Rapid and simultaneous detection of _Mycobacterium bovis_ and _Mycobacterium avium_ subsp. _paratuberculosis_ in invasive flies by duplex PCR

Jihong ZHANG1,2, Shijun ZHENG1, Tangming ZHU3, Siguo LIU4, Rusong LI2, Ting ZHU3, Suwen HUANG2, Jianfeng WANG5, Jianbo NI2, Xiuling ZHAO2, Jing SUN5, Shaotang HUANG2,*,

1 College of Veterinary Medicine, China Agricultural University, Beijing 100193, P. R. China
2 Ningbo Entry–Exit Inspection and Quarantine Bureau, Ningbo 315012, P. R. China
3 Yuyao Entry–Exit Inspection and Quarantine Bureau, Yuyao 315400, P. R. China
4 Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences, Harbin 150001, P. R. China
5 Hebei Entry–Exit Inspection and Quarantine Bureau, Shijiazhuang 050051, P. R. China

Abstract: A rapid and effective polymerase chain reaction (PCR) test, specific for the detection of _Mycobacterium bovis_ and _Mycobacterium avium_ subsp. _paratuberculosis_ in invasive flies, was developed. This duplex PCR technique was specifically designed to amplify the sequences of IS6110 of _M. bovis_ and IS900 of _M. avium_ subsp. _paratuberculosis_. The results of the interference test, simulated contamination test, and several other experiments indicated that this technique had high stability, specificity, and sensitivity with detection limits at 92.98 pg of _M. bovis_ and 110.27 pg of _M. avium_ subsp. _paratuberculosis_. Therefore, this duplex PCR test is a rapid and effective technique to simultaneously detect _M. bovis_ and _M. avium_ subsp. _paratuberculosis_.

Key words: Fly, _Mycobacterium bovis_, _Mycobacterium avium_ subsp. _paratuberculosis_, duplex PCR

1. Introduction

Bovine tuberculosis and paratuberculosis or Johne’s disease are caused by _Mycobacterium bovis_ and _M. avium_ subsp. _paratuberculosis_, respectively, and result in a consumption disease, clinically recognized as a wasting syndrome in ruminants such as cattle and goats. These diseases are distributed worldwide, primarily infecting domestic or wild ruminants, and they have a great impact on the dairy and beef industry (1–3).

Bovine tuberculosis and paratuberculosis are classified as type B epidemic diseases by the Office International des Epizooties and are ranked as type 2 epidemic diseases in China. They are regarded as 2 of the key quarantine diseases in inspection and quarantine, such as the entry–exit of large and medium animals, raw hides, and other animal products. Currently, in the field of entry–exit inspection and quarantine, techniques such as delayed type hypersensitivity, enzyme-linked immunosorbent assay (ELISA), and bacterial isolation are performed to identify these diseases. However, these techniques are not satisfactory in the aspects of specificity, sensitivity, and the inspection period, as they cannot satisfy the requirements of rapidness and disease control and quarantine.

It is known that flies can carry many pathogenic microorganisms and spread many diseases. This is a great health hazard to people and animals. Many flies and maggots can be captured from containers filled with wet salted cattle hides, especially in the summer. Exudate can also be found on the floor of these containers with wet salted cattle hides. Therefore, it is necessary that these materials carrying bovine tuberculosis and/or paratuberculosis be inspected and detected.

Risk analysis results have shown that the risk of infectious diseases being spread from the export country to the import country by the international trade of raw cattle hides inevitably exist. Bovine tuberculosis and paratuberculosis are high-risk diseases because it is difficult to inactivate or kill the relevant pathogens in the procedures of slaughter, storage, and international transportation (4).

The polymerase chain reaction (PCR) test, as a fast and effective pathogen identification technique, has been widely used in the quarantine area, especially for the detection of bovine tuberculosis and paratuberculosis pathogens that grow slowly and are difficult to culture (3,5,6). PCR and real-time PCR methods have been...
established for the detection of bovine tuberculosis and paratuberculosis (7–14). However, neither the method for detecting the flies and exudate samples nor a duplex PCR method for the simultaneous detection of bovine tuberculosis and paratuberculosis has been reported. In this study, a duplex PCR method was developed using primers designed according to the IS6110 gene sequence of bovine tuberculosis and the IS900 gene sequence of paratuberculosis. This test was then used for testing the flies, maggots, feces, wet salted hides, and exudate samples.

2. Materials and methods

2.1. Materials

2.1.1. Bacterial strains

The inactivated standard strain of *M. bovis*, *M. avium*, and *M. intracellulare* were gifted by the Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Sciences. The standard strains of *M. avium* subsp. *paratuberculosis* (strain no. C68604) were purchased from the Chinese Institute of Veterinary Drug Control. The standard strains of *E. coli*, *Salmonella*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Pseudomonas aeruginosa*, and *Photobacterium damselae* were provided by the Ningbo Entry–Exit Inspection and Quarantine Technology Center, China.

2.1.2. Reagent

Taq DNA polymerase and dNTPs were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). Lysozyme, protease K, and sodium perchlorate were purchased from Sangon Biotech Co., Ltd. (Shanghai, China).

2.2. Methods

2.2.1. DNA extraction

At least 1 loopful of solid bacterial cultures (*M. bovis* and *M. avium* subsp. *paratuberculosis*) was added to 400 µL of TE buffer (pH 8.0) and boiled at 80 °C for 20 min. After cooling to room temperature, 50 µL (10 mg/mL) of lysozyme was added to the tube and the tube was incubated at 37 °C for 1.5 h, oscillating the tube several times during the incubation period. As the next step, 70 µL of SDS (10%) and 5 µL of proteinase K (20 mg/mL) were added to the tube, mixed gently, and water-bathed at 50 °C for 1 h, oscillating the tube several times during the incubation period. Next, 100 µL of 5 M sodium perchlorate was added to the tube and water-bathed at 55 °C for 0.5 h, oscillating the tube several times during the incubation period. For purification, the same volume of phenol/chloroform (25:24, v/v) was added, mixed gently, and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new tube and the extraction was repeated. The supernatant was transferred to a new tube containing the same volume of chloroform and centrifuged at 12,000 rpm for 10 min. The supernatant was transferred to a new tube containing 0.7 times the volume of dimethyl carbinol and incubated at room temperature until DNA was precipitated, and it was then centrifuged at 12,000 rpm for 10 min. The DNA pellet was washed twice with 70% alcohol, dried, and dissolved in 50 µL of TE buffer. The DNA was stored at –20 °C until use (15–21).

2.2.2. Primer

The primers of the IS900 gene and IS6110 gene were designed with Oligo 6.0 software (Molecular Biology Insights, Cascade, CO, USA) according to the sequences of *M. avium* subsp. *paratuberculosis* (MAP) [gi:8919124] and *M. bovis* (TB) [gi:31742509] (Table). The amplicon sizes of IS900 and IS6110 were 331 bp and 558 bp, respectively.

2.2.3. PCR amplification

The PCR amplification reaction system and reaction conditions were determined as the Oligo 6.0 software recommended.

The test samples were assayed in a 50-µL reaction mixture containing 1 µL of *M. bovis* DNA template (0.9–92.98 ng), 1 µL of *M. avium* subsp. *paratuberculosis* DNA template (1.1 fg to 1.1 µg), 5 µL of 10X PCR buffer, 2 µL of dNTPs (2.5 mM), 2 µL of forward and reverse primers (20 µM), 0.5 µL of Taq DNA polymerase (2.5 U), and 38.5 µL of nuclease-free water.

The thermal profile for the PCR consisted of 1 cycle of Taq DNA polymerase activation at 95 °C for 10 min, followed by 30 cycles of PCR at 95 °C for 45 s, 60.5 °C for 45 s, and 72 °C for 1 min, followed by 1 cycle of extension at 72 °C for 7 min.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>Pathogen</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>IS900 P1</td>
<td>GAC GAC TCG ACC GCT AAT TGA</td>
<td><em>M. avium</em> subsp. <em>paratuberculosis</em></td>
<td>331 bp</td>
</tr>
<tr>
<td>IS900 P2</td>
<td>ATG AGC AAG GCG ATC AGC A</td>
<td><em>M. avium</em> subsp. <em>paratuberculosis</em></td>
<td>331 bp</td>
</tr>
<tr>
<td>IS6110 P1</td>
<td>CGG CTG GTC TCT GGC GTT GAG</td>
<td><em>M. bovis</em> (TB)</td>
<td>558 bp</td>
</tr>
<tr>
<td>IS6110 P2</td>
<td>GTC CCG CCG ATC TCG T</td>
<td><em>M. bovis</em> (TB)</td>
<td>558 bp</td>
</tr>
</tbody>
</table>
The PCR product was examined by 1.5% agarose gel electrophoresis at 120 V for 25 min and then detected using a UVI proplatinum gel imaging system.

2.2.4. Sensitivity experiment
The concentrations of a 10-fold dilution series of DNA of the standard strains of M. bovis and M. avium subsp. paratuberculosis were detected by spectrophotometer. The diluted DNA of the standard strains was used for PCR amplification to determine the sensitivity.

2.2.5. Specificity experiment
The duplex PCR was used to detect 2 Mycobacteria (M. avium and M. turtle) and other laboratory pathogens including Salmonella, Escherichia coli, and Staphylococcus aureus to determine the specificity of this test.

2.2.6. Interference experiment
The duplex PCR was used to detect 2 Mycobacteria (M. avium and M. tuberculosis) and other laboratory pathogens including Salmonella, Escherichia coli, and Staphylococcus aureus to determine the specificity of this test.

2.2.7. Simulated contamination experiments
A certain proportion of the inactivated bacteria of M. bovis and M. avium subsp. paratuberculosis was added to 400 µL of TE solution. Flies, maggots, feces, exudate, and raw hides were added to the solution and mixed thoroughly. The mixture was used as simulated contaminated samples. The DNA extraction of the mixture and PCR were performed with the methods described above. The fly and maggot samples were added to 400 µL of TE solution prior to the nucleic acid extraction and the DNA was extracted directly. After the feces and raw leather samples were added to 400 µL of TE buffer, the mixture was centrifuged at 12,000 rpm for 5 min, and the supernatant was then used for DNA extraction. Otherwise, the tissue fluid sample mixture was centrifuged at 12,000 rpm for 5 min, the supernatant was discarded, and the pellet was dissolved in 400 µL of TE for DNA extraction (22).

2.2.8. Comparison of SN/T 1907-2007 standard PCR with M. bovis detection methods
Duplex PCR, SN/T 1907-2007 standard PCR for the detection of tuberculosis mycobacterium (23,24), and the Mycobacterium bovis detection method (7) were used to detect simulated samples containing DNA extracted from M. avium subsp. paratuberculosis and M. bovis. The SN/T 1907-2007 standard PCR was carried out in accordance with the SN/T 1907-2007 standard protocol and the detection method for M. bovis was performed as described previously (7).

2.2.9. Repeatability experiment
Duplex PCR was used to detect the same simulated contaminated fly samples 3 times to determine its repeatability.

2.2.10. Detection of clinical samples
Duplex PCR was used to detect flies, maggots, feces, exudate, raw hides, and other clinical samples collected from the containers at the ports of entry.

3. Results
3.1. Confirmation of duplex PCR amplification conditions
As expected, a 331-bp amplicon and a 558-bp amplicon were produced by duplex PCR using the DNA extracted from M. bovis and M. avium subsp. paratuberculosis, respectively, which was consistent with the procedure developed (Figure 1).

3.2. Sensitivity of duplex PCR
The expected PCR products were detected by duplex PCR in 10-, 100-, and 1000-fold dilutions of M. bovis and M. avium subsp. paratuberculosis mixture, while no products could be obtained in other dilutions. The amount of DNA in M. bovis and M. avium subsp. paratuberculosis was about 92.28 pg and 110.27 pg, respectively, in a 1000-fold dilution sample (Figure 2).

3.3. Specificity of duplex PCR
To verify the specificity of the duplex PCR, 8 different common pathogens were tested with the same procedure, including Salmonella, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Vibrio parahaemolyticus, Vibrio alginolyticus, Pseudomonas aeruginosa, and Photobacterium damselae. No PCR products were detected (Figure 3). The same result was also obtained with M. intracellular and M. avium (Figure 4).

3.4. Interference test
Eight different common pathogens (Salmonella, Escherichia coli, Staphylococcus aureus, Listeria monocytogenes, Vibrio parahaemolyticus, Vibrio alginolyticus, Pseudomonas aeruginosa, and Photobacterium damselae) were mixed with the DNA from the standard strain; Lane 3: M. avium subsp. M. bovis standard strain; Lane 2: M. avium subsp. paratuberculosis standard strain; Lane 1: M. bovis standard strain; Lane 4: negative control.

Figure 1. Establishment of the duplex PCR. Lane M: marker; Lane 1: M. bovis and M. avium subsp. paratuberculosis standard strain; Lane 2: M. avium standard strain; Lane 3: M. avium subsp. paratuberculosis standard strain; Lane 4: negative control.
3.5. Stimulated contamination test by duplex PCR
All of the stimulated contamination samples could be amplified by duplex PCR and the expected PCR products were observed after the PCR amplification of these samples (Figure 6).

3.6. Comparison tests by duplex PCR
Specific PCR products produced by duplex PCR using *M. bovis* and *M. avium* subsp. *paratuberculosis* templates were detected, which was consistent with the results obtained from the SN/T 1907-2007 method and the MPB70 amplification method (Figure 7).

3.7. Repeatability test of duplex PCR
Under the optimized reaction mixture and amplification conditions, stimulated contamination samples obtained from the invasive fly were tested 3 times. The expected PCR products were consistently produced by duplex PCR (Figure 8).

3.8. Test of clinical samples by duplex PCR
Duplex PCR was used to test 50 invasive fly samples, 42 maggot samples, 26 feces samples, 15 exudate samples, and 25 raw hide samples. All of these samples tested negative. Blood samples from 4 *M. bovis*-positive deer diagnosed by ELISA and single PCR (1) also tested *M. bovis*-positive (Figure 9).

4. Discussion
*M. avium* subsp. *paratuberculosis*, *M. tuberculosis*, *M. bovis*, *M. avium*, and other *Mycobacteria* in animals and in the environment belong to the family *Mycobacterium*. Specific nucleotide sequences need to be located for molecular biology identification because their structures and molecular constitutions are very similar. In this study, the IS6110 insert sequence of *M. bovis* and the IS900 insert sequence of *M. avium* subsp. *paratuberculosis* were used as target genes. The IS6110 insert sequence and IS900...
insert sequence are often selected as molecular labels or PCR examination aim genes for *M. avium* subsp. *paratuberculosis* and *M. bovis* (8,14,25–28), respectively. A duplex PCR method for the identification of *M. avium* subsp. *paratuberculosis* and *M. bovis* in one test was developed based on these sequences. The multicopy of the insert sequence used as the target gene in these 2 mycobacteria ensured the sensitivity of the method in theory (29,30).

In this study, the SN/T1907-2007 standard method (24), “lysozyme-proteinase K-sodium perchlorate extraction” (21), and the modified “lysozyme-proteinase K-sodium perchlorate extraction method” were used for the template DNA extractions. DNA templates required for the experiment were successfully extracted using these 3 methods. The modified "lysozyme-proteinase K-sodium perchlorate extraction” method was not only more efficient than the SN/T1907-2007, but it could also be used to achieve the same efficiency as the lysozyme-proteinase K-sodium perchlorate extraction method in half of the time. Moreover, this test can be completed within 1 day and is suitable for the port of entry inspection and quarantine. Considering all the above factors in this study, the modified lysozyme-proteinase K-sodium perchlorate extraction method was selected for the preparation of the DNA templates of the samples.

The sensitivity, specificity, interference experiment, simulated contamination experiments, and repeatability of the duplex PCR were tested. These results showed that this method was specific, sensitive, and repeatable and could be widely used for the quarantine of imported raw cattle hides.

To develop the duplex PCR, many important parameters such as primer concentrations and annealing temperature need to be optimized to get the best PCR results. The sensitivity of the duplex PCR in this study was lower than that of the conventional PCR because it could

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**Figure 6.** Testing of simulated contaminated samples by duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–6: stimulated contamination samples with fly, maggot, bovine feces, tissue fluid, and raw cattle hides, respectively; Lane 7: negative control.

**Figure 7.** The comparison tests of duplex PCR. Lane M: DL2000 marker; Lane 1: PCR products from duplex PCR; Lane 2: PCR products from the guild standard SN/T 1907-2007; Lane 3: PCR products from MPB70; Lane 4, negative control.

**Figure 8.** The repeatability test of duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–4: simulated contaminated flies samples; Lane 5: negative control.

**Figure 9.** Testing of clinical samples by duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–13: clinical samples; Lane 14: negative control.
detect 2 different target genes from 2 different nucleotides in the same reaction system at the same time. The detection limitation of Mycobacterium avium subsp. paratuberculosis was 1.1027 pg and that of Mycobacterium bovis was 0.9298 pg. Two pairs of primers may competitively inhibit each other in the process of the PCR amplification to cause this problem. Studies on duplex PCR seem to have this phenomenon, as described by Farkas et al. (31), in which the sensitivity of real-time PCR using primers designed for detecting the avian influenza virus matrix protein gene and the Newcastle disease virus fusion F protein gene decreased.

The results of the duplex PCR in this study could effectively eliminate the interference caused by contamination with several bacteria. The same results were achieved using the duplex PCR test when compared to the SN standard and MPB70 method and it could be used for the detection of M. avium subsp. paratuberculosis and M. bovis at same time in one test. The negative results of 158 samples of imported raw hides by this method correlated well with the results of the port quarantine results, and 4 M. bovis-positive deer blood samples were M. bovis-positive by this test. Four deer blood samples were collected from TB-positive cases as confirmed by ELISA, which were culled without antibiotic treatment. Persistent infection with M. bovis existed in these deer as an intracellular parasitic bacterium, M. bovis (32). The method is quite useful and applicable for the import raw hide quarantine detection of M. avium subsp. paratuberculosis and M. bovis because it is quick, efficient, and could be completed within 24 h. This technique will provide a new approach for raw hide quarantine detection.

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References


