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Rapid and simultaneous detection of *Mycobacterium bovis* and *Mycobacterium avium* subsp. *paratuberculosis* in invasive flies by duplex PCR

Jihong ZHANG[1,2], Shijun ZHENG[1], Tangming ZHU[3], Siguo LIU[4], Rusong LI[2], Ting ZHU[4], Suwen HUANG[2], Jianfeng WANG[2], Jianbo NI[2], Xiuling ZHAO[2], Jing SUN[5], Shaotang HUANG[2,*]

[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

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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 IS*6110* of *M. bovis* and IS*900* 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

* Correspondence: huangst@nbciq.gov.cn

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 IS*6110* gene sequence of bovine tuberculosis and the IS*900* 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 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 IS*900* gene and IS*6110* 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 IS*900* and IS*6110* 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. Primers used for duplex PCR assays.

Primer name	Sequence (5′-3′)	Pathogen	Product size
IS900 P1	GAC GAC TCG ACC GCT AAT TGA	*M. avium* subsp. *paratuberculosis*	331 bp
IS900 P2	ATG AGC AAG GCG ATC AGC A		
IS6110 P1	CGG CTG GTC TCT GGC GTT GAG	*M. bovis* (TB)	558 bp
IS6110 P2	GTC CCG CCG ATC TCG T		

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

After the DNA extracted from *M. bovis* and *M. avium* subsp. *paratuberculosis* was mixed with the DNA from the *Salmonella*, *Escherichia coli*, *Listeria monocytogenes*, *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Pseudomonas aeruginosa*, *and Photobacterium damselae* at a proportion of 2:2:1, 2 µL of the mixture was used for PCR amplification.

2.2.7. Simulated contamination experiments

A certain proportion of the inactivated bacteria of *M. bovis* and *M. avium* subsp. *paratuberculosis* were 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 1000 rpm for 2 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. intracellulare* 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*

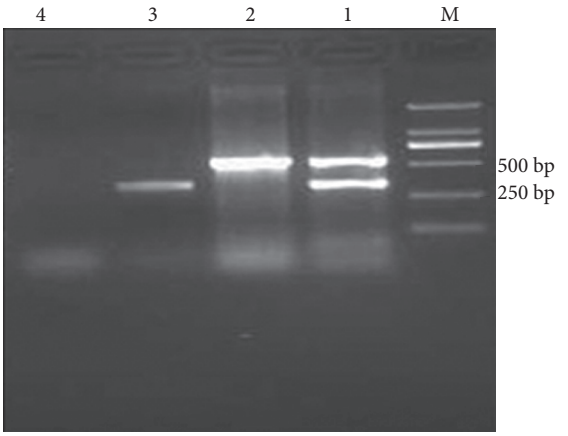

Figure 1. Establishment of the duplex PCR. Lane M: marker; Lane 1: *M. bovis* and *M. avium* subsp. *paratuberculosis* standard strain; Lane 2: *M. bovis* standard strain; Lane 3: *M. avium* subsp. *paratuberculosis* standard strain; Lane 4: negative control.

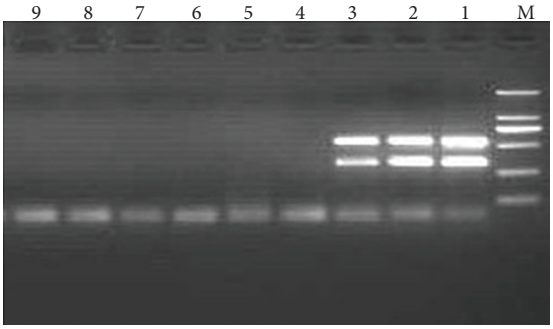

Figure 2. The DNA limit test of the PCR assay. Lane M: DL2000; Lanes 1–9, 10-fold to 10^9-fold diluted samples.

aeruginosa, and *Photobacterium damselae*) had no interference in the duplex PCR (Figure 5).

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

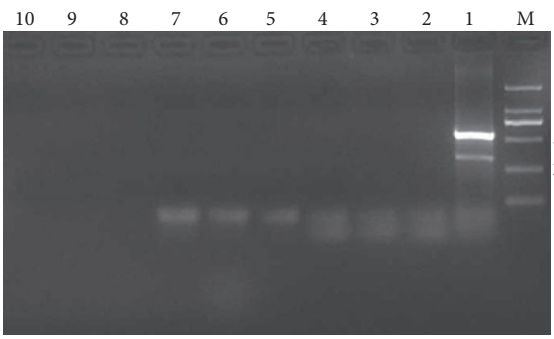

Figure 3. The specificity test of the duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: *Salmonella*; Lane 3: *Escherichia coli*; Lane 4: *Staphylococcus aureus*; Lane 5: *Listeria monocytogenes*; Lane 6: *Vibrio parahaemolyticus*; Lane 7: *Vibrio alginolyticus*; Lane 8: *Pseudomonas aeruginosa*; Lane 9: *Photobacterium damselae*; Lane 10: negative control.

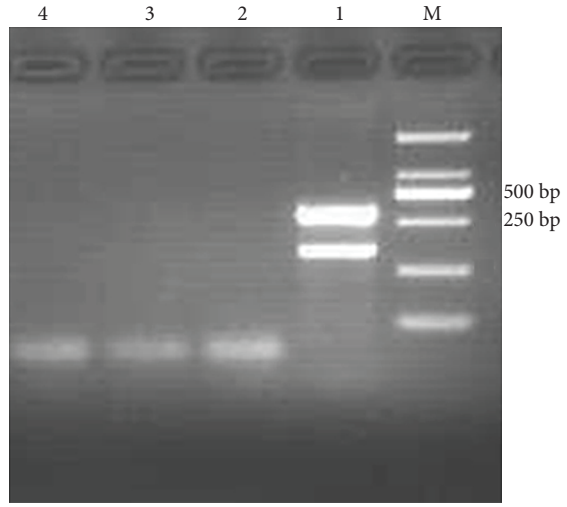

Figure 4. The specificity test of the duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: *Mycobacterium avium* subsp. *avium*; Lane 3: *M. intracellulare infection*; Lane 4: negative control.

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 IS*6110* insert sequence of *M. bovis* and the IS*900* insert sequence of *M. avium* subsp. *paratuberculosis* were used as target genes. The IS*6110* insert sequence and IS*900*

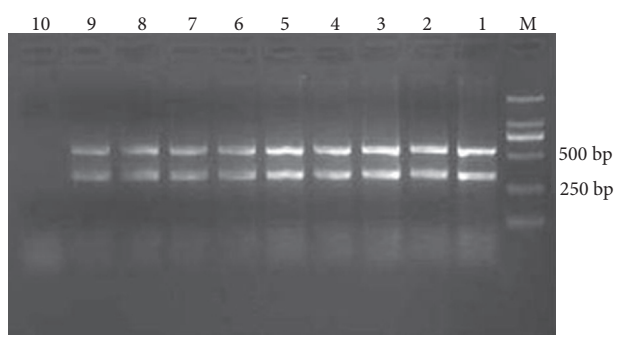

Figure 5. The interference test of the duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lane 2: *Salmonella*; Lane 3: *Escherichia coli*; Lane 4: *Staphylococcus aureus*; Lane 5: *Listeria monocytogenes*; Lane 6: *Vibrio parahaemolyticus*; Lane 7: *Vibrio alginolyticus*; Lane 8: *Pseudomonas aeruginosa*; Lane 9: *Photobacterium damselae*; Lane 10: negative control.

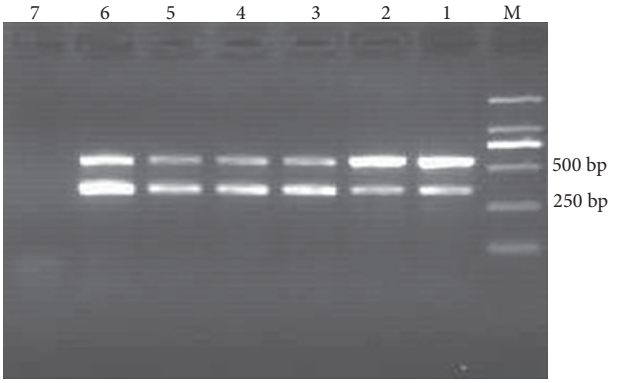

Figure 6. Testing of simulated contaminated samples by duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–6: stimulated contamination samples with fly, maggot, bovine feces, tissue fluid, and raw cattle hides, respectively; Lane 7: negative control.

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).

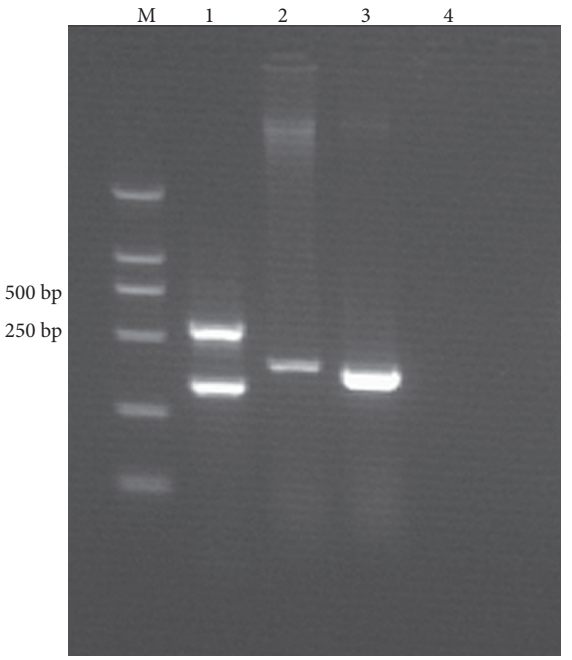

Figure 7. The comparison tests of duplex PCR. Lane M: DL2000 marker; Lane 1: PCR products from duplex PCR; Lane 2: PCR products from the guild standard SN/T 1907-2007; Lane 3: PCR products from MPB70; Lane 4, negative control.

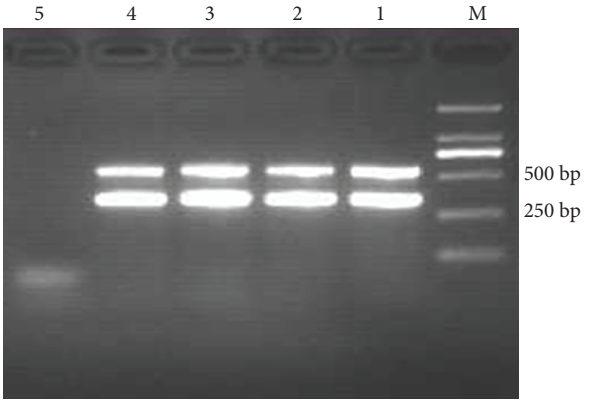

Figure 8. The repeatability test of duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–4: simulated contaminated flies samples; Lane 5: negative control.

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

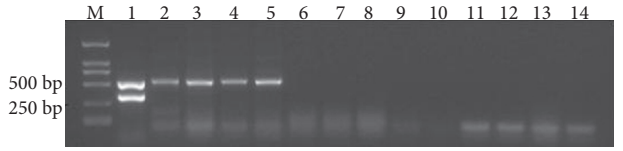

Figure 9. Testing of clinical samples by duplex PCR. Lane M: DL2000 marker; Lane 1: positive control; Lanes 2–13: clinical samples; Lane 14: negative control.

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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