Robustness of *Mycobacterium Bovis* Determination Using Fast and Simple qPCR Assay

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Abstract. *Most of the acute intestinal diseases are caused by food-borne pathogens. The robustness of qPCR-based fast and simple procedure for food safety detection of Mycobacterium bovis (M. bovis) DNA using EvaGreen real-time PCR for LightCycler was evaluated. Tm calling and Cp were used for analysis of PCR products. Tm calling showed better performance than Cp-based calculations for near limit of detection (LoD) positive samples. The studied qPCR M. bovis assay showed good sensitivity and excellent robustness, which allows using this assay during emergency or when this method is rarely used.*

Introduction

The frequency of outbreaks of food-borne infection cases worldwide is still extremely high (Fleckenstein et al., 2010; Postollec et al., 2011). Food pathogens are commonly found in the intestines of healthy food-producing animals, and can be transmitted to humans through contamination of the food chain. *Mycobacterium bovis*, a member of the *Mycobacterium tuberculosis complex* pathogen group (MTBC), is responsible for both bovine tuberculosis (bTB) in cattle and zoonotic tuberculosis (TB) in humans (Collins et al., 2022). The transmission of *M. bovis* is possible in a number of different ways, involving mechanisms such as respiratory secretions, environmental contamination, and consumption of contaminated food. Historical data indicate that in Europe among all bacteriologically confirmed TB cases the median proportion of *M. Bovis* or *M. Caprae* infections was 0.4% (range 0%–21.1%) (Müller et al., 2013). Beyond direct health consequences, bTB has significant global economic implications in both developed and developing countries (Waters et al., 2011; Smith et al., 2006). The notable risk of *M. bovis* transmission through drinking contaminated milk is in places where pasteurization is not mandatory (Smith et al., 2009).

Thus, a strict control of the whole food chain aimed to enforce contamination detection measures is required. Molecular methods for the detection and identification of food pathogens have significant benefits as compared to traditional methods due to speediness, sensitivity, specificity and accuracy. On the other hand, they often require dedicated instrumentation, highly trained personnel, and higher labour costs. Emerging molecular techniques are widely using isothermal amplification instead of temperature cycling like PCR but PCR still remains the most used one. All of these claims are valid for both beneficial and pathogenic bacteria (Severgnini et al., 2011; Jošić et al., 2016). The advantage of molecular methods is their universal character, i.e., most of them can be used on different platforms – capillary instruments, plate instruments or micro-arrays or can be easily adapted to them (Fukushima et al, 2007, 2010). There are two mainstreams in development of new PCR-based methods. One is multiplex qPCR with a simultaneous amplification of several microbes in a single reaction (Karus et al., 2017; Cremonesi et al., 2014). In this approach, the primer sets are designed with a similar annealing temperature but significantly differentiating melting temperatures of amplicons to distinguish between amplicons following thermal cycling (Karus et al., 2017; Zhao et al., 2014). Performing a multiplex assay instead of several singleplex analyses might reduce the total costs for testing (Binnicker, 2015). Still, multiplex methods are more complex. Thus, they are less robust and usually need well trained personnel to perform. Another approach is to identify different pathogens in different capillaries or PCR plate wells. This eliminates the possible effect of competition, and the primer concentration can be higher and sensitivity can be better. There are also mixed methods available (tandem-PCR) where the short conventional multiplex PCR is followed by nested single-well target amplification using qPCR (Ginn et al., 2017). All of these methods can be used in food safety as well as in clinical (human and veterinary medical) settings depending on sample material. Although PCR can reach high sensitivity and specificity, its introduction for routine detection has been frequently hampered by a lack of robustness (Van der Wolf et al., 2001).

The objective of the present study was to revise the analytical sensitivity and test the *Mycobacterium bovis* detection assay robustness on unexperienced users.

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Such an analysis can be used to analyse any kind of food, including but not limited to milk, cheese, meat products and any animal origin novel food (Karus and Karus, 2018). However, the outbreaks of *M. bovis* are not frequent, that means that this test is not used widely in routine and, thus, the robustness is crucial if this assay should be implemented in a limited time period. The aim of the study was to investigate whether the involvement of non-proficient users impacts the outcomes of analysis and to identify potential limitations in PCR-based assays under such circumstances.

Materials and methods

Samples and primer design

The concentration of *M. bovis* genomic DNA used for reference target strains varied from 1.0 till 1.9 ng/µL. Bacterial strains and accession number of target genes of isolated genomic DNA and primer sequences are listed in Table 1. Primers for real-time PCR amplification were designed using PRIMER EXPRESS (ver. 3.0). Primer pairs were ordered from Tib-MolBiol (Germany) and tested for EvaGreen assays on LightCycler 2.0® (Roche Diagnostics GmbH, Switzerland) using 5x HOTFIREPol® EvaGreen® qPCR Mix Plus(Capillary) with 7.5 mM $MgCl_2$ ready-to use mastermix (Solis BioDyne, Estonia). Samples were prepared using isolated bacterial DNA and NA-free water from Roche Diagnostics (Germany). No real food samples were used.

EvaGreen PCR assay

Quantitative PCR reactions followed by melting curve analysis were performed in a final volume of 20 µL. Capillaries content is given in Table 2. The reaction mix was prepared immediately (up to 30 min) before the run. Tenfold dilutions of the target genomic DNA were tested to determine the fair amount of template DNA detected by the assay (PCR sensitivity). The used testing thermal profile and full analysis protocol are given in Table 3.

In total, 38 positive samples with initial content of MB nucleic acids from 1E-9 g to 8E-9 g and 29 negative samples were performed by 2 proficient, and in total 63 fully instructed, users, who had no hands-on (first time) experience in qPCR. Two experienced laboratory technicians analysed one positive and one negative sample in each run in parallel to ensure the comparability of results.

Tm calling was performed at λ 530 nm using LightCycler480 SW1.5.0.

Results

Analytical sensitivity

The analytical sensitivity in testing was identified by analyses of tenfold dilution series of bacterial DNA. The 95% hit rate was calculated from log(g/analyse): hit rate plot at 5.6E-11 g (Figure 1) according to a hit rate calculated from positively identified amplification products by Tm calling. Tm of *M. bovis* in this assay was 84.32°C (sd 0.08°C).

User dependent assay robustness

To analyse the potential effect of a laboratory technician, we involved non-skilled personnel and made the analyses on different days, different runs and involved total of 63 persons for the analyses. Every non-skilled person prepared and performed only one sample and one analysis of a sample with blind content to avoid any effect of manual training or sample content concern.

The results show that all (100%) positive samples

Table 1. Reference target bacterial strain and primers designed using PRIMER EXPRESS **(ver. 3.0)**

Species	Strain	Gene target	Primer forward	Tm
Mycobacterium bovis	DSM 43990	hsp	GGGTCAAGCTCGACGTTGA	58°C
			Primer reverse	Tm
			CGGTGGTCCGTTTGGAACT	58°C

DSM – strains obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig (Germany)

Table 2. Reaction mix content

	c	V (µL)	C final
MM	5 x	4	1 x
Primer f	5 pmol/µL	1	250 nM
Primer r	5 pmol/µL	1	250 nM
Sample (DNA template)		5	
H_2O total		9	
TOTAL		20	

Table 3. qPCR program

Program Name		Hotstart		
Cycles	1	Analysis mode	None	
Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
95	None	00:15:00	20.00	
Program Name		Amplification		
Cycles	45	Analysis Mode	Quantification	
Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
95	None	00:00:05	20.00	
60	None	00:00:14	20.00	
72	Single	00:00:31	20.00	
Program Name		Melting		
Cycles	1	Analysis mode	Melting curves	
Target (°C)	Acquisition Mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
75	None	00:00:10	20.00	
95	Continuous		0.15	5
Program Name		Cooling		
Cycles	1	Analysis mode	None	
Target (°C)	Acquisition mode	Hold (hh:mm:ss)	Ramp rate (°C/s)	Acquisitions (per °C)
40	None	00:00:15	20.00	

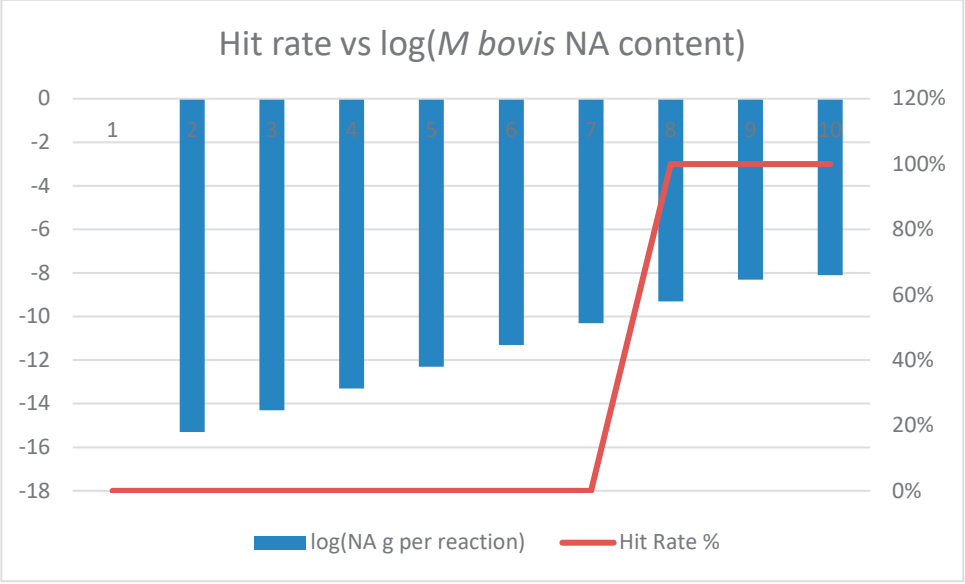


Figure 1. Hit rate vs log (M. bovis NA content g)

were finally identified as positive by melting curve analyses (Tm calling, Table 4). This is important, as the lowest content for analyses was near LoD, only one ng per reaction. To detect possible cross-contamination, a number of negative samples were analysed on all runs together with different concentration positive samples, and no cross-contamination was detected.

Tm calling versus quantification (Cp calculation from an amplification curve by 2nd derivative Max)

In robustness of the assay, we evaluated the hit rate by the 2nd derivative max calculation of an amplification curve in PCR cycling. The Tm calling

Table 4. Summary of robustness study based on Tm calling

Target g				
Reaction	N tested	N positive *M bovis*	N negative *M. bovis*	Hit rate
0	29	0	29	0%
1.00E-09	11	11	0	100%
2.00E-09	11	11	0	100%
4.00E-09	12	12	0	100%
8.00E-09	4	4	0	100%
Total	67	38	29	

Table 5. Summary of robustness study based on Cp value

Target g				
Reaction	N tested	N positive *M bovis*	N negative *M. bovis*	Hit rate
0	29	0	29	0%
1.00E-09	11	8	3	73%
2.00E-09	11	11	0	100%
4.00E-09	12	12	0	100%
8.00E-09	4	4	0	100%

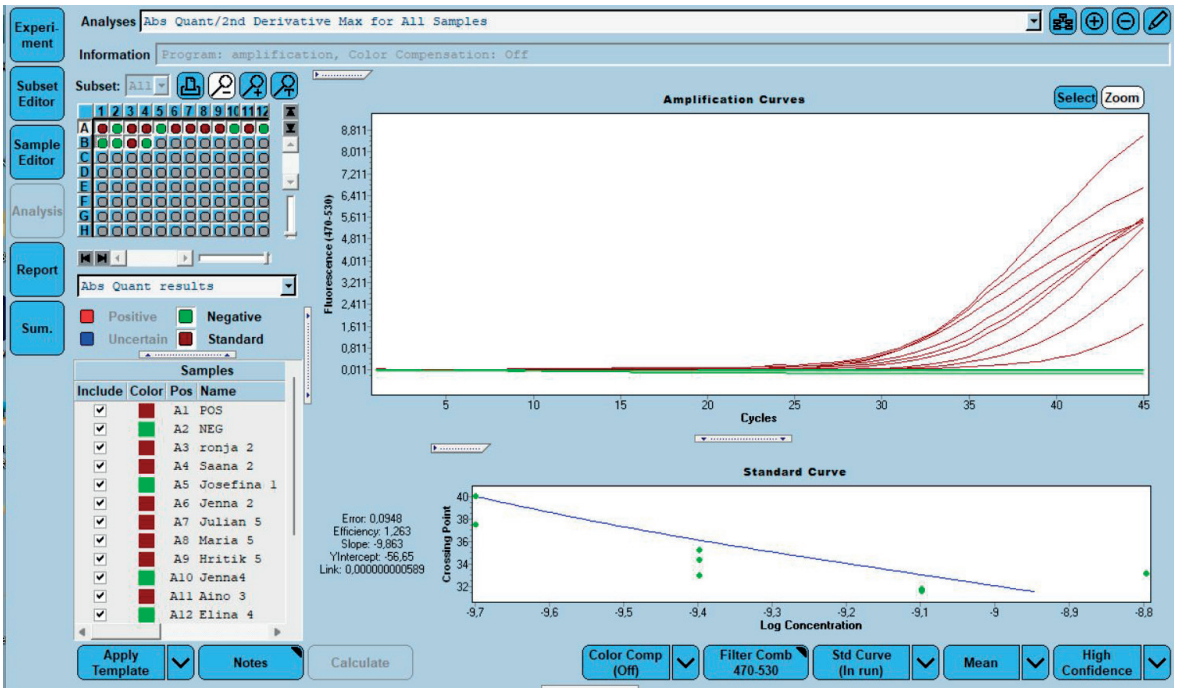
allows finding all positives by non-trained users, but not using simple Cp (quantification analyse, Table 5). This shows how important it is in such analyses not to rely only on Cp values, but also perform the melting curve analyses although this is mostly done for positive samples to prove the specificity of analysis by amplified product Tm.

Examples of quantification and melting curve analyses are given in Figure 2 and Figure 3.

The sensitivity calculations using Cp values were lower as LightCycler Software was not able to calculate Cp-values of low concentration samples and showed a status of "> - Late Cp call (last five cycles) has higher uncertainty".

Discussion and conclusions

It is generally accepted (Amagliani et al., 2006) that prevention of food-borne disease basically depends on surveillance and prompt identification of pathogens in food products. The major advantage of the current molecular method is simplicity, short time necessary to obtain the results and the robustness of an assay. To ensure better analytical sensitivity, we increased the sample volume to 5 µL compared to earlier protocols (Karus et al., 2017). This might be also relevant to compensate of pipetting errors in technicians. The limit of detection of our method is close to other latest methods with another target of *M. bovis* in singleplex qPCR analyses (0.01 ng *M. Bovis* BCG DNA) (Zeineldin et al., 2023). A crucial step for molecular assessment of microbial communities



Positive and/or spiked samples
Negative samples

Figure 2. Example of amplification curves of EvaGreen assays in a robustness test by unexperienced users

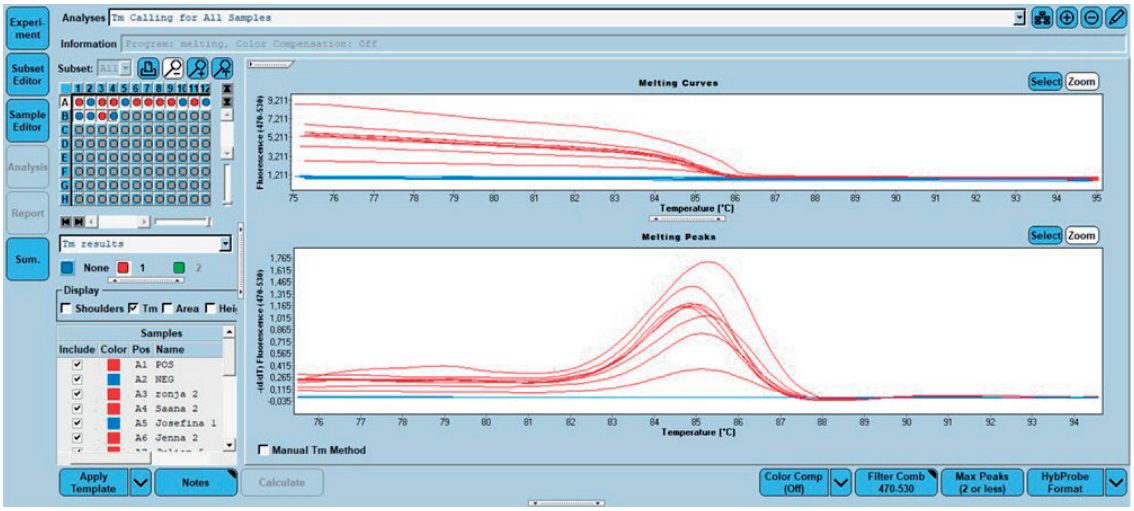


Figure 3. Example of melting curves of EvaGreen assays in a robustness test by unexperienced users

is the selection of a gene or genetic marker that can be used to differentiate a wide variety of organisms (Justé et al., 2008). Usually, the specificity of assays is ensured by using hydrolysis or hybridization probes (Severgnini et al., 2011). Indeed, any of all additional chemistries, even the most widely used TaqMan® chemistry, will increase significantly the costs of an assay. Several real-time PCR assays for a single reaction have been developed for the detection of the pathogens of our interest, and the trend has been moving towards strategies for a rapid identification of more than one pathogen through the development of multiple analysis platforms (Fukushima et al., 2010; Cremonesi et al., 2014). However, this trend does not improve the robustness of assays. Our results in *M. bovis* qPCR tests show that simple methods can be robust and have a good sensitivity when Tm calling is used. Analyses based on Cp values may cause caution due to the possibility of false negative results, but software also warns about late Cp, and, thus, prevents pathogen misdetection. Lázaro-Perona et al. (2021) showed lately, during Covid-19 pandemics, the importance of the method robustness (unexperienced user effect in nucleic acids purification), but also, that robust methods can be implemented quickly and efficiently (Lázaro-Perona et al., 2021).

There are still several concerns and limitations due to several risks in *M. bovis* analysis in real potential outbreak conditions by less experienced technicians. Efficient DNA extraction from complex matrices (such as food) can be challenging. Inhibitors present in food samples may affect PCR efficiency. Molecular methods can be highly sensitive, but false negatives can occur due to low bacterial load in samples or inhibitory substances. In our study, we did not find any cross-contamination, but when the target concentration is extremely high, there is still a danger of getting false positive results by cross-contamination of a sample itself or purified DNA by less trained technicians. Handling of live *M. bovis* poses also health risks. Proper inactivation methods are essential to protect laboratory personnel. Biosafety precautions are essential. These precautions will not diminish the importance of robustness of methods. In opposite, there is no guarantee that there is always enough laboratory capacity or no fully automated on-site testing methods available.

The real-time PCR assay described in this study has the potential to be a fast-screening assay for *M. bovis* enabling simultaneous processing of many food samples. Because the assay development does not include the sample preparation steps, the only prerequisite is to obtain good quality (good purity and sufficient concentration) purified DNA samples from various matrixes and can also be used for HACPP (analysing surfaces, etc) risk analysis, or other goals. It showed excellent performance even if used by 63 non-experienced technicians. This means that the assay does not require highly skilled molecular biology specialists to implement it, but requires only careful following of good laboratory practice. This assay may be used for accurate and rapid diagnosis of food-borne outbreaks as it has the potential to be used in routine diagnostic laboratories providing a simple, fast, cheap and sensitive alternative method to culture-based or TaqMan qPCR methods, especially if there is a need to implement this method quickly.

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