

Development of one-tube multiplex polymerase chain reaction (PCR) for detecting *Mycobacterium bovis*

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ABSTRACT. A multiplex PCR (m-PCR) with primers targeting the 16S rRNA, Rv3873 and a 12.7-kb fragment in the genomes of a *Mycobacterium tuberculosis* complex was designed for the differential diagnosis of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG and non-tuberculosis *Mycobacterium* (NTM). The specificity of this assay was 100%, and the detection limit was 15 pg of genomic DNA. Of the 206 blinded clinical samples, the detection rate of *M. bovis* infection by m-PCR was lower than that of the interferon gamma (IFN- γ) release assay; however, the false-positive rate by the tuberculin skin test and false-negative samples in the IFN- γ release assay were reduced. Our findings indicated that our m-PCR method is a useful tool for complementation to differentiate *M. bovis* from *M. tuberculosis* and NTM species.

KEY WORDS: differential diagnosis, multiplex PCR, *Mycobacterium bovis*, *Mycobacterium tuberculosis*

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Mycobacterium bovis belongs to the *Mycobacterium tuberculosis* complex (MTC) (which includes *M. bovis*, *M. tuberculosis*, *M. microti*, *M. africanum*, *M. pinnipedii*, *M. caprae*, *M. canetti* and *M. mungi*) [6, 9]. *M. bovis* primarily infects cattle, causing bovine tuberculosis (bTB), and occasionally infects other species of mammals [7]. *M. bovis* is an important veterinary disease that can spread to humans through the inhalation of aerosols or ingestion of unpasteurized dairy products [13]. The eradication of bTB is based on reliable diagnostic methods.

Over past several decades, the most commonly utilized diagnostic method for bTB identification is the tuberculin skin test (TST), which is economical and easily applied. However, one disadvantage of using bovine-purified protein derivative (PPD-B) is the shared antigenic components in PPD-B and nonpathogenic environmental mycobacterium, which can reduce the specificity of the TST [20]. Although the interferon gamma (IFN- γ) release assay showed higher specificity than the TST, the high cost of commercial ELISA kits limits its use in developing countries, such as China. PCR, including real-time PCR and nested PCR, has been shown to be effectively and easily applied in the detection of *M. tuberculosis*, requiring only 3–5 hr with higher specificity and sensitivity compared to culture methods [5, 8, 10, 15, 16]. However, some of these methods yield false-negative results, as the target sequences (such as IS6110) are not uniformly present in all clinical isolates [20]. Moreover, the sensitivity of PCR amplification ranges from 59 to 90% in sputum specimens

[2], but varies from 53 to 78% in non-respiratory specimens [1]. Therefore, the development of more sensitive methods is required.

In this study, we present a single-step multiplex PCR (m-PCR) assay targeting the 16S rRNA, Rv3873 and a 12.7-kb fragment as a rapid and simple assay for differentiating *M. bovis*, *M. bovis* BCG and *M. tuberculosis* as well as members of the MTC and non-tuberculosis *Mycobacterium* (NTM).

M. tuberculosis strain C68503, *M. bovis* strains C68001, C68004 and C68010, *M. bovis* BCG strain C68017, *M. avium* strains C68201 and C68203, *M. intracellulare* strain C68226 and *M. paratuberculosis* strain C68787 were obtained from the China Institute of Veterinary Drug Control (Beijing, China). *Escherichia coli* strain 0702 was preserved in our lab. Genomic DNA was extracted as previously described [17].

The primers for m-PCR in this study were based on previously described sequences [21]. The primers targeted a 229-bp sequence in *M. bovis*, which in the case of *M. tuberculosis*, is interrupted at position 197 by a unique 12.7-kb fragment. Oligonucleotide sequences of the primers were as follows: common forward primer, CSB1 (5'-ttccgaatcccttgta-3') and two reverse primers, including *M. bovis*-specific CSB2 (5'-ggagagcgcgcttgta-3') and *M. tuberculosis*-specific CSB3 (5'-agtcgctggcttctcttta-3'). Primers targeting Rv3873 (5'-tcgcgttgaccgagatggattat-3', 5'-cgctcatctcaccagttggc-3') may distinguish *M. bovis* BCG from *M. tuberculosis* and *M. bovis* [4]. Primers targeting 16S rRNA (5'-acggtggtactag-tgtgggttc-3', 5'-tctgcgattagcgaacta-3') with a unique fragment of 575 bp, which may distinguish *Mycobacterium* species from non-*Mycobacterium* species, were also used in this m-PCR system.

PCR conditions, such as annealing temperature, primer concentration, amplification cycle and Mg²⁺ concentration, were optimized. Finally, the m-PCR reaction mixture (25 μ l) consisted of 5 pmol of each primer, 0.5 μ l of Taq

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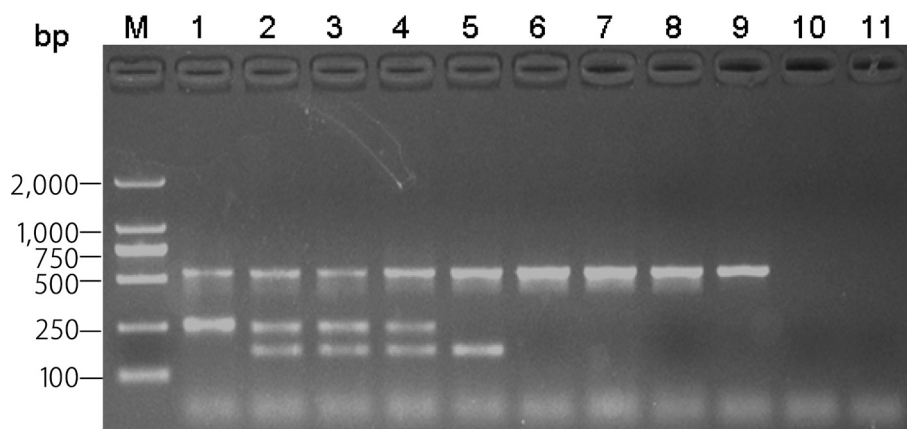


Fig. 1. Detection specificity of multiplex PCR. M. DL2000 Maker, 1. *M. tuberculosis* C68503, 2–4: *M. bovis* C68001, C68004, C68010, 5. *M. bovis* BCG C68017, 6. *M. avium* C68201, 7. *M. avium* C68203, 8. *M. paratuberculosis* C68787, 9. *M. intracellulare* C68226, 10. *E. coli* 0702, 11. negative control.

DNA polymerase (5 U/ μ l, Takara, Otsu, Japan), 2.5 μ l of 25 mM MgCl₂, 2 μ l of 2.5 mM dNTP mixture and genomic DNA. The PCR procedure involved 5 min of initial enzyme activation at 95°C, followed by 30 cycles of denaturation at 95°C for 45 sec, annealing at 57°C for 45 sec, extension at 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were electrophoresed in a 1.5% agarose gel and visualized by Gold View fluorescence. Individually, the primer pair of 16S rRNA (575 bp), CSB1/CSB2 (262 bp), CSB1/CSB3 (168 bp) and Rv3873 (255 bp) all present the expected bands in the appropriate strains.

A 2-fold dilution of *M. bovis* C68001 DNA was made from 1 ng to prepare 1 ng, 500 pg, 250 pg, 125 pg, 60 pg, 30 pg, 15 pg, 8 pg, 4 pg, 2 pg, 1 pg and 500 fg DNA template in each reaction. DNA from *E. coli* was used as a negative control. The detection limit of the assay was defined as the lowest concentration of DNA that could be amplified and visually detected.

The TST was performed according to the Chinese standard diagnostic technique for tuberculosis in animals (GB/T 18645-2002). PPD-B antigens (2,500 IU/animal) were injected into cattle in a volume of 0.1 ml. The skin thicknesses at injection sites were measured with calipers before and 72 hr after the skin test by the same operator. For the TST, if the difference in skin thicknesses was equal to or greater than 4 mm, the animal was considered to be TB-infected, while if the difference was less than 2 mm, the animal was considered to be free of TB infection [19]. A total of 206 cattle were blind-selected for the TST in Jiangsu province. Additionally, all 206 cattle were also diagnosed using the *M. bovis* IFN- γ test kit for cattle (Bovigam, Prionics AG, Schlieren, Switzerland) according to the manufacturer's instructions. Briefly, the anti-coagulant blood from each animal was dispensed into a 24-well tissue culture tray (200 μ l/well, using 3 wells for each animal) and stimulated with 50 μ l PPD-A (avian tuberculin PPD, 300 mg/ml, AsureQuality, Tullamarine, Australia), PPD-B (bovine tuberculin PPD, 300 mg/ml,

AsureQuality) and PBS. After incubation at 37°C in 5% CO₂ for 24 hr, the plasma in each well was collected for IFN- γ detection. Cattle with PPD-B-stimulated blood plasma and an optical density (OD) greater than 0.1 compared to PPD-A and PBS were considered to be TB-infected.

Nasal swabs of all 206 cattle were sampled and immersed in 2 ml sterile phosphate-buffered saline; the pellets, which potentially contained mycobacterium, were centrifuged and submitted to genomic DNA extraction as previously described. Next, genomic DNA was detected using the established m-PCR.

As shown in Fig. 1, the m-PCR reaction using the template DNA from *M. bovis* showed three bands (575 bp, 255 bp and 168 bp), while the reaction using the DNA from *M. bovis* BCG showed two bands (575 bp and 168 bp). The DNA from *M. tuberculosis* showed three bands (575 bp, 262 bp and 255 bp); the bands for 262 bp and 255 bp are very close in size, causing them to appear as one band on the agarose gel. In addition, DNA from NTM strains showed a unique amplicon of 575 bp, while DNA from *E. coli* showed no bands in this reaction system. These results suggest that m-PCR could effectively differentiate *M. bovis*, *M. tuberculosis*, *M. bovis* BCG and NTM. Figure. 2 shows that the lowest limit of detection for *M. bovis* was 15 pg DNA, with three clear PCR product bands (168, 255 and 575 bp).

To evaluate the detection specificity of this m-PCR, *M. bovis* infection in 206 cattle was detected in the m-PCR, TST and IFN- γ release assays simultaneously. Of the 206 cattle, 54 (26.2%) showed positive results in the TST based on Chinese standard criteria (GB/T 18645-2002). In contrast, 8.25% (17 cattle) showed positive results in the IFN- γ release assay from anti-coagulant blood. Only 7 cattle were found to be positive for *M. bovis* infection by m-PCR, and no *M. tuberculosis* or *M. bovis* BCG were detected (Table 1). In addition, genomic DNA from nasal swabs of 19 cattle showing positive results in the TST, and 25 cattle showing negative results in the IFN- γ release assay exhibited a unique

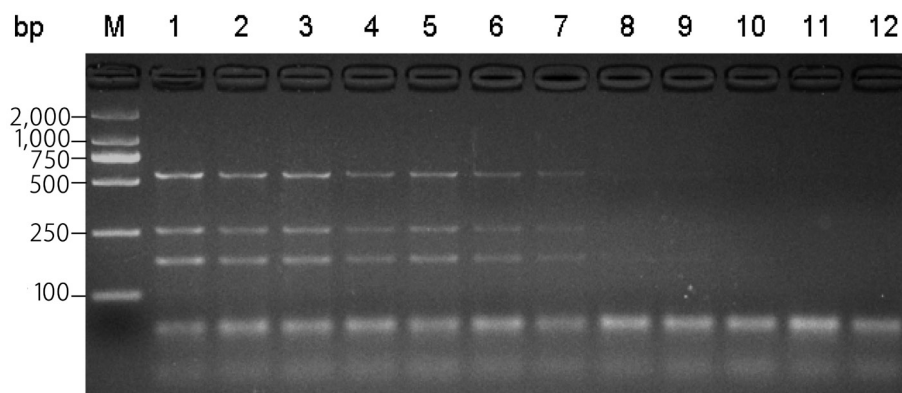


Fig. 2. Sensitivity of multiplex PCR for detecting *M. bovis*. Lanes 1–12 represent the PCR amplification results for different sample dilutions. The amounts of *M. bovis* in lanes 1–12 were as follows: 1: 1 ng, 2: 500 pg, 3: 250 pg, 4: 125 pg, 5: 60 pg, 6: 30 pg, 7: 15 pg, 8: 8 pg, 9: 4 pg, 10: 2 pg, 11: 1 pg, 12: 500 fg, M. DL 2000 Marker.

Table 1. Detection rate of *M. bovis* infection in 206 cattle by multiplex PCR, tuberculin skin test and IFN- γ release assay

	No. positive	%
Multiplex PCR test	7	3.34 (n=206)
Tuberculin skin test	54	26.2 (n=206)
IFN- γ release assay	17	8.3 (n=206)

band at 575 bp in m-PCR, indicating that the cattle may have been infected by NTM species (data not shown).

Various genes have been targeted in numerous PCR assays designed to differentiate between MTC and NTM [1, 11, 15]. However, most of the assays have been invalidated, because of their lack of specificity or sensitivity. The development of a species-specific PCR assay requires that a target gene or DNA fragment is present in all isolates from the particular species of interests and absent from all other unrelated species [3]. One of these molecular approaches is m-PCR, which targets a number of different genes, including insertion sequences (e.g., IS6110, IS6100 and IS1081). However, some of these methods yield false-negative results, as the target sequences (such as IS6110 targets) are not uniformly present in all clinical isolates [14]. In the current study, primers targeting the 229-bp sequence polymorphism generated in the presence of a 12.7-kb fragment in the *M. tuberculosis* genome, provided an effective method for definitive detection of these two closely related species [21]. This designed primers correctly identified *M. tuberculosis* (262 bp) and *M. bovis* (168 bp) at the species level in a single reaction tube. However, this reaction system based on a 12.7-kb fragment polymorphism could not differentiate *M. bovis* isolates from *M. bovis* BCG. Because the Rv3873 gene is absent from most or all vaccine strains of *M. bovis* BCG [12], primers targeting the Rv3873 gene are included in this multiplex PCR, which may distinguish *M. bovis* from *M. bovis* BCG. Furthermore, primers targeting species-specific 16S rRNA

are also included in the m-PCR system to identify NTM infection in cattle. Currently, the detection limit of this established m-PCR assay is 15 pg genomic DNA, which is lower than that of PCR targeting a single gene and may contribute to a lower *M. bovis* detection rate compared to the TST and IFN- γ release assay. In addition, some undefined materials in the nasal swab may affect the detection specificity and sensitivity. Consistent with our results, a novel PCR with two pairs of primers targeting the Type I signal peptidase gene for simultaneous detection of *M. tuberculosis* complex and *M. bovis* was established. Based on tuberculosis and non-tuberculosis strain DNA, this PCR method was highly specific and sensitive. However, the sensitivity and specificity in sputum samples were 84 and 76%, respectively [18]. Several factors may contribute to this low detection rate; for example, one of the critical factors is that infected cattle did not shed *Mycobacterium* through the respiratory tract. Additionally, other factors including excreted mycobacterium levels were very limited, and DNA was lost during the extraction process. These limitations should be overcome to improve tuberculosis detection sensitivity and specificity by m-PCR directly from clinical samples.

Taken together, our m-PCR assay targeting 16S rRNA, Rv3873 and a 12.7-kb fragment appears to be useful for the rapid differentiation of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG species and NTM. Our m-PCR protocol is highly species-specific and can be easily used as a supplemental method for routine monitoring in veterinary and medical microbiology laboratories, particularly in endemic areas where bovine and human TB coexist. Distinguishing *M. bovis* from *M. tuberculosis* is required for monitoring the spread of *M. bovis* to humans.

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