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Differentiation of live and dead *Mycobacterium tuberculosis* complex in meat samples using PMA qPCR

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ABSTRACT

The causative agents of zoonotic bovine tuberculosis (bTB), *Mycobacterium bovis* and *M. caprae*, are members of the *M. tuberculosis* complex (MTC). Wildlife such as red deer infected with bTB are often without pathological findings, thus meat thereof may be classified as safe for human consumption. The culturing of MTC is time consuming and inappropriate to be applied with fresh meat and food. Therefore, a rapid method "PMA qPCR" to differentiate living and dead cells of MTC was developed in this study. By treating with 50 μM PMA™ dye, dead *M. bovis* BCG ($\leq 10^4$ cells/ml meat suspension) could be completely discriminated and was not detected by specific MTC PCR. The limit of detection of MTC without treatment with PMA™ dye was 10 cells/ml. All 50 venison samples obtained for field study purposes were negative for MTC. However, 40% were slightly PCR positive for non-TBC mycobacteria. By culturing using selective enrichment, one single colony of *M. avium* was isolated. This is the first report on the isolation of *M. avium* from venison. Considering the difficulties of diagnosing mycobacteria in various matrices, the developed PMA qPCR is applicable for the differentiation of dead and living cells of MTC in meat samples.

1. Introduction

The members of the *Mycobacterium tuberculosis*-complex (MTC) are causative agents of human and animal tuberculosis. The species of this complex are *Mycobacterium tuberculosis, M. bovis, M. caprae, M. africanum, M. microti, M. pinnipedii, M. mungi, M. orygis, M. sunicattae, M. canettii* and *Dassie Bacillus* (Huard et al., 2006; Alexander et al., 2010). *M. tuberculosis* is the main causative agent of tuberculosis in humans, while *M. bovis* and *M. caprae* are causative agents of the bovine tuberculosis (bTB), a notifiable disease in Germany (TIERSEUCHANZV, 2014) and of the World Organization for Animal Health (OIE, 2013). The latter two species have the highest zoonotic potential among all MTC members (Muller et al., 2013). Humans can be infected with both species by direct contact with infected animals or by consumption of contaminated milk, meat and meat products (CFSPH, 2009; Torres-Gonzalez et al., 2013). The infection with bTB is reported in humans (Thakur et al., 2012; Rodriguez et al., 2009) and in many animal species such as cattle, goats (Bezos et al., 2012), pigs, sheep and foxes (Rodriguez et al., 2011), wild boar (Duarte et al., 2008; Matos et al., 2014), red deer (Lopez-Olvera et al., 2013; Schoepf et al., 2012), roe deer (Zanella et al., 2008), badgers (Tomlinson et al., 2015), camels (Pate et al., 2006) and elephants (Yoshida et al., 2018).

Infection of bTB in red deer is reported worldwide, e.g. up to 50% in farmed deer and 20% in wild red deer in New Zealand (Martin-Hernando et al., 2010). In Europe, the prevalence of bTB in red deer is between 1% and 27%, wherein the highest prevalence is reported in France and in Spain, namely 24% and 27% (Wilson et al., 2009). In the Alpine countries, the prevalence of bTB (*M. caprae*) in red deer ranges from 1% to 4% in Southern Upper Bavaria, Germany to ≥20% in the nearby located area Lechtal, Austria (Domogalla et al., 2013; Schoepf et al., 2012; EMIDA, 2013; Büttner et al., 2013).

During the monitoring program of Coordination of European Research on Emerging and Major Infectious Diseases in Livestock Animals (EMIDA) - European Research Area - Net (ERA-Net) over "Tuberculosis in Alpine Wildlife", it was also observed that the typical pathological-anatomical alterations in infected red deer are often absent (nonvisible lesions, NVL) (EMIDA, 2013). As a consequence, the carcasses of the infected animals were classified as safe for human consumption. Subsequently, the consumption of this MTC infected meat might pose a risk for humans. In Germany, the consumption of game meat and the number of hunted wild animals such as red deer and wild boar is continually increasing since years (BfR, 2006; Deutscher Jagdverband (Germany Hunting Association), 2018). The Federal Institute for Risk Assessment (BfR, Germany) has issued a press release that game meat can pose a health risk potential due to zoonotic agents. Thus, proper handling of these types of products is recommended (BfR,

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2006).

However, if the MTC agents in meat were dead already, one might argue that these agents actually do not pose a risk to human health. Therefore, ISO-methods for the detection of zoonotic pathogens regularly request cultural approaches. The detection of living mycobacteria in meat is difficult due to their special cell wall properties which cause fastidious growth; as such the differentiation between living and dead bacteria is even more complex. Wong et al. (2016) described the differentiation between living and dead *M. smegmatis* by using autofluorescence, which is also applicable for direct smears of specimens containing high concentrations of mycobacteria. Culturing, the current gold standard method to detect MTC is time consuming (3–4 weeks or more), thus it is an inappropriate method for investigation of fresh meat. Rapid, PCR-based methods to detect DNA are widely used, but cannot differentiate between living and dead MTC. The use of messenger RNA (mRNA) as a marker for viability was described, since mRNA is a highly labile molecule with a very short half-life, and should therefore provide a better information on the viability status than DNA-based methods (Keer and Birch, 2003). However, it has been shown in several studies that mRNA may persist in a detectable form for many hours after cell death, confirming the potential for poor correlation between mRNA detection and cell viability (Keer and Birch, 2003).

The detection of membrane integrity of living and dead cells by using a photoreactive DNA binding dye "Propidium Monoazide" (PMA™ dye, Biotium, USA) and a subsequent quantitative PCR (qPCR) amplification has been carried out by several studies. Principally, the membranes of dead cells are usually permeable, so that the PMA dye can diffuse into dead cells and bind with nucleic acids. After treatment with light, the DNAs bound with PMA dye are permanently modified and cannot be amplified by DNA polymerase (Nocker et al., 2006). Thus, only DNA from viable cells will be amplified and quantified by qPCR. There are several reports on the application of PMA dye to differentiate living and dead microorganisms such as *Campylobacter* spp. in chicken carcasses (Josefsen et al., 2010; Pacholewicz et al., 2013), pure cultures of *Listeria innocua* (Lovdal et al., 2011), *Salmonella enterica* in lettuce (Liang et al., 2011) and in cooked ham (Martin et al., 2013), *Staphylococcus aureus* in wipe-samples (Schmidlin et al., 2010), milk powder and meat products (Zhang et al., 2015), *Mycobacterium avium* subsp. *paratuberculosis* in milk (Ricchi et al., 2014) and in a cellular sample (Pooley et al., 2016) and *Mycobacterium tuberculosis* in clinical samples (de Assunção et al., 2014; Kim et al., 2014). However, the evaluation of the results of these studies are variable and therefore not sufficient to apply for the purpose of the present study.

There are only few reports about the contamination with MTC, regardless of whether living or dead, in game meat. Thus, the aim of this study was to develop a fast and sensitive technique for quantification and differentiation of living and dead MTC in venison by using the PMA dye and a subsequent qPCR amplification. Primers and probes specific for MTC were developed in this study. The results were evaluated and validated by means of commercially provided qPCR methods and the results of PMA dye techniques from other studies.

2. Material and methods

2.1. Samples

Bacterial species used to validate the specificity of qPCR systems are listed in Table 1, namely *M. tuberculosis* complex (MTC, n = 5), non-tuberculous mycobacteria (non-TBC, n = 10) and non-mycobacterial species (non-Mb, n = 25). The reference species *Mycobacterium bovis* strain Bacille Calmette-Guérin-Pasteur (*M. bovis* BCG) was provided by the National Reference Center (NRC) for Mycobacteria, Research Center Borstel, Germany. Afterwards venison and fecal samples were investigated for testing the specificity of all qPCR systems (see Section 2.6).

Venison (n = 50) were obtained from local butchers located in a high prevalence area for bTB in red deer in Alpine region, South of Bavaria, Germany. Fecal samples of red deer (n = 25) were also found in the same area as the venison and were collected from descending colons of animals by hunters. Meat and fecal samples of red deer were processed for laboratory investigation on the day of delivery; for some cases, they were stored at 4 °C for maximum 24 h. Fecal samples from wild boar (n = 25), also collected from descending colon by hunters, were obtained from the previous study (Dorn-In et al., 2016). They came originally from the Northwest of Poland and were stored at −20 °C prior laboratory investigation.

Table 1
Bacterial species for validation of the specificity of qPCR systems.

Mycobacterium tuberculosis complex (MTC)	Non-mycobacterial species (Non-Mb)
M. bovis BCG (from NRC, Germany)	*Campylobacter jejuni* (DSM 4688)
M. bovis (V-08-402)[a]	*Clostridium perfringens* (DSM 756)
M. bovis (D 911/43990)[a]	*Clostridium estertheticum* (DSM 14864)
M. africanum (6817)[a]	*Corynebacterium mycetiodes* (DSM, 20148)
M. microti (13-0086913-001-01)[a]	*Enterococcus faecalis* (DSM 2570)
Non-tuberculous mycobacteria (Non-TBC)	*Enterobacter cloacae* (DSM 30054)
M. avium (10-0165715-001-01)[a]	*Escherichia coli* (DSM 787)
M. chelonae (ERA 150)[a]	*Klebsiella pneumoniae* (DSM 26371)
M. kansasii (13-000617-003-01)[a]	*Lactobacillus iners* (DSM 13335)
M. intracellulare (DSM 43223)	*Micrococcus luteus* (DSM 1790)
M. terrae (DSM 43227)	*Mycoplasma bovis* (DSM 22781)
M. scrofulaceum (DSM 43992)	*Rhodococcus hoagii* (DSM, 20307)
M. avium subsp. *avium* (DSM 44156)	*Salmonella* Enteritidis (DSM 14221)
M. avium subsp. *silvaticum* (DSM 44175)	*Staphylococcus aureus* (DSM 2569)
M. avium subsp. *asiaticum* (DSM 44297)	*Streptococcus agalactiae* (DSM 2134)
M. avium subsp. *paratuberculosis* (DSM 44133)	*Streptococcus infantis*[b]
M. avium[b]	*Pseudomonas aeruginosa* (DSM 1117)
Non-mycobacterial species (Non-Mb)	*Pseudomonas fragi* (DSM 346)
Arcanobacterium pyogenes[b]	*Pseudomonas fluorescens* (DSM 5014)
Bacillus cereus (DSM 4312)	*Pseudomonas lundensis* (DSM 6252)
Bacillus licheniformis (DSM 13)	*Pseudomonas putida* (DSM 291)
Brevibacterium linens[b]	*Weissella hellenica* (DSM 7378)

DSM/DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.

[a] DNA samples were provided by the Bavarian Health and Food Safety Authority (LGL), Germany. The numbers in brackets are internal reference numbers.

[b] Isolated by the Chair of Food Safety, LMU, Germany.

2.2. Enumeration of M. bovis BCG

Mycobacteria usually stick together during growth, which leads to distorted results of both plate counting on solid medium as well as cell counting via microscope. To obtain single cells, *M. bovis* BCG was cultured in Middlebrook broth M7H9 (+Supplement BBL™ Middlebrook ADC Enrichment, Becton Dickinson, USA) and incubated aerobically at 37 °C for about 3 weeks. The culture was shaken daily for 5 min. Before harvesting, the culture was allowed to rest overnight at 37 °C. Then, only the supernatant of the culture was collected and filtered by using sterile gauze. The filtered supernatant was centrifuged at 500 × *g* for 5 min, then transferred into a new tube and shaken for 2 min. The number of cells/ml was microscopically determined (400×) using a Thoma cell counting chamber. If bacterial cell clumps with more than 6 cells were still present, a centrifugation at up to 1000 × *g* for 10 min followed and the enumeration of *M. bovis* BCG cells was re-assessed as described above.

Since a suspension of the fresh *M. bovis* BCG culture may contain also dead cells, culturable living cells were additionally enumerated by plate count technique using solid medium BD BBL™ Stacker™ Plate

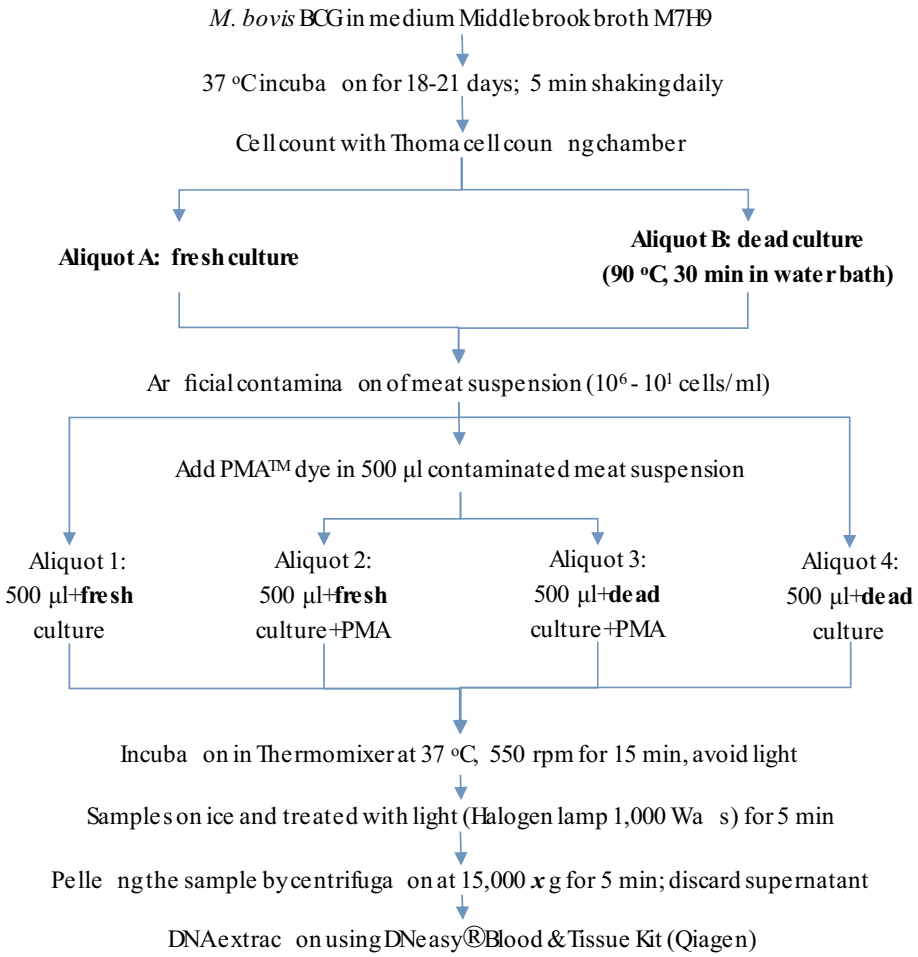

Fig. 1. Treatment with PMA™ dye.

(Becton Dickinson). Plates were incubated aerobically at 37 °C. Colony counting was performed at day 14, 20, 24 and 28 of incubation. The procedure of culturing, cell and colony counting of *M. bovis* BCG was repeated independently 3 times. All 3 replicates were processed independently for artificial contamination and PMA treatment (see Section 2.3 and 2.4).

2.3. Artificial contamination of M. bovis BCG in meat suspension

Ten Grams of venison and 90 ml NaCl (0.9%) were homogenized in a stomacher for 90 s. The preparation of meat suspension was similar to the culturing method. Meat suspension was used in order to include the effect of meat matrix in the DNA extraction and PCR amplification. For one experimental replicate, an aliquot of 10^7 cells/ml of *M. bovis* BCG (from Section 2.2) was treated at 90 °C for 30 min in a water bath to kill the cells. The dilutions of fresh and dead cultures of *M. bovis* BCG 10^7 cells/ml NaCl were artificially contaminated and further serially diluted in meat suspension. The final concentration of *M. bovis* BCG was 10^6–10^1 cells/ml meat suspension (corresponding to 10^7–10^2 cells/g meat). All artificially contaminated meat suspensions were subjected to DNA extraction (see Section 2.5). The artificial contamination was performed independently in 3 experimental replicates by using 3 replicates of *M. bovis* BCG suspension prepared as described in section 2.2.

2.4. PMA™ dye treatment

The toxicity of "Propidium Monoazide" (PMA™ dye, Biotium, USA), with the final concentrations 25, 50, and 100 µM, to living cells and its efficiency to differentiate living and dead cells of *M. bovis* BCG (10^6 cells/ml meat suspension) was evaluated. In total, 3 independent experimental replications from 3 independent enumerations and artificial contamination in meat suspension were performed for the test of PMA efficiency and its toxicity. The resulting most effective and less toxic concentration was applied to further serial dilution of *M. bovis* BCG, which was also performed in 3 replications. Fig. 1 shows the procedure of PMA dye treatment. Four aliquots of each of serial dilutions of *M. bovis* BCG (10^6 to 10^1 cells/ml meat suspension) were prepared: fresh cultures as well as dead cultures with and without PMA. 500 µl of each aliquot were transferred in a 1.5 ml transparent reaction tube (Eppendorf, Germany). The tubes were laid on an ice block, horizontally to the halogen lamb (1000 Watts) to obtain optimal exposure to the light. The distance between tubes and halogen lamp was 30 cm. First light treatment was 2.5 min, then the tubes were turned to the other side and exposed to the light source again for 2.5 min, which means 5 min in total. After treating with PMA, samples were subjected to DNA extraction using DNeasy® Blood and Tissue kit (Qiagen, Germany).

2.5. DNA extraction

In total, three DNA extraction kits were applied: DNeasy® Blood and Tissue Kit (Qiagen, Germany) for meat suspension, artificially contaminated meat suspensions and bacterial pure cultures. The PowerSoil® DNA Isolation Kit (Mo Bio Laboratories, USA) was applied for fecal samples of wild boars and the QIAmp® Viral RNA Mini Kit

Table 2
qPCR systems with primer pairs and probes.

qPCR systems, primer and probe: sequence (5′-3′)			Fragment	Specificity	Reference
1	Ingenetix (with probe)	not provided	129 bp	ITS of MTC	Ingenetix, Austria
2	Mb-1250F	ATGCCGCGAGGTTAAGCGAATC	535 bp	16S rRNA & ITS region of MTC	this study*
	Mtc-ITS251R	CACAAAGAACACGCCACCGCTA			Park et al. (2000) (modified)*
	Mtc-ITS129-Probe	FAM - CACTCGGACTTGTTCCAGG TGTTGT - BHQ1			this study*
3	Mb-1023-F	CCTGTGTGCAGGTGGTGCAT	760 bp	16S rRNA & ITS region of MTC	Mendum et al. (2000)
	Mtc-ITS251R	see qPCR system 2			
	Mtc-ITS129-Probe	see qPCR system 2			
4	Mb-571F	GGTGGTTTGTCGCGTTGTTC	473 bp	16S rRNA gene of mycobacteria	Mendum et al. (2000)
	Mb-1023R	ATGCACCACCTGCACACAGG			

* Primers and probes developed and modified in this study are based on their 16S rRNA and ITS regions of the MTC species provided in Genbank (NCBI), namely *M. africanum* (NR025238.1), *M. bovis* BCG (GU142937.1), *M. bovis* (BX248333.1, AB026693.1), *M. canettii* (NR074836.1), *M. caprae* (AJ131120.1), *M. microti* (NR025234.1, AB026700.1), *M. pinnipedii* (NR025249.1), *M. tuberculosis* (NC000962, AL123456.3) and other non-tuberculous mycobacteria, e.g. *M. asiaticum* (NR041901.1, AF191087.1), *M. avium* (AF410479.1), *M. avium* subsp. *paratuberculosis* (NR026082.1, NZCP010113.1), *M. bohemicum* (NR026054.1), *M. chelonae* (NR114659.1), *M. gastri* (AB026697.1), *M. fortuitum* (X52933.1), *M. heckeshornense* (NR028759.1), *M. indicus* (NC018612.1), *M. intracellulare* (X52927.1), *M. kansasii* (NR114662.1, CP006835.1), *M. kubicae* (HM022200.1), *M. riyadhense* (EU274643.2), *M. scrofulaceum* (AB026702.1), *M. shottsii* (NR025636.1), *M. simiae* (JX266703.1), *M. smegmatis* (NR115233.1, AJ291599.1), *M. szulgai* (KT168285.1), *M. terrae* (NR115677.1) and other bacterial species (data not shown).

(Qiagen, Germany) for fecal samples of red deer. Meat and fecal samples were suspended with 0.9% NaCl 1:10. Ten Grams of meat in 90 ml NaCl was mixed for 90 s in a stomacher, while fecal suspensions (containing 1 g feces and 9 ml NaCl) were vortexed for 2 min. 500 µl of meat suspension and 100 µl of fecal suspension were used for DNA extraction. The methods of DNA extraction followed the instruction manuals of the DNA extraction kits. For fecal samples extracted with the QIAmp® Viral RNA Mini Kit, the step of DNA digestion was excluded.

2.6. qPCR protocol

Four quantitative PCR (qPCR) systems (Table 2) were applied in this study. System 1 is a commercial qPCR kit (BactoReal® kit *Mycobacterium tuberculosis* complex, Ingenetix, Austria) and specific for the Internal Transcribed Spacer (ITS) region of MTC. Primers and probes for the qPCR systems 2 and 3 were developed and modified in this study based on the 16S ribosomal RNA (16S rRNA) gene and the ITS region of MTC, non-TBC mycobacteria and other bacterial species provided in Genbank (NCBI, see Table 2). They are specific to a partial sequence of the 16S rRNA and the ITS region of MTC. The 4th qPCR system (473 bp) using a primer set described by Mendum et al. (2000) is specific to the genus *Mycobacterium* and was used as an additional reference qPCR system.

All qPCR runs were performed in a Biorad CFX96 Touch™ Cycler (Bio-Rad Laboratories, USA). For qPCR system 1, each reaction contained a 20 µl mixture that included 10 µl DNA reaction mix (2×), 1 µl MTBC assay mix (contains primers and the probe for detection of MTC), 1 µl CR assay mix (contains primers and probe for detection of internal positive control, IPC), 3 µl H_2O and 5 µl DNA sample. A 2-step qPCR was applied, starting with incubation at 50 °C for 2 min, followed by initial denaturation 95 °C for 5 min and 45 cycles in a series of denaturation at 95 °C for 5 s; the annealing and elongation was at 60 °C for 60 s. For qPCR systems 2 and 3, each 20 µl reaction mix contained 0.5 µM of each primer and 0.20 µM probe, 10 µl of SensiFAST™ Probe No-ROX Kit (Bioline, Germany) and 5 µl DNA template, filled up with H_2O. The 2-step qPCR for the systems 2 and 3 started with initial denaturation at 95 °C for 5 min, followed by 45 cycles in a series of denaturation at 95 °C for 10 s; the annealing and elongation was at 65 °C for 60 s. For qPCR system 4, a 20 µl reaction contained 0.2 µM of each primer, 10 µl of SensiFAST™ SYBR No-ROX Kit (Bioline) and 2 µl DNA template, filled up with H_2O. A 3-step qPCR was applied, starting with initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 5 s, the annealing was at 65 °C for 5 s and elongation was at 72 °C for 10 s. Two formerly developed primer pairs (335F & Com 2 and 769F & 1492R, Dorn-In et al., 2015) were used for sequencing the 16S rRNA gene of suspicious colonies isolated from meat samples. The PCR reactions and PCR protocols are similar to qPCR system 4.

2.7. Isolation of mycobacterium spp. from venison samples

Fifty venison were additionally investigated for the presence of *Mycobacterium* species by official culturing methods describe by FLI (2015). Briefly, 20 g of meat were homogenized with 180 ml sterile NaCl (0.9%) in a stomacher for 1 min. To decontaminate the accompanying microorganisms, 10 ml meat suspension was mixed with 10 ml *N*-Acetyl-L-Cystein-NaOH solution (NALC-NaOH), then shaken by 250 U/min for 20 min at room temperature. In order to neutralize NALC-NaOH, the suspension was mixed with 20 ml Sorensen's buffer and centrifuged at 3300 × *g* for 20 min. The pellet was re-suspended in 1 ml Sorensen's buffer. A 150 µl aliquot of the suspended pellet was spread on Loewenstein-Jensen-agar with glycerol and PACT (contains antibiotics amphotericin, carbenicillin and malachite green), Stonebrink agar with PACT and added into Kirchner-Bouillon with PANTA (contains antibiotics amphotericin, nalidixin acid and azlocillin), then aerobically incubated at 37 °C. All 3 media are specific for mycobacteria and are commercially available from Artelt-ENCLIT GmbH & Co., Germany. The growing of colonies was investigated weekly until 8 weeks of incubation. Suspicious colonies were subcultured and subjected to DNA extraction and PCR amplification using 4 qPCR systems described in Section 2.5 and 2.6.

2.8. Statistical analysis

The statistical difference (in $\log_{10}$ equivalence) of efficiency of PMA dye concentration (25, 50, and 100 µM) to discriminate the dead cells and their potential toxicity to living cells was evaluated using one-way analysis of variance (One-way ANOVA) provided by a statistical analysis program OriginPro 2019 (OroiginLab®, USA).

3. Results

3.1. Enumeration of culturable M. bovis BCG

All 3 replicates of fresh cultures of *M. bovis* BCG suspension (1.0×10^6 cells/ml) enumerated using Thomas cell counting chamber and microscope were further enumerated for culturable cells on solid medium BD BBL™ Stacker™ Plate. The results were 3.3×10^5 (replicate 1), 3.5×10^5 (replicate 2) and 3.2×10^5 (replicate 3) cfu/ml. This means, the other 6.5–6.8 × 10^5 cells in each milliliter of *M. bovis* BCG suspensions were dead, clumped or impaired cells.

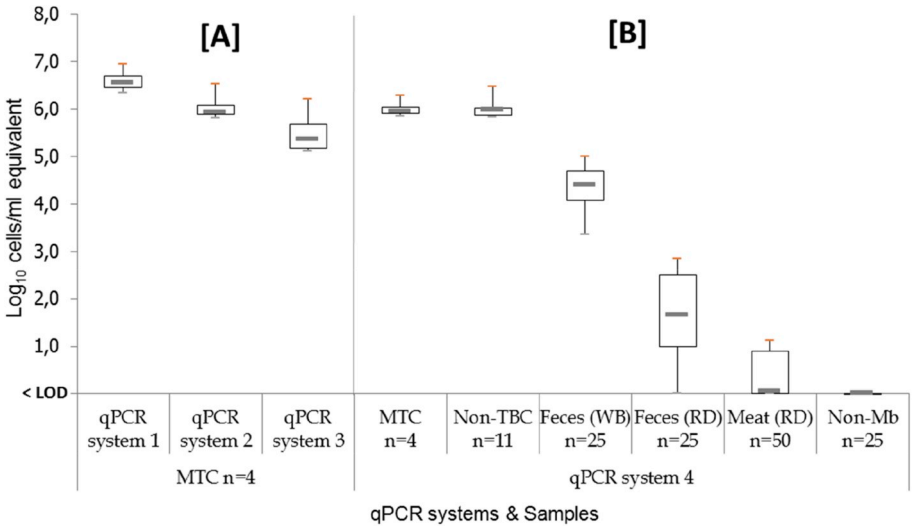

Fig. 2. Specificity of all 4 qPCR systems: **[A]** Results of qPCR systems 1, 2 and 3. All amplify exclusively DNA from *Mycobacterium tuberculosis* complex (MTC); other sample types (see **[B]**) are negative. **[B]** Results of qPCR system 4: MTC and non-tuberculous mycobacteria (Non-TBC) were amplified in DNA extracts from pure cultures, feces from wild boar (WB), feces and of some meat samples from red deer (RD), but not from non-mycobacterial species (Non-Mb). < LOD = no PCR amplification (negative). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Sensitivity and specificity of qPCR systems

For sensitivity testing, 500 µl of each serial dilution (10^6–10^1 cells/ml) of both fresh and dead cultures of *M. bovis* BCG in meat suspension were subjected to DNA extraction using the DNeasy® Blood and Tissue Kit. After that, PCR amplification of all 4 qPCR systems was carried out. The limit of detection (LOD) was 10 cells/ml by using qPCR systems 1, 2 and 4, whereas the LOD by using qPCR system 3 was 10^3 cells/ml meat suspension (see Section 3.4).

Fig. 2 shows the specificity of the tested qPCR systems. All four qPCR systems did not amplify non-mycobacterial DNA (non-Mb) listed in Table 1 (data not shown). qPCR systems 1, 2 and 3 amplified exclusively DNA from all tested MTC (*M. bovis*, *M. africanum* and *M. microti*) and not from non-tuberculous mycobacteria (non-TBC). The genus-specific qPCR system 4 was used to assure the sensitivity and specificity of qPCR systems 1, 2 and 3. It amplified DNA of all mycobacterial DNA samples (MTC and non-TBC).

3.3. Quantification of the relative amount of mycobacterial DNA in samples

The relative amount of DNA in field samples (venison and feces, see Fig. 2) was quantified using a standard curve obtained from a fresh culture (10^6–10^2 cells/ml meat suspension) of *M. bovis* BCG. The Ct-values were converted into $\log_{10}$ level equivalents (Fig. 2). It has to be mentioned that the standard curve was the serial dilution of *M. bovis* BCG in meat suspension. Therefore, the amount of mycobacterial DNA in fecal samples of wild boar and red deer quantified by using this standard curve was only approximate, as they are not from the same matrix type. All field samples were negative for MTC, tested by qPCR systems 1, 2 and 3 (data not shown).

By using the genus-specific qPCR system 4, the amount of mycobacterial DNA from all fecal samples of wild boar (n = 25) was equivalent to 3.5–5.0 $\log_{10}$, and from 19 fecal samples of red deer it was between 1.0 (LOD) to 2.9 $\log_{10}$. DNA of mycobacteria in 6 fecal samples of red deer were lower than the LOD. In total, 40% (n = 20/50) of venison showed a positive signal by using this qPCR system. However, the amount of DNA was equivalent to or lower than the LOD ($\leq$1.0 $\log_{10}$). Positive PCR samples resulting from qPCR system 4 from 2 fecal samples of wild boar, 2 fecal samples of red deer and 6 venison samples were submitted for sequencing (Eurofins, Germany). All 4 DNA samples from feces were 99–100% identical to the 16S rRNA gene sequence of variable non-TBC *Mycobacterium* species (e.g., *M. bacteremicum*, *M. frederiksbergense*, *M. haemophilum*, *M. fortuitum*, *M. smegmatis* and *M. avium*) provided in GenBank (NCBI). Further PCR products of 6 venison samples could not be sequenced due to too low DNA concentrations. The sequencing results from fecal samples of wild boars and red deer confirm the specificity of qPCR system 4 to DNA of the genus *Mycobacterium*. However, a species identification is not possible by using this qPCR system.

3.4. Toxicity and the efficiency of the PMA™ dye

Three concentrations of PMA dye (25, 50 and 100 µM per reaction) were tested for their toxicity to living cells and for their efficiency to discriminate between dead and living cells of *M. bovis* BCG. The concentration of the reference strain *M. bovis* BCG was 10^6 cells/ml meat suspension (equivalent to 10^7 cells/g meat). A standard curve of *M. bovis* BCG fresh cultures (10^6–10^1 cells/ml meat suspension) was used in order to quantify the amount of the target DNA in the samples. Fig. 3 shows the box plots of $\log_{10}$ cells/ml equivalent of 3 independent replicates of the 4 sample types, fresh and dead cultures with and without PMA.

The PCR amplification results of the DNA from fresh and dead cultures without PMA were similar, independently of the qPCR systems. By using qPCR system 1, the $\Delta\log_{10}$ cells/ml equivalent between fresh cultures with and without PMA was 1.0 $\log_{10}$, whereas there was no statistical difference between the $\log_{10}$ level of fresh and dead cultures treated with PMA. By using qPCR systems 2, 3 and 4, the $\Delta\log_{10}$ cells/ml equivalent between fresh cultures with and without PMA was 1.5–1.8 $\log_{10}$. Between dead cultures with and without PMA it was 3.0–3.5 $\log_{10}$, and between fresh and dead cultures with PMA it was about 1.5–1.8 $\log_{10}$ (see Fig. 3).

A higher concentration of PMA (100 µM as well as 50 µM) has a tendency to decrease the amount of amplified DNA from both living and dead cultures, but not statistically significant from the concentrations 25 µM (Fig. 3). The final concentration of 50 µM of PMA dye was applied for further serial dilution of *M. bovis* BCG (10^6–10^1 cells/ml meat suspension, see Section 3.4) due to the cost factor and to avoid unknown effects by using higher concentrations than recommended.

3.5. Differentiation of living and dead cells of M. bovis BCG

The final concentration of 50 µM PMA dye per reaction was subsequently applied for the differentiation of dead cells from living cells of *M. bovis* BCG from further serial dilutions of meat suspensions containing 10^5 - 10^1 cells/ml (corresponding to 10^6 - 10^2 cells/g meat). The amount of amplified DNA determined with qPCR was converted into $\log_{10}$ cells/ml equivalent using a standard curve complied from fresh cultures of *M. bovis* BCG (10^6–10^2 cells/ml). The ΔCt-value between each $\log_{10}$ level of this standard curve ranged from 3.0 to 3.5 PCR

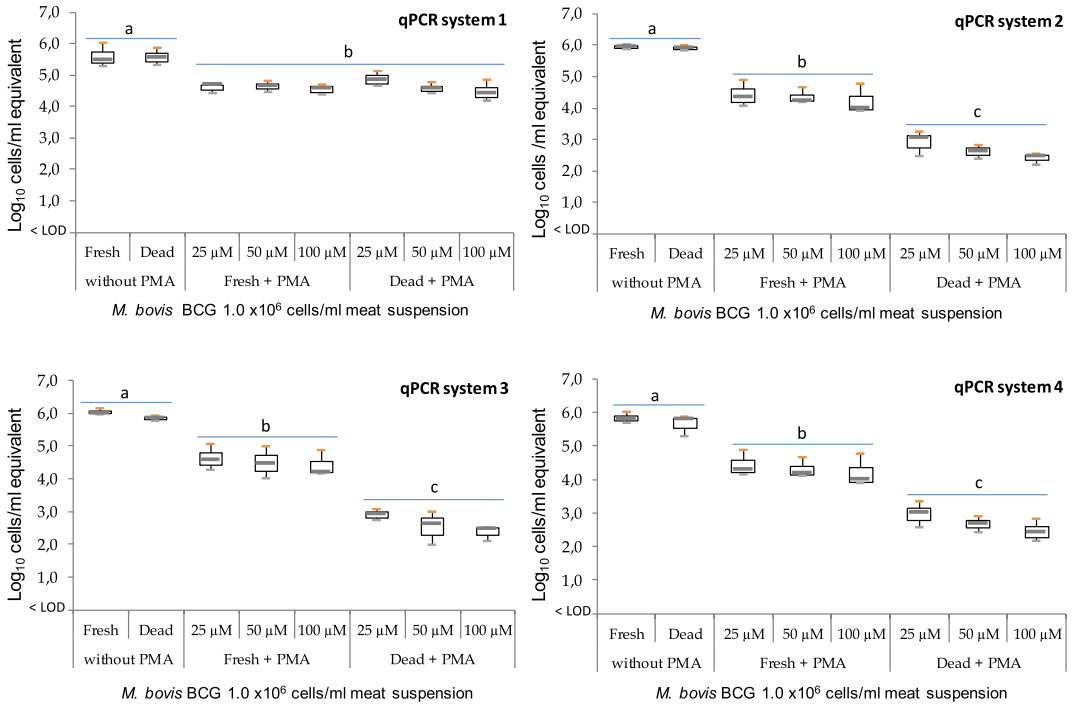

Fig. 3. PMA dye with final concentrations 25, 50 and 100 µM to test toxicity to living cells and efficiency to discriminate dead cells. DNA extracts were tested with 4 qPCR systems. Y-axis shows the $\log_{10}$ cells/ml equivalent of 3 independent replicates. < LOD = no PCR amplification (negative). Samples with the same letters (a, b, c) show no statistically significant difference ($p > 0.05$), whereas the differences between a vs. b, a vs. c and b vs. c are statistically significant ($p < 0.05$).

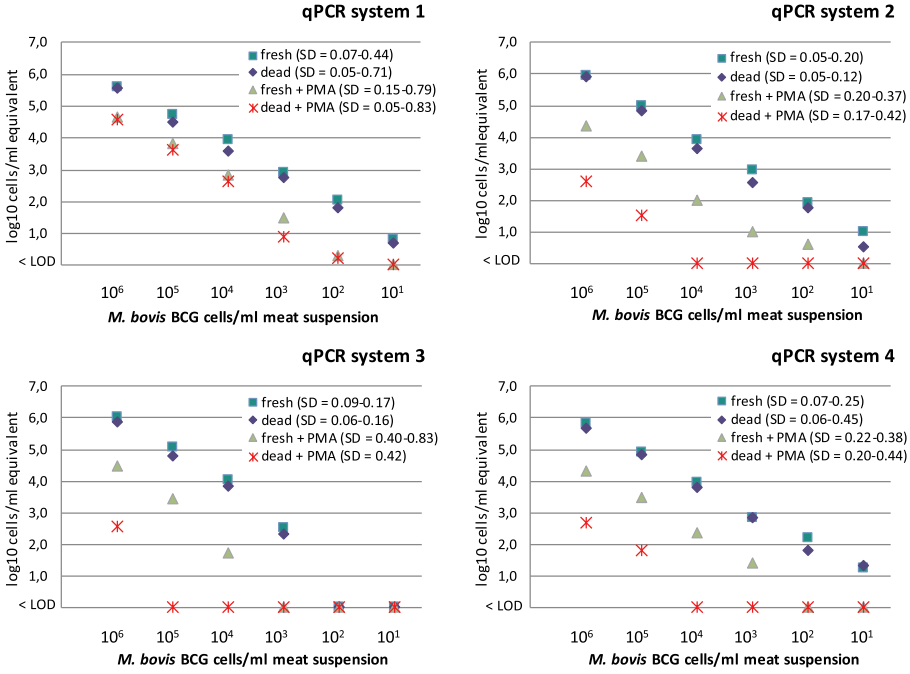

Fig. 4. Discrimination of living and dead cells *of M. bovis* BCG (10^6-10^1 cells/ml meat suspension) using PMA dye with a final concentration of 50 µM. SD: standard deviation of all 3 independent experimental replicates. $\log_{10}$ cells/ml equivalent < LOD means no PCR amplification (negative). Slope/PCR efficiency of the standard curve was −3.433/95.6% (qPCR system 1), −3.428/95.8% (qPCR system 2), −3.908/80.3% (qPCR system 3) and −3.375/97.8% (qPCR system 4).

cycles for all PCR systems. Fig. 4 shows the mean value of the $\log_{10}$ cells/ml equivalent (with standard deviation: SD) of all 3 independent experimental replicates of each sample type (fresh and dead cultures with and without PMA, respectively). The amount of amplified DNA from fresh and from dead cultures is similar when using the same qPCR system. The limit of quantification (LOQ) of both culture types was down to 10^2 cells/ml and the limit of detection (LOD) was 10 cells/ml meat suspension, tested with the qPCR systems 1, 2 and 4. It was observed that the standard deviation (SD) of all sample types from all 3 independent experimental replicates had by tendency a wide range, if the concentration of *M. bovis* BCG was low.

For qPCR system 1, the PCR amplification of fresh and dead cultures with PMA was similar. The LOD of cultures treated with PMA (both fresh and dead cultures) was 10^2 cells/ml meat suspension. By using qPCR systems 2 and 4, the LOD of fresh cultures treated with PMA was 10^2 cells/ml and 10^3 cells/ml, respectively. By contrast, the LOD of dead cultures treated with PMA and tested with both qPCR systems was 10^4 cells/ml. The PCR amplification by using qPCR system 3 was impaired, since the LOD of both fresh and dead cultures was only at the concentration 10^3 cells/ml meat suspension, while the LOD of fresh and

dead cultures treated with PMA was 10^4 and 10^6 cells/ml meat suspension, respectively (see Fig. 4).

3.6. Isolation of mycobacteria from meat of red deer

A single suspicious colony was isolated from one of 50 venison. The DNA extract from the isolated colony was submitted for PCR amplification using all qPCR systems (Table 2). It was positive only with qPCR system 4 which is specific for the genus *Mycobacterium*. The sequence of the 16S rRNA gene, amplified with universal primer pairs for bacteria (335F & Com 2 and 769F & 1492R, Dorn-In et al., 2015) shows an identity of 99% to a DNA sequence of *Mycobacterium avium* provided in GenBank (accession no. CP016396.1, NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

4. Discussion

This study intended to develop a method to differentiate living and dead cells of the *Mycobacterium tuberculosis*-complex (MTC) in venison by using Propidium Monoazide (PMA™ dye) and a subsequent quantitative PCR (qPCR). The mycobacterial species *M. bovis* BCG was used as a reference strain for the target organisms MTC. Three independent experimental replicates of 1.0×10^6 cells/ml meat suspension determined by microscopy contained about $3.2–3.5 \times 10^5$ culturable cells/ml, which means about $6.5–6.8 \times 10^5$ dead, culturable clumped or impaired cells/ml were present in one ml meat suspension.

The sensitivity testing of qPCR system 1, 2 and 4 showed that the limit of detection (LOD) of *M. bovis* BCG was down to 10 cells/ml meat suspension. This means, the large target fragment of 473 bp (qPCR system 4) and 535 bp (qPCR system 2) provided similar results as the short target fragment of qPCR system 1 (129 bp). The LOD of qPCR system 3 was 10^3 cells/ml, which is probably caused by a too large amplicon size (760 bp) which impedes the efficiency of PCR amplification (Thornton and Basu, 2011). As specificity test, the qPCR systems 1, 2 and 3 amplify exclusively the DNA from MTC, while qPCR system 4 could also amplify non-TBC mycobacteria. Thus, qPCR system 4 was further applied to quantify the amount of mycobacterial DNA in all field sample types.

The amount of non-TBC mycobacterial in venison was ≤ LOD. Fecal samples of wild boars contained higher mycobacterial DNA than feces of red deer. This result may be attributable to the different region of sample origins (North Poland vs. South Germany) as well as the DNA extraction method (PowerSoil DNA Isolation kit vs. QIAmp Viral RNA Mini kit). In this study, fecal samples were intended to be used for the validation of the qPCR systems 1, 2 and 3 only. Therefore, the LOD of mycobacterial DNA in fecal samples by using both DNA extraction kits, PowerSoil DNA Isolation kit and QIAmp Viral RNA Mini kit, was not evaluated in this study.

Afterwards, the efficiency and toxicity of PMA dye were evaluated. A higher final concentration of PMA dye is reported to increase the efficiency to discriminate the dead cells from living cells (Fittipaldi et al., 2012). The most often used PMA dye concentration is between 50 and 100 μM (Nkuipou-Kenfack et al., 2013). The PMA dye is considered nontoxic, however, different bacterial species response differently to the dye (Nocker et al., 2006).

In this study, the group of dead cultures (10^6 cells/ml) treated with the PMA concentrations 25 μM, 50 μM and 100 μM showed no statistically significant difference ($p > 0.05$) of PCR amplification, although the concentration of 100 μM PMA showed by tendency of a higher efficiency to discriminate dead cells from living cells. The toxicity of PMA was tested with fresh cultures (10^6 cells/ml). The results of all qPCR systems show that the $\Delta\log_{10}$ levels between fresh culture treated with 25 and 50 μM PMA were up to 0.1 $\log_{10}$ and between 25 and 100 μM PMA they were from 0.1 to 0.2 $\log_{10}$. These differences were marginal; however, this could be the result of the toxicity of PMA in higher concentrations.

Further, the amount of DNA of fresh cultures (10^6 cells/ml) treated with PMA was about 1.0–1.5 $\log_{10}$ levels (ΔCt-value about 3.2–5.2 PCR cycles, dependent on PCR system) lower than fresh cultures without PMA, independently of the final concentration of PMA dye. This difference is statistically significant ($p < 0.05$) and might partly result from a concentration of dead cells up to $6.5–6.8 \times 10^5$ cells/ml present in fresh cultures as described above, not only from the toxicity of PMA.

Therefore, to avoid unknown adverse effects by using higher concentrations, the final concentration of 50 μM PMA was further applied to the serial dilutions of fresh and dead cultures of *M. bovis* BCG (10^6 to 10^1 cells/ml meat suspension). All serial dilutions were further tested with the qPCR systems 1, 2, 3 and 4.

A commercial qPCR kit (Ingenetix, qPCR system 1) could not differentiate between dead cells and living cells by using PMA dye, since the DNA of 10^3 and 10^2 dead cells/ml treated with PMA could be detected. qPCR system 2 provided a better result compared with the other qPCR systems, namely, no PCR signal is detected if the concentration of dead cells is ≤ 10^4 cells/ml meat suspension. However, the LOD of fresh cultures with PMA was only down to 10^2 cells/ml (containing about 35 culturable cells/ml), which was higher than the LOD of fresh culture without PMA (10 cells/ml).

By using qPCR system 3, there was an absolute suppression of PCR amplification of samples containing ≤ 10^5 dead cells/ml meat suspension. However, this qPCR system was not optimal due to low PCR efficiency and low sensitivity as described before. qPCR system 4 provided a similar result as system 2, except the LOD of fresh cultures with PMA was 10^3 cells/ml meat suspension (about 350 culturable cells/ml) which was 1.0 $\log_{10}$ higher than the LOD obtained with qPCR system 2. According to the results, the toxicity of PMA are most obviously seen in samples containing a low amount of bacteria such as between 10^2 vs. 10^1 cells/ml (qPCR system 2) and between 10^3 vs. 10^2 cells/ml (qPCR system 4). Although the toxic effect remains present, it becomes more and more negligible in higher concentrations.

A report of de Assunção et al. (2014) shows that about 2.3×10^2 dead cells of *M. tuberculosis* treated with 50 μM PMA cannot be absolutely discriminated by subsequent qPCR amplification of a 70 bp target fragment of the intergenic region. Similar with a report of Kim et al. (2014) who used the commercial Anyplex MTB/NTM Real-Time Detection (Korea), the ΔCt-values of dead cells of *M. tuberculosis* with and without treating with 50 μM PMA are not constant and range between −0.8 and 17.7 PCR cycles. Ricchi et al. (2014) used 50 μM PMA to discriminate living and dead *M. avium* subsp. *paratuberculosis* in milk by subsequent PCR amplification of 140 bp of the IS900 gene, resulting in a ΔCt-value between dead cells with and without PMA < 5.0 PCR cycles. In this study, the ΔCt-value between dead MTC bacterial cells with and without PMA obtained from qPCR system 2 (535 bp) was constantly between 12.0 and 13.0 PCR cycles, corresponding to about 3.5–4.0 $\log_{10}$ cells/ml, independent from the concentration of the total MTC in suspension. The ΔCt-value between fresh culture of MTC cells with and without PMA was 5.0–6.5 PCR cycles, corresponding to about 1.5–2.0 $\log_{10}$ cells/ml. This Ct-difference was partially according to dead cells present in fresh cultures and additionally to the toxicity of PMA to the living cells as discussed above.

The light treatment is applied in order to induce cross-linking of PMA to dsDNA, so that the dsDNA can not be amplified by subsequent PCR. In this study, we did not compare the efficiency of halogen lamp (1000 Watts) with other light sources provided by supplier e.g. PMA-Lite™ LED Photolysis Device (Biotium) or PMA-BLU-V System photoactivation device (Dynex). The 1000 Watts halogen lamp additionally produces excessive heat, which may cause the unknown effect to the efficiency of PMA or even to living cells, that they may die during heat treatment. Therefore, the sample tubes had to be put on ice to reduce the temperature during light treatment. However, the halogen lamp was commonly used for this purpose by other studies with different Watt, time of exposure and distance to the samples e.g. 650-W, 2 min, 20 cm (de Assunção et al., 2014), 600-W, 5 min, 20 cm (Kim et al.,

2014) and 650-W, 2 min, 15 cm (Ricchi et al., 2014). In this study, we used a 1000-W halogen lamp, an exposure time of 2.5 min (2 times), and the distance between sample tubes and light source was 30 cm.

Apart from the toxicity effect of PMA and light treatment procedure with the halogen lamp, the different PMA concentrations (25, 50 and 100 μM) applied in each study was not a crucial point, but the subsequent PCR amplification and the amplicon sizes of the target DNA may highly affect the efficiency of differentiation between living and dead MTC. Once inside the cells, the dye intercalates into the nucleic acids with a certain stoichiometry and inhibits the PCR amplification of the target sequence. The frequency in which the dye binds to the nucleic acids within the large target fragment is higher than within the short fragment. Thus, the efficiency of PMA dye to discriminate dead microorganisms is higher when using a larger target sequence (Soejima et al., 2008; Contreras et al., 2011; Martin et al., 2013). On the other hand, a short amplicon size is recommended in order to increase the efficiency of qPCR (Thornton and Basu, 2011). Thus, most specific primer pairs for MTC produce only small fragments such as in the qPCR kit that is commercially available from Ingenetix (Austria, 129 bp) or provided by other studies such as Park et al. (2000; 70 bp) or by the Friedlich-Loeffler Institute, Germany (FLI, 2015: HELI + EK- and S1081 + EK-System; 91 bp and 85 bp, respectively). However, to increase the efficiency of the differentiation of dead cells by using PMA dye, a primer set producing a large amplicon size is required. Therefore, qPCR systems 2 and 3 were developed in this study. While the PCR efficiency was impaired by using primer pairs targeting to too large target fragment (760 bp, qPCR system 3), the qPCR system 2 targeting a fragment of 535 bp generated the highest PCR efficiency to discriminate dead cells from living MTC among the tested qPCR systems in this study.

Although all venison samples were negative for MTC, tested by qPCR system 1, 2 and 3, 40% thereof showed slightly positive signals (≤LOD) for non-tuberculous mycobacteria using qPCR system 4. As a result of culturing, a single suspicious colony was isolated from one venison. The sequence consisting of about 1000 bp of the16S rRNA gene of this colony aligns with *Mycobacterium avium* provided in Genabank (NCBI). This is the first report of the detection and isolation of non-tuberculous mycobacteria in game meat obtained from red deer hunted in Bavaria, Germany. The subspecies differentiation of this isolate was not performed. However, some subspecies of *M. avium* have been linked to certain human diseases, for example pulmonary diseases and lymphadenitis, especially in immunocompromised patients (Sangari et al., 1999).

For culturing, a total volume of 10 ml meat suspension was concentrated by centrifuge, then the pellet was dissolved with 1 ml medium suspension and spread on specific solid medium. Thus the result of culturing represents sample volume of 10 ml meat suspension, while the sample volume used for DNA extraction was 0.5 ml. Additionally, the contaminating mycobacteria may not disseminate equally on the surface of meat. Thus, in order to increase the probability of detecting mycobacteria in meat sample by DNA extraction and PCR amplification, the pelleting of large sample volume (e.g. 10 ml) as performed by culturing method may be required. The detection of DNA of MTC and other non-tuberculous mycobacteria in food by using qPCR based methods and culturing was previously reported by Sevilla et al. (2017), namely, *M. bovis*, *M. avium*-, *M. fortuitum*- and *M. terrae*-complex in cow milk and cow milk products (e.g. cheese, yogurt and milk powder). In one ground beef and one chicken sausage, *M. avium* was detected by culturing, but not by direct PCR amplification of products. While another chicken sausage and one horst hamburger sample were positive for *M. avium* by direct PCR detection, but not by culturing (Sevilla et al., 2017).

In this study, although the LOD of MTC was down to 10 cells/ml, it was not possible to exclude the presence of MTC in meat samples, as observed by culturing of *M avium* with a negative qPCR result from one venison. The application of PMA for the differentiation between living and dead MTC has to be carefully performed and interpreted regarding to toxicity potential, especially if only a low amount of living cells is present in samples. The method of light treatment by halogen lamp should also be standardized in order to achieve optimum results. Since MTC may be present only in a low amount and may not spread equally on the surface of meat, optimization of sample preparation in order to obtain sufficient MTC cells (at least 10 cells/reaction, if present) for the DNA extraction is required for further studies. Nevertheless, optimized rapid detection and differentiation between living and dead cells by using PMA dye and specific qPCR as described in this study may be useful to exclude a potential risk for the consumer.

5. Conclusions

According to the report of the increasing number of natural infections with bTB in wild animals (EMIDA, 2013) and the increasing consumption of game meat (BfR, 2006) in Germany, the risk of contamination with living MTC in game meat should be taken into consideration. The molecular biological method described in this study allows rapid detection, quantification and, to a certain extent, differentiation of dead and living cells of bacterial members of MTC. The method may be useful for screening and monitoring the level of contamination of living or dead cells of MTC in meat and meat products and, after appropriate adjustment, in other sample types such as milk, feces and environmental samples from areas with a high prevalence of bovine tuberculosis and other MTC agents.

Declarations of interest

None.

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