ACTCGTAGGGAATAAACCGTaaataagaatagagagagaaagttGACAACMGR GTGYTRGAYATG-3′	65 nt
RVA_MOLigo_1		PHO-5′-CTCAAGATGGAGTCTACGCAACATCTCACTTCTTACTACCGCG-3′	43 nt
RVA_MOLigo_2	A028	5′-ACTCGTAGGGAATAAACCGTgatagatttagaatgaattaagtgATTTAATGCT TTTCAGTGGTTGATG-3′	69 nt
EAC_MOLigo_1		PHO-5′-ATTAGCACAATGAATAATCATCGTCTCACTTCTTACTACCGCG-3′	43 nt
EAC_MOLigo_2	A014	5′-ACTCGTAGGGAATAAACCGTattgtgaaagaaagagaagaaattTATACACA CGCAATCACCAC-3′	64 nt
Universal forward primer		5′-**CGCGGTAGTAAGAAGTGAGA**-3′	20 nt
Universal reverse primer		*5′-*ACTCGTAGGGAATAAACCGT*-3′	20 nt

Target-hybridizing complementary sequence is underlined, universal forward primer in bold, universal reverse primer in italics, and xTAG sequence is differentiated by lowercase.

*Fluorescent label.

AdV_40/41, adenovirus 40/41; EAC, external control; HAV, hepatitis A virus; HEV_G1, hepatitis E virus genotype 1; HEV_G3, hepatitis E virus genotype 3; NoV_GI, norovirus genogroup I; NoV_GII, norovirus genogroup II; nt, nucleotide; PHO, phosphorylation; RP, reverse transcription primer; RVA, rotavirus A.

Information's BLAST program to find possible interactions with nontarget sequences. MOLigo probes and primers were synthesized by Generi-Biotech with a standard desalting purification method.

Both universal primers for singleplex PCR were adopted from Thierry et al.[21] The forward primer without labeling was synthesized by Generi-Biotech, the labeled (BODIPY-TMRX) reverse primer was provided by Eurofins Genomics.

Reverse Transcription

Because the majority of targets of the system have an RNA genome, reverse transcription with specific primers had to be performed prior to the MOL-PCR. The reaction mix included PrimeScript Reverse Transcriptase (Moloney Murine Leukemia Virus; Takara, Kyoto, Japan), PrimeScript reaction buffer (Takara), RNase Inhibitor (Human Placenta; New England Biolabs, Ipswich, MA), dNTP PCR Mix (Serva, Heidelberg, Germany), and Ultra-PCR water (Top-Bio, Prague, Czech Republic). The composition of the reaction mixture corresponds to the manufacturer's recommendations for the standard protocol with PrimeScript Reverse Transcriptase.

All experimental schemes were always performed at least in duplicate and included no-template controls (negative control) to monitor possible cross-reactions or contamination. The signal values for these controls were also used as the basis for making a call for target positivity or negativity.

Table 3 Sensitivity of the Assay on the Concentration Series of Genome Equivalents in Water

Number of GE per reaction (quantification by qPCR)	Number detected (positive samples/analyzed samples)						
	HAV	HEV-1	HEV-3	NoV GI	NoV GII	AdV	RVA
5×10^5	8/8	8/8	8/8	8/8	8/8	8/8	8/8
5×10^4	8/8	8/8	8/8	8/8	8/8	8/8	8/8
5×10^3	8/8	8/8	8/8	8/8	8/8	8/8	8/8
5×10^2	8/8	8/8	8/8	8/8	8/8	8/8	8/8
5×10^1	8/8	8/8	8/8	8/8	8/8	8/8	8/8
5×10^0	6/8	8/8	8/8	0/8	4/8	8/8	8/8

AdV, adenovirus 40/41; GE, genome equivalents; HAV, hepatitis A virus; HEV-1, hepatitis E virus genotype 1; HEV-3, hepatitis E virus genotype 3; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II; RVA, rotavirus A.

RT products were immediately used for MOL or stored at 4°C to be used within a day. Prolonged storage or freezing is feasible; however, it can lead to an undesired detection signal drop, thus affecting the sensitivity of the analysis.

MOL

The ligation reaction is the step leading to actual detection. The ligation step was physically separated from the following singleplex PCR to avoid interference between reaction mixtures.[21,24,27] To find the proper functional pair of MOLigos for each target, all reaction mixtures were initially prepared in singleplex. Only one pair for one target and one DNA/cDNA template was tested. After this selection, all functional pairs were gradually mixed together into one ligation reaction (eight different pairs of MOLigos: HAV, HEV-1, HEV-3, NoV GI, NoV GII, AdV, RVA, and external control). If an undesirable interaction occurred between individual systems, optimization, redesign, elimination, or replacement of individual targets followed.

As previously described,[20] the optimized ligation reaction combines 5 nmol/L of each MOLigo probe with 2.5 μL of 10× Hifi *Taq* DNA ligase reaction buffer, 0.5 μL of Hifi *Taq* DNA ligase (New England BioLabs), and 2.5 μL of template DNA/cDNA from reverse transcription.[20] With the addition of PCR H_2O (Top-Bio), the reaction volume was increased to 25 μL. The thermal cycling protocol (DNA Engine Dyad; Bio-Rad, Hercules, CA) included initial

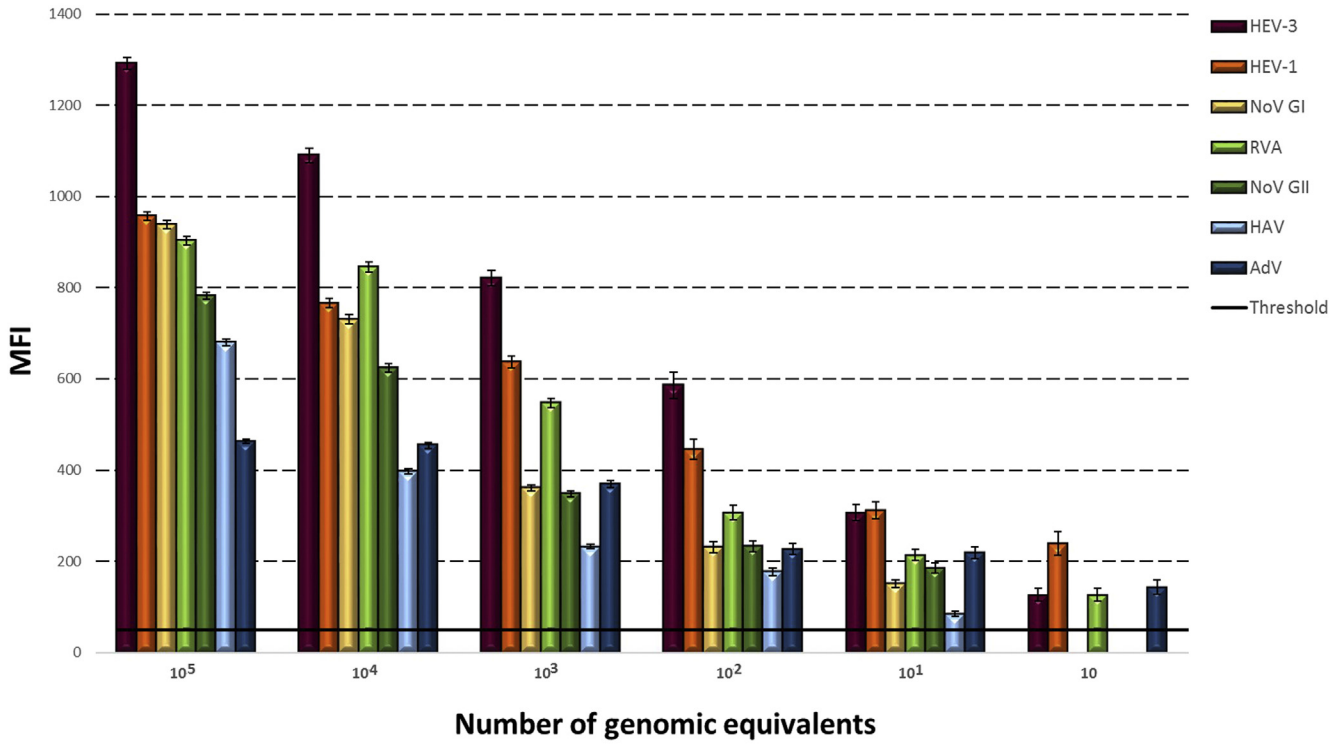

Figure 2 Sensitivity of the assay. The threshold determines the limit for positive samples whose MFI values exceed 50 after subtracting the negative control signal (background). All MFI values given are thus already reduced by the MFI values of the relevant negative controls. AdV, adenovirus 40/41; HAV, hepatitis A virus; HEV-1, hepatitis E virus genotype 1; HEV-3, hepatitis E virus genotype 3; MFI, median fluorescence intensity; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II; RVA, rotavirus A.

Table 4 Interlaboratory Validation of the Assay

DNA/RNA sample	Qualitative detection of HAV, HEV-1, HEV-3, NoV GI, NoV GII, AdV, RVA			
	A	B	C	D
S	+	+	+	+
5×	+	+	+	+
10×	+	+	+	+
TA	+	+	+	+
Ex	−	−	−	−

+, all targets correctly detected; −, no target detected; 5×, fivefold diluted concentration of isolated RNA/DNA; 10×, 10-fold diluted concentration of isolated RNA/DNA; A, the Veterinary Research Institute; AdV, adenovirus 40/41; B, University of Veterinary and Pharmaceutical Sciences Brno; C, Military Veterinary Institute Hlucin; D, Biological Defense Center of the Army of the Czech Republic at Techonin (Military Health Institute); Ex, undiluted isolated DNA of *Staphylococcus aureus* (CAPM 5719); HAV, hepatitis A virus; HEV-1, hepatitis E virus genotype 1; HEV-3, hepatitis E virus genotype 3; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II; RVA, rotavirus A; S, stock concentration of isolated RNA/DNA ($1 \times 10^5 - 1 \times 10^7$ genome equivalents per μL); TA, trace amount of isolated RNA/DNA ($1 \times 10^0 - 1 \times 10^1$ genome equivalents per μL).

denaturation for 10 minutes at 95°C, 20 cycles of 30 seconds at 95°C, and 1 minute at 60°C. Samples were immediately used for the following amplification in singleplex PCR. If necessary, the samples were held at 4°C until further processing.

Singleplex PCR

The final protocol for PCR was established previously, considering all critical points of the processs.[20] The best performance was achieved with the use of EliZyme HS Robust MIX (Elisabeth Pharmacon, Brno, Czech Republic), with a concentration 0.0625 μmol/L of universal forward primer and 0.25 μmol/L of universal reverse primer labeled by BODIPY-TMRX.

The thermal cycling protocol for the PCR reaction was as follows: 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds. Samples were either immediately used for hybridization or stored at 4°C until further processing.

Magnetic Microspheres Coating

For detection on the MAGPIX instrument, commercially available magnetic microspheres (12.5×10^6 microspheres/mL; Luminex Corporation) were used. These beads were coupled with anti-TAG sequences by a carbodiimide coupling method. The bead were coated according to adapted Bio-Plex Bead Coupling protocol by Bio-Rad with minor modifications.[20] An in-house coupling approach was chosen to achieve the best possible signal-to-noise ratio.

Hybridization

The PCR products were hybridized to magnetic microspheres through their TAG sequences. The following set of beads was used: HAV (region 25), HEV-1 and HEV-3 (region 34), HEV-3 only (region 18), NoV GI (region 38), NoV GII (region 15), AdV (region 43), RVA (region 28), and external control (region 36). The hybridization protocol was adopted from Reslova et al[20] with a modification in the thermal cycler program. Better results were obtained with a program following the standard instructions for hybridization from the xMAP Cookbook,[41] although it was primarily designed for detection assays based on product labeling by the interaction of a streptavidin-phycoerythrin conjugate with biotin. Hybridization was performed accordingly at 96°C for 90 seconds, followed by 37°C for 30 minutes. The other parameter that was changed was the number of microspheres used per reaction. A total of 1,250 beads was determined to be sufficient to reach a minimum of 50 detected beads per well for analysis in 100% of samples. Immediately after the end of hybridization, the reaction volume was increased to 60 μL by addition of 45 μL of analysis buffer at room temperature containing 10 mmol/L Tris-Cl (pH 8.0; Sigma-Aldrich, St. Louis, MO), 0.1 mmol/L EDTA (VWR International, Radnor, PA), 90 mmol/L NaCl (Carl Roth, Karlsruhe, Germany), and 0.02% Tween 20 (Alpha Diagnostic, San Antonio, TX).

MAGPIX Analysis

Beads with hybridized MOL-PCR products were analyzed in the MAGPIX Instrument using the original software, xPONENT software version 4.2 for MAGPIX (both Luminex Corporation). Median fluorescence intensity (MFI) values were calculated from the analysis of at least 50 microspheres of each region per sample.[20,22] Results were subsequently evaluated. If the fluorescence signal (MFI value) of a given sample in a respective region of microspheres was higher than 50 after subtracting the value of the negative control, the sample was declared positive. If needed, a semiquantitative evaluation could also be performed. MFI values decrease with lowering numbers of viral genome equivalents present in the sample.[22,27] However, only one target/region can be evaluated this way. It is not feasible to compare targets/regions with each other in a semiquantitative analysis.

Sensitivity of the System

Principles similar to those used for qPCR evaluation were used to determine the sensitivity of the detection system.[42,43] Sensitivity was tested using a range of target genome copies diluted in Ultra-PCR water (Top-Bio). The concentration series included the following dilutions: 1×10^5, 1×10^4, 1×10^3, 1×10^2, 1×10^1, and 1×10^0 genome equivalents per μL in eight repetitions. qPCR

Table 5 Analyzed Biological Samples

Sample type	Number of samples	Number detected						
		HAV	HEV-1	HEV-3	NoV GI	NoV GII	AdV	RVA
Stool	28	5	5	2	4	5	4	2
Human serum	2		2					
Wild boar feces	4			4				
Wild boar bile	37			9				
Wild boar liver	32			9				
Water	10				2	2	2	

Blank cells indicate that the samples did not have the pathogen.

AdV, adenovirus 40/41; HAV, hepatitis A virus; HEV-1, hepatitis E virus genotype 1; HEV-3, hepatitis E virus genotype 3; NoV GI, norovirus genogroup I; NoV GII, norovirus genogroup II; RVA, rotavirus A.

systems mentioned above were used for verification and quantification. The limit of detection (LOD) was defined as the lowest concentration of viral genome equivalents (final dilution) that always gave a positive result for all replicates.

Specificity of the System

All targets (primers, MOLigos, nucleic acid) were tested for possible interactions with each other; a singleplex reaction was run in comparison with multiplex reactions. During testing and development of the complete system, feces, bile, serum, water, parasite, and cell and bacterial culture samples were analyzed for the presence of target pathogens. These samples served for the specificity testing, and the respective RT-qPCR/qPCR was considered as the reference method.

Isolated genetic material from bacterial and parasitic foodborne pathogens was used to further verify the specificity of the system. These agents may appear in the tested matrices, and possible undesired interactions may interfere with the usability of the system. Six bacterial species and four parasites were tested: *Yersinia enterocolitica* CAPM 6154, *Escherichia coli* CAPM 5358, *Listeria monocytogenes* CAPM 5879, *Campylobacter jejuni* CAPM 6316, *Salmonella enterica* CAPM 5445, *Staphylococcus aureus* CAPM 5719, *Toxoplasma gondii, Trichinella spiralis, Taenia saginata,* and *Giardia intestinalis*. Genomic DNA of these was extracted as previously described.[20,44] All bacterial isolates were provided by the CAPM. Oocysts of *To. gondii* and a part of a *Ta. saginata* body segment were obtained from the National Reference Laboratory for Parasites at the University of Veterinary and Pharmaceutical Sciences (Czech Republic). Larvae of *Tr. spiralis* were provided by the International Trichinella Reference Center (Italy). Genomic DNA of *G. intestinalis* was purchased from American Type Culture Collection (30888D; ATTC, Manassas, VA).

Interlaboratory Validation

To test the reproducibility and robustness of our assay, interlaboratory testing was performed in four specialized laboratories: the Military Veterinary Institute Hlucin, the Biological Defense Center of the Army of the Czech Republic at Techonin (Military Health Institute), the University of Veterinary and Pharmaceutical Sciences in Brno, and the Veterinary Research Institute.

All experimental material was transported either on dry ice or wet ice to maintain the optimal storage conditions. All participating laboratories received detailed operation instructions how to perform the whole analysis and how to process the results obtained. All laboratories were asked to strictly follow the instructions and report any possible deviations. None of the participating laboratories was familiar with the order or content of the individual samples. Laboratory testing and initial analysis of the results were performed blindly. The final evaluation and comparison of the obtained results with the actual layout of the interlaboratory ring test was performed independently of the previous testing.

With negative controls included, the interlaboratory validation test comprised a total of 43 samples. A concentration series of 5 samples was made for each of the seven targets/pathogens. The isolated nucleic acid stock was diluted in Ultra-PCR water (Top-Bio) as follows: one non-diluted sample ($1 \times 10^5 - 1 \times 10^7$ genome equivalents per μL), one 5× diluted sample, two 10× diluted samples, and one sample with the concentration of the target nucleic acid reaching the detection limit ($1 \times 10^0 - 1 \times 10^1$ genome equivalents per μL). The whole test set was completed with four negative controls (RNase free water) and four excess samples. The excess samples contained the undiluted isolated DNA of *St. aureus* CAPM 5719 and were used to verify and eliminate the possibility of undesired interactions to ensure the validity of interlaboratory testing.

Results

Sensitivity

The sensitivity of the detection system was verified on a prepared gradient of genome equivalents of the detected targets diluted in water (Table 3). For systems targeting nucleic acid of AdV, HEV, and RVA, genome equivalents as low as 1×10^0 per μL of isolated nucleic acid were

detected. The LOD of 1×10^1 genome equivalents per μL of isolated nucleic acid was set for the remaining systems (Figure 2). For standardization of the assay, a general LOD has been set at 1×10^1 genome equivalents per μL of isolated nucleic acid.

Specificity

All analyzed samples in this study tested using the complete functional RT-MOL-PCR system were also characterized by the RT-qPCR/qPCR systems described above. The results obtained corresponded. No sample was identified as positive by one method and negative by another, hence the false-positive and false-negative rates were both 0%. No interactions between targets (primers, MOLigos, nucleic acids) were observed. This concordance of results confirms comparable usability of this detection tool to routine qPCR methods.

There was no false identification of the presence of the agent in any of the matrices tested (feces, bile, serum, water, and cell and bacterial culture), suggesting that the MOLigo probes do not interact with the background material. Also, there were no interactions between individual viral targets when creating the multiplex system.

Additionally, bacterial and parasitic agents connected to food or water contamination were tested to verify sufficient specificity of the detection system. Also, in this case, no undesirable interaction or specific amplification of non-targets were shown (no false-positive samples).

Interlaboratory Validation

According to the records from the collaborating laboratories, all protocol requirements were met during testing, and there was no deviation from the standard protocol during experimental work. Required experimental data were obtained in a timely manner and sent to our laboratory for further evaluation (Supplemental Figure S1). Differently high MFI values were obtained for individual systems in different laboratories. However, these deviations did not affect the final evaluation. The comparison of data shows 100% agreement among all participating laboratories as regards the determination of positivity or negativity of samples (Table 4).

Biological Samples

During the development of the detection system, a total of 113 biological samples of different origin were analyzed for further validating detection reliability. The evaluated multiplex RT-MOL-PCR system for detection of food- and waterborne viruses confirmed the outcomes of analyzes performed by RT-qPCR/qPCR methods. The results obtained with both methods were in agreement; 52 samples analyzed were determined as positive for the presence of at least one target pathogen (Table 5).

Discussion

Food- and waterborne viruses pose medically and economically significant threats to the general public because they are associated with considerable morbidity and mortality worldwide. In recent years, there is an increasing need to introduce control mechanisms for the presence of these pathogenic viruses. To implement effective control strategies, reliable methods for detection of these viruses are essential. Early diagnosis is needed to track and handle possible disease outbreaks. This study describes the development of a detection system combining RT-MOL-PCR with xMAP technology. This novel tool offers a reliable, rapid, and robust method for the detection of pathogenic agents.

In general, detection assays using xMAP technology and targeting nucleic acid of pathogens connected to food or water contamination have already been described, and some are even commercially available.[45−47] The xTAG Gastrointestinal Pathogen Panel[48−50] is a product provided directly by the Luminex Corporation. This diagnostic kit serves for the identification of multiple viral, parasitic, and bacterial nucleic acid in samples of human stool. However, being a commercial product, its exact composition and parameters are unknown, and thus possible adaptation to the laboratory's own needs is not feasible. Another significant limitation is that it only detects three enteric viruses (NoV, rotavirus, and AdV 40/41). Most of the already published methods exploiting xMAP technology apply detection during the PCR phase, which can be problematic due to unequal preference between individual templates.[23,27,51] The assay described in this article bypasses this problem by moving the detection step to the ligation phase with no interference between individual targets. Therefore, MOL-PCR is not susceptible to amplification bias or cross-hybridization. Albeit the principle and use of a combination of MOL-PCR and xMAP technology has been described previously,[21,22] significant optimization has been performed in the case of this study. Changes such as the different labeling of the amplified products (BODIPY-TMRX; Eurofins Genomics) and the modification of the hybridization protocol have been employed to develop a more sensitive and less laborious method. The main advantages of the fluorescent product labeling over the standard recommended biotin labeling typical for xMAP technology assays are reduction of the financial burden and the saving of time by eliminating the washing and incubation steps. It should be noted that these additional steps may also be critical because of the risk of possible cross-contamination by PCR products. Eliminating them therefore results in more reliable results.[20] A significant problem with detection systems exploiting xMAP technology and targeting the viruses mentioned here is the low sensitivity. In a study by Liu et al,[52] the LOD for the simultaneous detection of different human enteric viruses was between

10^3 and 10^4 genome equivalents per reaction. In a study by Hamza et al,[46] another similar detection system was described to have a LOD between 10^2 and 10^3 genome equivalents per reaction. The LOD of the multiplex assay presented here is one to two orders of magnitude lower (sensitivity comparable to the above-mentioned International Organization for Standardization methods using RT-qPCR). A possible reason for the low sensitivity of the compared systems may be the use of one-step RT-qPCR, which is generally considered to be more variable and less sensitive than the two-step protocols.[53–55] Another reason may be the use of a different detection method associated with the xMAP technology. In these cases, biotinylated PCR products were directly hybridized. Similar to the study by Hamza et al,[46] LOD in this study was set for dilutions of genome equivalents in water. LOD for samples of different matrices may vary and should be determined. Sufficiently high sensitivity is a critical factor for the detection of discussed viruses, because most of them are characterized by a very low infectious dose.[56,57]

The novel detection system presented here is capable of the simultaneous detection and identification of several unique DNA/RNA targets of viruses in a high-throughput reaction with good sensitivity and specificity. The study addresses the limitations of the qPCR assays, especially when it comes to the creation of robust multiplex systems. It is not necessary to choose which individual assay to run. In one reaction, the sample can be tested for a wide range of pathogens. Turn-around time is thus significantly improved. The results of the analysis of the sample for the presence of HAV, HEV-1, HEV-3, NoV GI, NoV GII, AdV, and RVA are available within 5 hours. In a comparable time, it would be possible to perform about two to three runs of RT-qPCR/qPCR, that is, detection of two or three viruses.[36,38,39] Moreover, the costs of performing one analysis are significantly lower compared with several qPCR runs. The method is easy to perform and does not require any special knowledge or skills compared with normal laboratory practice. It is designed to be used on samples of various origin (clinical samples, environment, food or water) to detect the presence of the pathogenic viruses most commonly associated with contamination of food or water. It is a flexible and adaptable assay allowing the addition of other signatures as they are identified to be relevant.

To demonstrate the validity, robustness, and applicability of the method, 113 real biological samples were analyzed during the method's development, and interlaboratory tests were also performed at four different independent laboratory workplaces. The Food and Drug Administration guidelines were used for the validation of the ring tests.[42] The results obtained in individual laboratories showed minor deviations; however, all positive and all negative samples were correctly identified within the evaluation criteria. Different signal heights (MFI values) for individual systems can be explained by the use of different laboratory equipment, processing speed, or by the skills and experience of the workers. According to the obtained homogeneity of the results from this collaborative method validation, it can be stated that this designed detection system shows a high degree of robustness, sufficient sensitivity, and specificity, and after the necessary optimizations, it may be provided to official and third-party detection institutions for outbreak investigations, survey studies, or routine screening of food, environment, or clinical samples for the presence of all included pathogenic viruses.

To allow better comparison with other methods,[46,52] the LOD of this detection tool was tested on a prepared gradient of genome equivalents of the detected targets diluted in water. The functionality of the method has been verified on a limited number of possible matrices (human stool and serum, animal feces, liver, serum, and bile, and drinking and service water). Introduction of the method for specific routine diagnostics will require testing of other desirable matrices and subsequent determination of the parameters of the final system.

This assay is not yet intended to serve as the main decisive method for final determination. Rather, this high-throughput tool allows testing of a larger number of samples of which the preselected ones can then be subjected to further verification and more detailed analysis. It is evident that further tests for more detailed classification of individual positive or disputed samples will be necessary. However, the obtained results strongly demonstrate that this system has the potential as a cornerstone for a complex screening system and rapid source tracking of food- and waterborne viruses.

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Supplemental Data

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Author Contributions

J.H. performed molecular analysis, created data output, and wrote the manuscript. P.V. and P.K. secured the biological material, provided financial support, interpreted data, and revised the manuscript. M.N., J.N., and T.M. performed interlaboratory testing. All authors approved the final version of the manuscript. J.H. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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