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REGULAR ARTICLES

Development of real-time PCR assay for the detection of *Mycoplasma bovis*

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Abstract

Mycoplasma bovis is one of the important bovine mycoplasma involved in economically important clinical conditions like respiratory diseases, otitis media, and mastitis. The present study was undertaken with the objective of developing a SYBR Green dye-based real-time PCR assay targeting *uvr*C gene for the diagnosis of *M. bovis*. The analytical sensitivity and specificity of the assay were evaluated. The test showed 10^3-fold more sensitivity than conventional PCR and detected down to 100 fg level of DNA. It was found to be specific, as no cross reactivity was shown with other related bacteria and *Mycoplasma* species. The developed assay was able to detect down to 40 copies of *uvrC* gene from spiked bovine milk samples. At present, this developed assay may be used as a valuable diagnostic tool for the detection of *Mycoplasma bovis*.

Keywords *Mycoplasma bovis* · *uvrC* gene · Real-time PCR

Introduction

Mycoplasmas are the smallest free-living micro-organisms capable of self-replication, highly fastidious bacteria, difficult to culture and are slow growing. Important pathogenic bovine mycoplasmas include *Mycoplasma mycoides* subsp. *mycoides* SC (*Mmm* SC), *Mycoplasma bovis* (*M. bovis*), *Mycoplasma bovigenitalium* (*M. bovigenitalium*), *Mycoplasma leachii* (*M. leachii*), *Mycoplasma bovirhinis* (*M. bovirhinis*), *Mycoplasma dispar* (*M. dispar*), and *Mycoplasma bovoculi* (*M. bovoculi*). Mycoplasmas appear to be increasingly involved in bovine mastitis, respiratory disease, and reproductive disorders worldwide (Nicholas et al. 2008). *M. bovis* is one of the most important pathogens causing respiratory disease, otitis media, mastitis, arthritis, and a variety of other disease in cattle worldwide, and is the second most pathogenic mycoplasma after *Mmm* SC (now known as *Mycoplasma mycoides* subsp. *mycoides Mmm*) (Maunsell et al. 2011; Fox 2012). Prevalence of *M. bovis*-associated mastitis both in cattle and buffaloes has been increasingly recognized as a problem globally (Fu et al. 2011; Eissa et al. 2012; Mustafa et al. 2013; Eissa et al. 2016). High morbidity rates and consequential economic losses are devastating to the affected cattle and buffalo farms, especially in developing countries (Wiggins et al. 2011; Eissa et al. 2012).

Diagnosis can be achieved by isolation and identification of the organism from the clinical samples. However, the fastidious nature of the organism, overgrowth of other contaminant bacteria, and inconclusive biochemical tests (observed routinely by initial isolation) make conventional bacterial identification methods unsuitable for routine diagnostic purpose. The real-time PCR is a very useful way of detecting active infection in a herd. Further, nucleic acid-based diagnostic tests have been developed to identify the organisms directly from clinical samples (Cremonesi et al. 2007). The most commonly used DNA-based techniques are PCR, Multiplex PCR, Nested PCR, PCR-RFLP, and LAMP.

During last decade, various probe-based real-time PCR assays have been developed for the diagnosis of *M. bovis* taking into account its advantages over traditional PCR-based detection systems. Fluorescent probe-based real-time PCR assay target 16S rRNA gene exhibited cross reactivity with *M. agalactiae* (Cai et al. 2005). DNA repair gene *uvrC*, which

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had certain unique sequences differing from *M. agalactiae*, was taken as target for designing new primer probe pairs in subsequently developed assay (Clothier et al. 2010). Sachse et al. (2010) used *oppD* as target gene for probe-based real-time PCR assay to detect *M. bovis* from milk, nasal, and conjunctival swabs whereas Boonyayatra et al. (2012) targeted the *fusA* gene encoding for elongation factor G of *M. bovis* in their developed assays. Ziv and Rajala (2012) designed a dual target TaqMan probe-based assay, targeting the housekeeping genes *fusA* and *oppD/F* of *M. bovis.* As the incorporation of probes increases the cost of real-time PCR assays, the present study was focused on developing a new SYBR Green dye-based real-time PCR assay targeting *uvrC* gene.

Materials and methods

Standard UK NCTC 10131 strain of *Mycoplasma bovis* was obtained from Referral Laboratory on Mycoplasma, Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar (U.P). *Mycoplasma bovis* was grown in modified PPLO broth medium (PPLO broth 30 g, yeast extract 2.5 g, glucose 2.0 g, sodium pyruvate 2 g, phenol red 0.5%, thallium acetate 5%, Benzyl penicillin 1000 IU/ml, DNA 0.2%, horse serum 10%), pH adjusted to 7.6–7.8, which was filtered through 0.22 μm Seitz filter and then incubated at 37 °C for 48 to 72 h to check sterility. The solid medium was prepared by adding 1.2% bacto agar (Difco) in PPLO broth medium. Prior to the real-time PCR assay development, *M. bovis* isolate was also biochemically characterized according to Aloutto et al. (1970).

Sample collection and processing

Fifty-one samples (30 buffalo lung tissue from local slaughter house and 21 milk samples from local dairies) were randomly collected from the areas of Bareilly District, Uttar Pradesh, India. The geographical location belongs to the agro-climatic zone of upper gangetic plains with a humid sub tropical climate (28.36° N; 79.43° E). All samples were enriched in modified PPLO broth for 24 h.

DNA extraction and PCR

Genomic DNA was extracted from standard *M. bovis* culture and clinical samples using Qiagen DNeasy Blood and Tissue kit, according to manufacturer's protocol. Primers targeting *uvrC* gene were designed using OligoAnalyzer software in DNASTAR Lasergenev6. Primers used in this study were Forward 5′-AAGTTGAAGTTGACCGGTTTG-3′ and Reverse 5′-TCCATATTTGGACCTAGTCCTTT-3′. Thermal cycling conditions were as initial denaturation at 94 °C for 5 min followed by 35 cycles of cyclic amplification-denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72 °C for 30 s with the final elongation at 72 °C for 10 min.

Real-time PCR assay

Real-time PCR assay was performed with the similar primer set as used for conventional PCR using Mx3000P spectrofluorometric thermal cycler operated by MxProTM PCR software. Reactions were run in duplicate, i.e., two sets at same time. Each PCR mix comprised of the total volume of 10 μl

Table 1 List of bacteria and *Mycoplasma* spp. used for evaluation of specificity of real-time PCR and PCR

Sl no	Bacteria	Strain/isolate (each single)	Source
1	*Staphylococcus aureus*	Field isolate	Division of Veterinary Public Health, IVRI
2	*Pasteurella multocida*	Field isolate	Hemorrhagic septicemia laboratory, IVRI
3	*Escherichia coli*	NZ1503–95	Division of Veterinary Public Health, IVRI
4	*Clostridium chauvoei*	Field isolate	Anaerobe lab, IVRI
5	*Brucella abortus*	NCTC10093	Genetic Engineering of Bacteria Laboratory, IVRI
6	*Salmonella Typhi*	Field isolate	National Salmonella Centre, IVRI
7.	*M.arginini*	NCTC 10129	Maintained with Referral Laboratory on Mycoplasma, Division of Veterinary Bacteriology & Mycology, IVRI
8.	*M. mycoides* subsp. *capri*	NCTC10137	
9.	*M. agalactiae*	NCTC 10123	
10.	*M. leachii*	NCTC 10133	
11.	*M. bovis*	NCTC 10131	
12.	*M. bovigenitalium*	NCTC 10122	

containing 0.25 μl each of forward and reverse primers (5 pmol each), 1 μl template DNA, and 5 μl 2× SYBRGreen QPCR Master Mix and 3.3 μl nuclease-free PCR grade water, 0.2 μl ROX (Low). The reaction parameters for denaturation time were optimized ranging from 1 to 3 s.

For calculation of copy number and generation of standard curve, obtained PCR product was cloned into pTZ57R/T vector (InsTA cloning kit, Fermentas) according to manufacturer's protocol. Recombinant plasmid DNA was isolated using GeneJET Plasmid Miniprep Kit. The plasmid concentration was adjusted to 100 ng concentration and copy number was calculated using the calculator found at: http://www.scienceprimer.com/copy-number-calculator-for-realtime-pcr. . Reactions were carried out using seven serial 10-fold dilutions for the generation of standard curve.

Detection and quantification of *M. bovis* in spiked milk samples

A serial tenfold dilution (ranging from 10^{-1} to 10^{-6}) of culture *M. bovis* standard UK NCTC 10131 (2.5×10^6 cfu/ml) was made in sterile PPLO broth. A 0.5 ml

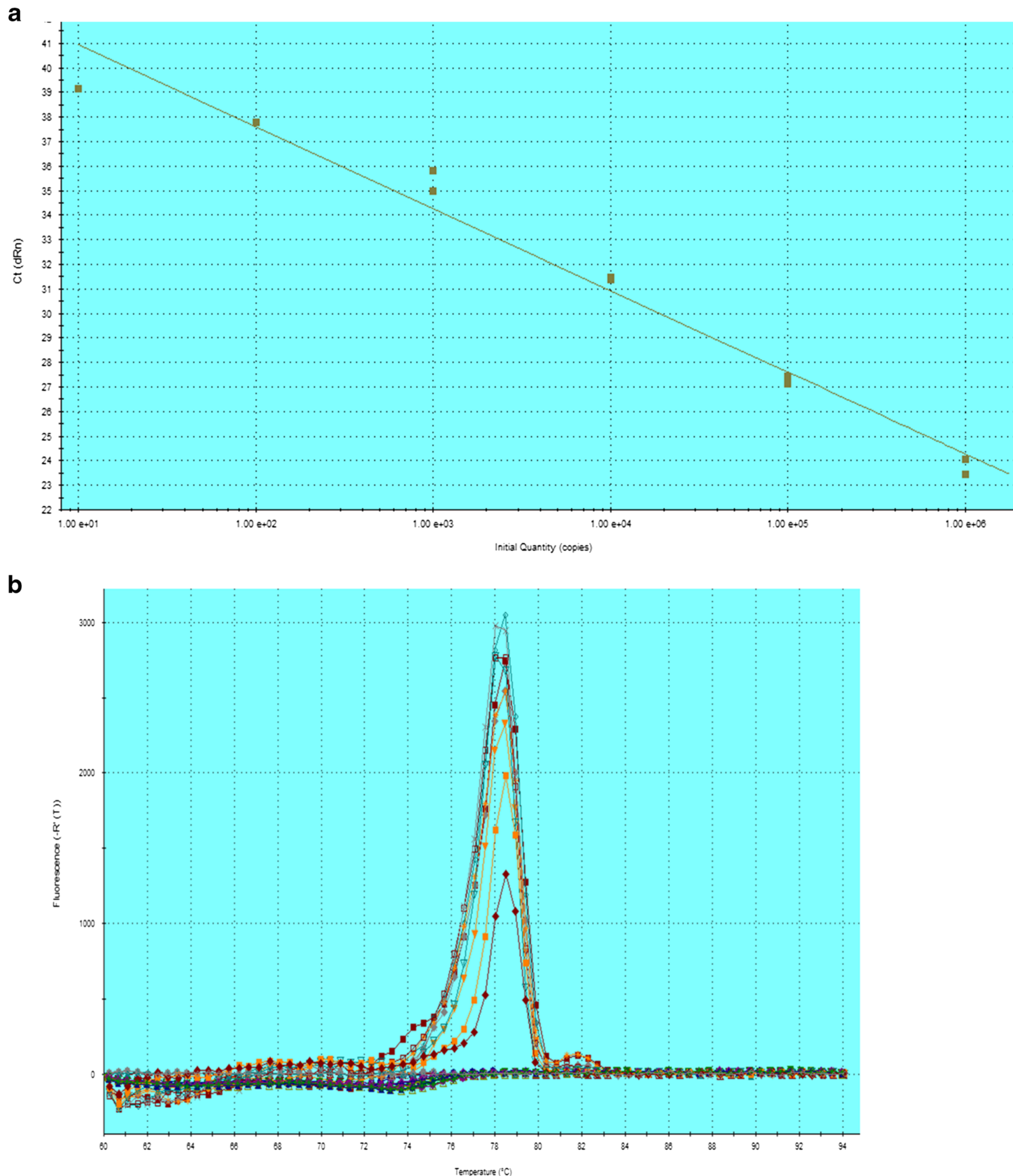

Fig. 1 **a** Standard curve of real-time PCR with 99.6% efficiency. **b** Melt curve showing specific product with a melting peak of 78.46 °C

volume of fresh cows milk was artificially contaminated with equivalent volumes (0.5 ml) of the mentioned serial dilutions of *M. bovis* culture. Before spiking, cow milk was checked for its sterility in modified PPLO broth and agar. Further, DNA was extracted from this serially diluted artificially contaminated milk using Qiagen DNeasy Blood & Tissue kit using manufacturer's protocol. The extracted DNA samples were kept in the constant volume of nuclease-free water and stored at − 20 °C (to minimize degradation) till further use in the real-time PCR and conventional PCRs assay.

Comparison of analytical sensitivity and specificity of real-time PCR with PCR

Real-time PCR and conventional PCRs were carried out under optimal reaction conditions using same primers set and their sensitivities were evaluated. Specificity of the developed real-time PCR and conventional PCR assays were assessed by performing the reaction with *M. bovis*, as well as other closely related mycoplasma and bacterial DNA (Table 1).

Results

M. bovis (NCTC 10131) showed typical small fried egg colonies on modified PPLO solid medium. In biochemical tests, it exhibited negative reaction for glucose fermentation, arginine hydrolysis, and serum digestion. However, the organism showed positive reaction for phosphatase test, tetrazolium reduction, and disc growth inhibition so confirming the specific characteristic of *M. bovis*.

Polymerase chain reaction

M. bovis-specific PCR was done using self-designed primers (as mentioned earlier in this text) which yielded a product size of 106 bp confirmed by agarose gel electrophoresis.

Real-time PCR assay

The cyclic conditions were optimized as per the KAPA SYBR FAST qPCR kit (KAPA BIOSYSTEMS, USA) with some modifications as follows: Enzyme activation (hold) at 95 °C for 3 min, followed by 40 cycles of cyclic amplification-denaturation at 95 °C for 3 s, annealing/extension at 60 °C for 20 s. A standard curve of real-time PCR of 99.6% efficiency was obtained (Fig. 1a) with a melting peak of 78.46 °C, the height of which may vary slightly higher or lower due to different *M. bovis* concentrations (Fig. 1b).

Detection and quantification of *M. bovis* in spiked milk samples

Real-time PCR could detect down to 40 copies of *uvrC* gene in case of *M. bovis* spiked milk samples (Fig. 2). This corresponded to 250 cfu of *M. bovis.*

Comparison of analytical sensitivity and specificity of real-time PCR with conventional PCR

Real-time PCR could detect down to 100 fg DNA of standard *M. bovis* strain (Fig. 3a), while detection limit of the conventional PCR was 100 pg (Fig. 3b). Therefore, the developed real-time PCR assay was found to be 10^3-fold more sensitive.

On evaluation of analytical specificity using uniform DNA concentration of 10 ng per reaction (minimum detection limit observed in real time PCR) for all the six bacteria and six *Mycoplasma* species (Table 1), the amplification with real-time assay could only be observed in *M. bovis* (Fig. 4a, b).

Screening of collected bovine samples by real-time PCR assay

On screening of 51 randomly collected bovine samples, 1 milk sample (2%) was found to be positive showing a Ct value of 36.66 with a melting peak at approximately 78.5 °C which is consistent with that expected for *M. bovis*, and an estimated 191 copies of *uvrC* gene.

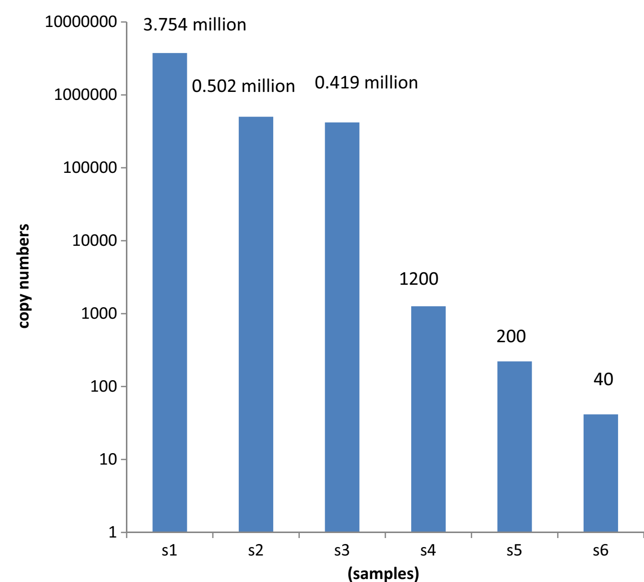

Fig. 2 Graph depicting the copy number of *uvr*C gene of *Mycoplasma bovis* in spiked milk sample

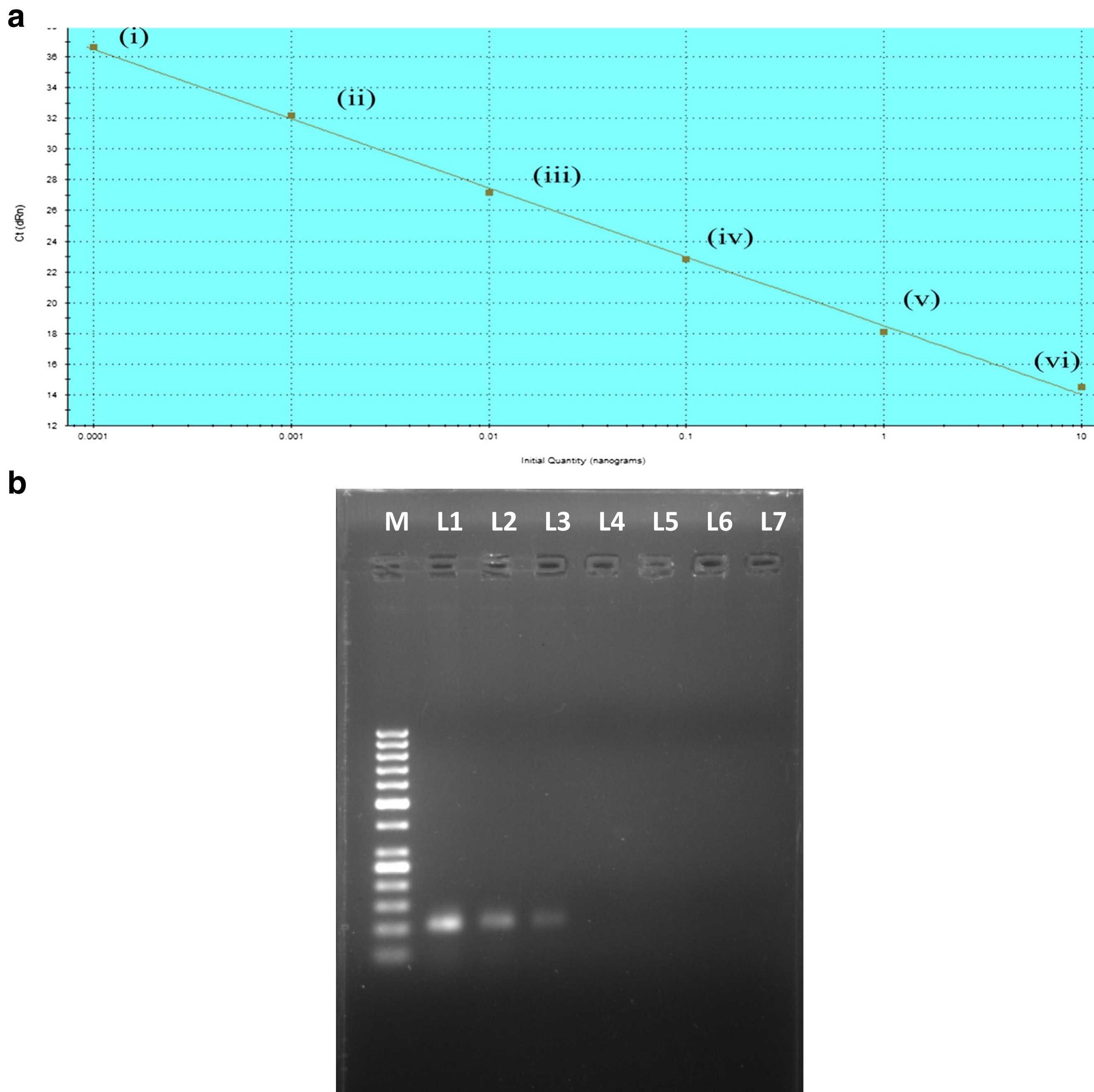

Fig. 3 Analytical sensitivity of real-time PCR and conventional PCR. **a** Standard curve showing sensitivity of real-time PCR up to 100 fg with low Ct value in (i), high Ct value in (vi) due to 10 ng conc. of template. **b** Agarose gel electrophoresis of PCR product showing sensitivity up to 100 pg. DNA concentration in L1 = 10 ng, L2 = 1 ng, L3 = 100 pg, L4 = 10 pg, L5 = 1 pg, L5 = 100 fg, L6 = 1 fg, L7 is negative control, M = 50 bp ladder

Discussion

Mycoplasma bovis is responsible for severe economic losses worldwide. The prevalence of the organism is usually underestimated due to difficulties in isolation and identification by conventional microbiological methods. Chronically infected animals continue to shed the organism and act as a source of infection in dairy herds (Nguyen and Truong 2015). Lack of detectable immune response in carrier animals at an acute infection stage with no clinical symptoms necessitates the use of molecular detection assays for screening purpose (Szacawa et al. 2016). In the current study, a new real-time PCR assay was developed based on the *uvrC* gene of *M. bovis*. This real-time PCR assay was found to be more rapid, sensitive and involves less labor than that of conventional PCR. Despite the reported genetic variability in mycoplasmas, the

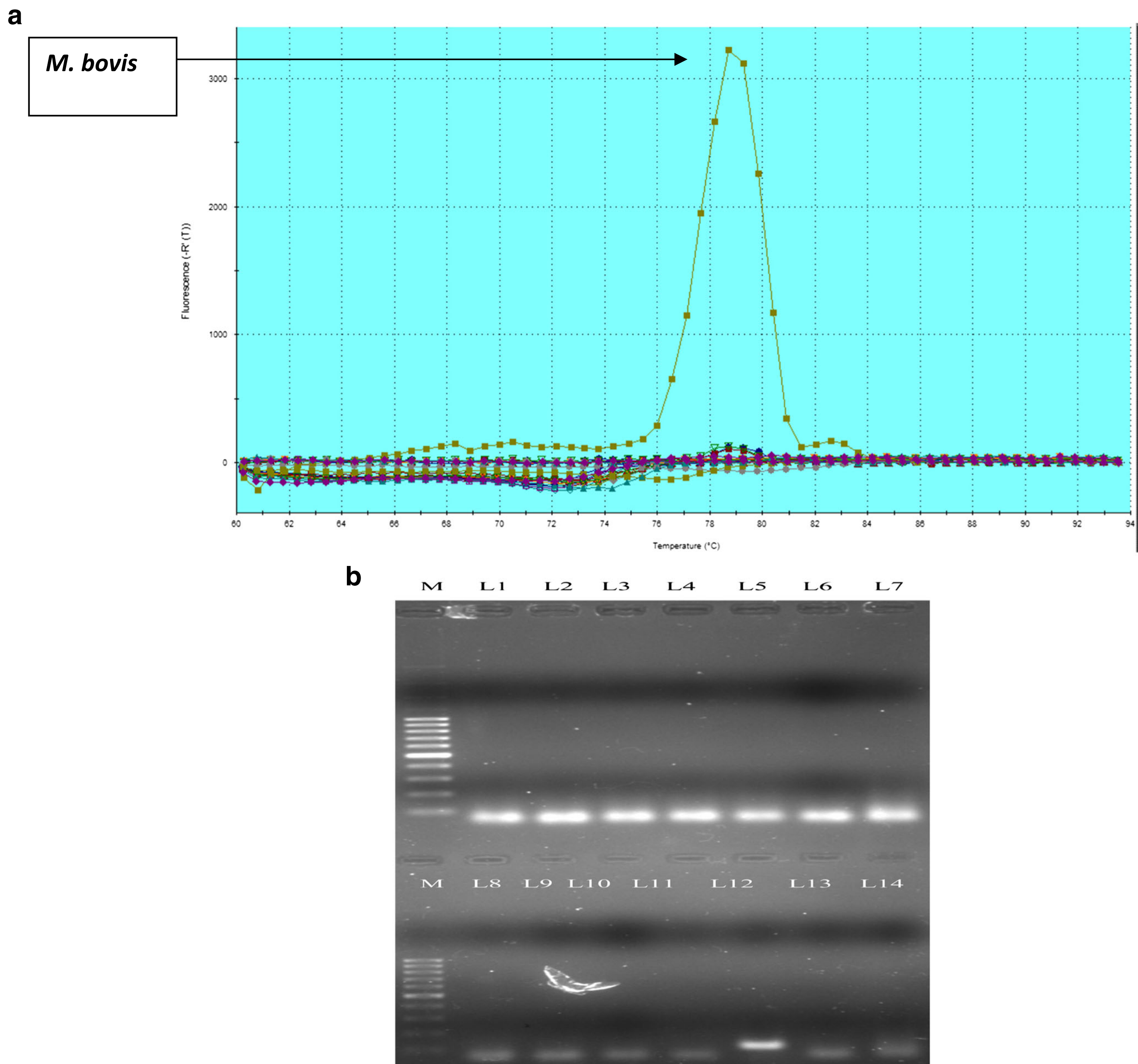

Fig. 4 Evaluation of specificity of real time PCR and conventional PCR. **a** Melt curve depicting presence of single specific product of *M. bovis*. **b** Agarose gel electrophoresis showing specific PCR product of 106 bp in lane 12, L1—*Staphylococcus aureus*, L2—*Pasteurella multocida*, L3—*E. coli*, L4—*Clostridium chauvoei*, L5—*Brucella abortus*, L6—*Salmonella typhi*, L7—*M. arginini*, L8—*M. mycoides* subsp. *capri*, L9—*M. agalactiae*—L10—*M. leachii*, L11—*M. bovigenitalium*, L12—*M.* bovis, L13 and L14—negative control

uvrC gene in *M. bovis* is stable and resists normal mutation pressures. This underlines the usefulness of the *uvrC* gene in *M. bovis* diagnosis with regard to the sequence stability of this essential gene (Thomas et al. 2004).

On the basis of observed Ct values and the standard curve, the assay demonstrated high sensitivity and was estimated to have a detection limit of 10 copies of *uvrC* gene of *M. bovis*. Sensitivity of this assay was on at par with previously reported TaqMan probe assays (Yoshida et al. 2002; Boonyayatra et al. 2012). It was superior in sensitivity and the detection time as compared to Naikare et al. (2015), which demonstrated detection limit of 83 copies of *uvrC* gene, i.e., approximately equal to 4.1×10^2 cfu/ml in 2.5 h.

For assessing the suitability of the developed assay for detection from clinical samples, spiking was done in freshly collected bovine milk sample using serial tenfold dilutions of *M. bovis* culture. Detection limit of 40 copies of *uvrC* gene (2.5×10^2 cfu/ml of milk) was consistent with the previous report of Clothier et al. (2010) who could detect 100 cfu/ml in milk. Cai et al. (2005) revealed sensitivity of real-time PCR

test to the level of 550 cfu/ml from spiked milk sample. Moreover, in our study, the detection level was up to 250 cfu from biological samples. In current study, we were able to detect up to 100 fg level of DNA; however, Naikare et al. (2015) recorded the sensitivity limit of 900 fg of genomic DNA. On evaluation of specificity using genomic DNA with 11 bacterial (*S. aureus*, *P. multocida*, *E. coli*, *C. chauvoei*, *B. abortus*, *Salmonella typhi*) and *Mycoplasma* species (*M. capri*, *M. agalactiae*, *M. bovigenitalium*, *M. arginini*, and *M. leachii*), real-time amplification was seen only in *M. bovis*. No cross reaction was recorded with closely related *M. agalactiae*, indicating its suitability to differentiate between *M. agalactiae* and *M. bovis*, which are cross reactive in most of the serological tests due to their high degree of homology.

Total 51 bovine (Buffalo) samples (21 milk and 30 lungs tissue) were screened to detect and quantify *M. bovis*. Out of these 51 clinical samples, only 1 milk sample (2%) was found to be positive with 1.91×10^2 copies/ml milk by the real-time PCR; however, the same was undetectable with conventional PCR test.

The probable reason of this poor detection number (only one animal) may be due to the lesser sample size in our case or else may be due to resistance in buffaloes to the diseases (Dua 2003). Further, the higher percentage of incidence in buffaloes (2/22 in clinical herd and 5/37 in sub-clinical herd) in earlier studies (Eissa et al. 2012) may be because of high endemicity of the disease (Sachse et al. 2010). Eissa et al. (2016) also observed 14.3% of *M. bovis* infection in clinical mastitis cases in Egyptian buffaloes.

The real-time PCR test described here was shown to be effective for the specific detection of *M. bovis* and as a potential tool for diagnosis of infection. The test compared well with the reported sensitivity of TaqMan probe-based assays and is rapid and inexpensive to use in a diagnostic setting. Moreover, the developed real-time PCR was 10^3 times more sensitive compared with that of traditional gel-based PCR, having an estimated detection limit of 40 copies of the *uvr*C gene of *M. bovis* in spiked milk samples.

This point to the suitability of developed assay as a rapid and economic diagnostic tool. As the sample size was relatively small, this promising assay warrants being applied on a wider scale to further assess its suitability in the field to enable its adoption for the screening of diseased/carrier animals in a healthy herd and further to minimize the cost of treatment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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