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Development of a multiplex PCR to identify *Salmonella, Leptospira* and *Brucella* species in tissue samples

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Abstract : We have developed and optimized a multiplex polymerase chain reaction (mPCR) for simultaneous detection of *Brucella*, *Salmonella* and *Leptospira* with high sensitivity and specificity. Three pairs of oligonucleotide primers were designed to specifically amplify the targeted genes of *Salmonella, Leptospira* and *Brucella* species with sizes of 521, 408 and 223 bp, respectively. The mPCR did not produce any nonspecific amplification products when tested against 15 related species of bacteria. The sensitivity of the mPCR was 100 fg for *Brucella* and 1 pg for both *Salmonella* and *Leptospira* species. In the field application, kidney, liver and spleen were collected from wild rats and stray cats and examined by mPCR. The high specificity and sensitivity of this mPCR assay provide a valuable tool for diagnosis and for the simultaneous and rapid detection of three zoonotic bacteria that cause disease in both humans and animals. Therefore, this assay could be a useful alternative to the conventional method of culture and single PCR for the detection of each pathogen.

Keywords : *Brucella*, *Leptospira, Salmonella* species, multiplex PCR, tissue samples

Introduction

Brucellosis, Salmonellosis and Leptospirosis are serious diseases that occur worldwide and affect wild and domestic animals as well as humans [5, 9, 11]. Although these diseases were once largely eradicated, they are now a continuing and apparently increasing global problem. Epidemiological studies have shown that rodents and other wild animals often act as reservoirs and vectors for a number of infectious agents that cause disease in farm livestock and domestic pets [12]. Recent evidence indicates that *Brucella, Salmonella and Leptospira* infections have also been reported in wildlife, rodents and cats [3, 10, 19, 25, 28]. *Brucella, Salmonella and Leptospira* species are intracellular, slow growing and facultatively fastidious. Bacteriological isolation is usually employed for diagnosis. However, the isolation of these pathogens, with the exception for *Salmonella*, is usually difficult, time-consuming and laborious. These diseases can be diagnosed by serology, but many factors may cause false positive and negative results [1, 2, 5, 15].

To overcome these diagnostic problems, molecular techniques such as polymerase chain reaction (PCR) have been used for increased sensitivity and specificity. Numerous PCRs have been developed for the detection of *Brucella* [1, 13, 15, 16, 21, 28], *Salmonella* and *Leptospira* [2, 5, 6, 14, 26, 29] have been applied to DNA extracted from bacterial cells, blood and tissue samples. In diagnostic laboratories, the use of PCR is limited by cost, time and sometimes the availability of an adequate test sample volume. mPCR diagnostics could help to overcome these difficulties and increase the diagnostic capacity of PCR. mPCR is cost effective and has the potential to save considerable amounts of time. Several mPCRs for the detection of *Brucella*, *Salmonella* and *Leptospira* have been described and successfully applied; examples include mPCRs for the simultaneous detection of *Brucella* spp. and *Salmonella abortus ovis* [23], the detection of both *Brucella* spp. and *Leptospira* spp. from aborted bovine fetuses [20], and the detection of both *Brucella canis* and *Leptospira*

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interrogans in canine semen [11].

We have attempted to detect these three pathogens in various tissues of rodents and cats. Because single-PCR detection of each pathogen is too laborious, time consuming and wasteful of reagents, we attempted to develop mPCR for this purpose. The aim of this investigation was to develop an mPCR assay that could be used for the simultaneous detection of *Salmonella*, *Leptospira* and *Brucella* in a single reaction with highly sensitivity and specificity, using as template either DNA extracted directly from tissue samples or bacterial culture. The availability of such a rapid test should simplify and streamline diagnostic testing for these three important pathogens of humans and animals.

We report the development of the first mPCR assay for the sensitive and reliable detection of *Salmonella*, *Leptospira* and *Brucella* based on the combination and optimization of published primer sets. The specificity and sensitivity of the mPCR were tested, and the results were compared with those of single-PCR detection. We also report the application of mPCR for the direct detection of these pathogens in tissue samples of stray cats and rodents.

Materials and Methods

Bacterial strains

All reference strains of *Salmonella* (*S.* Enteritidis and Typhimurium), *Leptospira* (*L. interrogans* serovar Pomona, *L. interrogans* serovar Icterohemorrhagiae, and *L. interrogans* serovar Bratislava), *Brucella* species (*B. abortus*, *B. abortus* vaccine strain RB51, *B. ovis* and *B. canis*) and another 15 bacterial strains were used in this study (Table 1).

Genomic DNA extraction

Total DNA was extracted from bacterial cells and tissue specimens using the Genomic DNA extraction kit (YGT100 and YGB100; RBC, Taiwan) according to the manufacturer's protocol. Briefly, for tissue samples, 3 types of organ tissues (kidney, liver and spleen) were taken out and homogenized by Tissue Lyzer (QIAGEN, Germany). Cultured bacterial cells were transferred to microcentrifuge tubes and centrifuged for 1 min at 14,000 rpm. The supernatant was then discarded. DNA concentration and purity were determined by a spectrophotometer at the wavelengths of 260 nm (GENESYS 10S UV; Thermo Scientific, USA) Extracted DNA was stored at –20°C for further use.

Table 1. Bacterial strains used in this study and the results of mPCR

Genera/species	Strain	mPCR
Brucella abortus	ATCC 17385	+
Brucella abortus RB51	RB51	+
Brucella ovis	ATCC 25840	+
Brucella canis	ATCC 23365	+
Leptospira i. Pomona	ATCC 23478	+
Leptospira i. Icterohemorrhagiae	ATCC 43642	+
Leptospira i. Bratislava	ATCC 23578	+
Salmonella Enteritidis	ATCC 13076	+
Salmonella Typhimurium	ATCC 14028	+
Salmonella Gallinarum	ATCC 9184	+
Salmonella Choleraesuis	ATCC 13312	+
Streptococcus suis	ATCC 43765	–
Bacillus subtilis	ATCC 6633	–
Bordetella bronchiseptica	ATCC 10580	–
Clostridium tetani	ATCC 19406	–
Escherichia coli	ATCC 10536	–
Enterobacter	ATCC 35028	–
Shigella flexineri	ATCC 12023	–
Haemophilus parasuis	ATCC 19417	–
Achloplasma laidawi	ATCC 23206	–
Listeria monocytogenes	ATCC 19117	–
Mycoplasma pneumoniae	ATCC 15492	–
Mycoplasma hyopneumoniae	ATCC 25934	–
Mycoplasma gallisepticum	ATCC 1961	–
Pasteurella multocida	ATCC 43137	–
Staphylococus aureus	ATCC 29213	–

Primer selection and optimization of mPCR

The target genes, oligonucleotide primers and expected product sizes for each pathogen are shown in Table 2. Based on size differences of approximately 100 between amplicons, we selected each primer as follows: the *BCSP31* primers amplified a 223 bp fragment of a gene encoding a cell surface protein (*BCSP31*) of *Brucella* spp. [1]; the *LipL41* primers amplified a 408 bp fragment from the gene encoding *LipL41* of *Leptospira* spp. [27]; and the *invA* primers amplified a 521 bp fragment of the *invA* gene encoding a 54-kDa protein of *Salmonella* spp. [26]. Selected oligonucleotide primers were synthesized by Bioneer (Korea).

For optimization of the mPCR, the amplification was carried out in a 50 μL reaction mixture containing 10 μL of 5× PCR buffer (50 mM NaCl, 50 mM Tris-HCl, pH 9.0); 250 μM each deoxynucleotide triphosphate; 2

Table 2. List of oligonucleotide primers used in this study

Genus/species	Target gene	Primer set (5' → 3')	Length of PCR product (bp)	References
Leptospira spp.	*LipL41*	GGCTATCTCCGTTGCACTCTTTG ATCGCCGACATTCTTTCTACACG	408	[27]
Brucella spp.	*BCSP31*	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCTTTCAAGGTCTG	223	[1]
Salmonella spp.	*InvA*	TTGTTACGGCTATTTTGACC CTGACTGCTACCTTGCTGAT	521	[26]

mM $MgCl_2$; and 1 U of GoTaq Flexi DNA polymerase (Promega, USA). Each primer (Table 2) was added at the following final concentrations: 15 pmol for *BCSP31* and 20 pmol for *invA* and *LipL41*. One microliter of diluted template DNA from reference strains or 5 μL of template DNA from a tissue sample was added to each reaction mixture. The final volume of the reaction mixture was adjusted to 50 μL with sterile deionized distilled water. The magnesium chloride concentration (Mg^{2+} 1, 1.5, 2, 2.5 and 3 mM), primer concentration and template DNA volume were tested as variables for optimal mPCR results. All mPCR reactions were performed in a thermocycler (PC100; MJ Research, USA) with the following temperature cycling parameters: initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 30 sec, primer annealing at various temperatures from 54 to 60°C for 30, 40, 50 or 60 sec for optimization, primer extension at 72°C for 30 or 60 sec, and a final extension at 72°C for 15 min.

Ten microliters of the amplified product was evaluated for expected size by electrophoresis on a 1% agarose gel in 1X Tris-boric acid-EDTA buffer [TBE: 0.089 M Tris-base, 0.089 M boric acid, and 0.002 M EDTA (pH 8.0)] at 100 volts. After electrophoresis, amplification products were detected by visualization of the bands under UV light after staining with Gel Red (Komabiotech, Korea). A 100-DNA ladder (Bioneer, Korea) was used as molecular marker to indicate the size of the amplicons.

Specificity and sensitivity of optimized mPCR

To determine the specificities of the optimized mPCRs, genomic DNA prepared from *Salmonella, Leptospira, Brucella* spp. and 15 reference strains (Table 1) was used in mPCR. To study the influence of DNA template from tissue samples on mPCR amplification of *Salmonella, Leptospira,* and *Brucella* spp., extracted DNA from kidney, liver and spleen tissues of rodents and stray cats was used for testing the mPCR. The sequences of the amplicons were conrmed by sequence analysis and a BLAST search of the GenBank database. The DNA used as sequencing templates consisted of PCR products purified with the HiYield Gel/PCR DNA mini kit (YDF100; RBC, Taiwan).

The detection limit of the mPCR assay was determined using limiting dilutions of DNA. DNA from *B. abortus*, *S.* Enteritidis and *L.* Pomona was quantified spectrophotometrically and diluted to 100 ng/μL with sterile deionized distilled water. Serial dilutions from 100 ng to 10 fg were made, and 1 μL of each dilution was analyzed in a 50 μL PCR and mPCR. The lowest concentration visible on an agarose gel was set as the detection limit.

Results

Optimal condition of mPCR

Three pairs of oligonucleotide primers were designed to amplify specific regions of BCSP31, LipL41 *and* invA from the *Brucella, Leptospira* and *Salmonella* genomes, respectively. Primer *BCSP31* was designed to amplify an approximately 223 bp fragment from *Brucella*. Primer *LipL41* was designed to amplify an approximately 408 bp fragment from *Leptospira*. The invA primer pair amplifies approximately 521 bp of the *invA* gene encoding a 54-kDa protein in *Salmonella* spp. (Fig. 1).

To optimize the mPCR, we tested all of the primers with several combinations of annealing temperature, extension time, number of cycles and primer concentration. The concentration of magnesium chloride was varied from 1 to 3 mM. At low magnesium concentrations, some of the expected PCR fragments were faint or non-detectable, while nonspecific PCR products of various sizes were amplified at high magnesium concentrations. From these results, the optimum magnesium concentration for the assay was determined to be 2 mM. The concentrations of all primers were adjusted to 20 pmol, which resulted in differences in the intensities of the

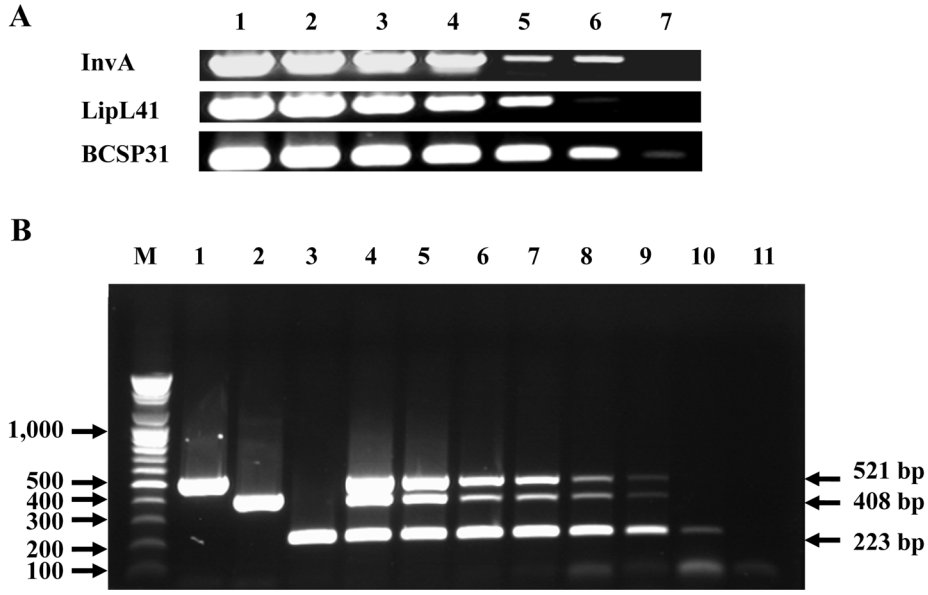

Fig. 1. Sensitivity of single PCR for *Salmonella*, *Leptospira* and *Brucella* target genes (A) and the multiplex PCR for simultaneous amplification of all bacterial target DNA (B). Multiplex PCR products were separated in a 1% agarose gel and stained with Gel Red. Each target DNA fragment was extracted, and 10-fold serial dilutions were prepared at concentrations of 100 ng to 100 fg. One microliter of each concentration of each target DNA was subjected to single PCR for *Salmonella*, *Leptospira* and *Brucella* using specific primer pairs (A, lanes 1~7), and mixed DNA was subjected to mPCR. (B) Lanes 1~3: single PCR for each pathogen, *S.* Enteritidis (521 bp), *L.* Pomona (408 bp) and *B. abortus* (223 bp), respectively; Lanes 4~10: mPCR with mixed DNAs at each concentration; Lane 11: negative control; Lane M: 100 bp DNA ladder.

amplified DNA fragments. Initially, the intensity of the *BCSP31*-specific amplicon was higher than other PCR products; however, the intensities of all the amplicons became almost identical after the concentration of the *BCSP31* primers was decreased to 15 pmol. The amplification condition showing the best resolution of all amplicons included an annealing step at 57°C for 40 sec, an extension step at 72°C for 30 sec and 40 cycles of amplification (Fig. 1B).

Specificity of mPCR

The specificity of primer pair for each pathogen was analyzed by single PCR. As shown in Fig. 1A, each primer pair amplifies the corresponding target gene, clearly yielding the amplicon sizes of 521 bp for *Salmonella*, 408 bp for *Leptospira* and 223 bp for *Brucella*. Three amplification products were observed simultaneously in an mPCR in which a mixture of chromosomal DNA of *Brucella*, *Leptospira* and *Salmonella* was used as a template (Fig. 1B). To determine the specificity of the primers selected, genomic DNA from 15 different strains of 13 bacterial species were assayed. None of the amplification products were generated from any of the other bacterial strains (Table 1). This result indicates that the mPCR assays are highly specific for the simultaneous detection of these three pathogens. No PCR product was obtained from a negative control with no template.

Sensitivity of mPCR

Comparisons of the sensitivity of single PCR and mPCR detection using *BCSP31*, *inv*A and *LipL41* primers for detecting purified *Brucella*, *Salmonella* and *Leptospira* DNA are shown in Fig. 1. The threshold sensitivity of the PCR assay was determined by testing serial dilutions of *Brucella*, *Salmonella* and *Leptospira* DNA from 100 ng to 100 fg. The detection limits of single PCRs and mPCR were found to be the same; as little as 100 fg of *Brucella* DNA was detected after 40 cycles of amplification, in comparison with 1 pg of *Salmonella* and *Leptospira* DNA.

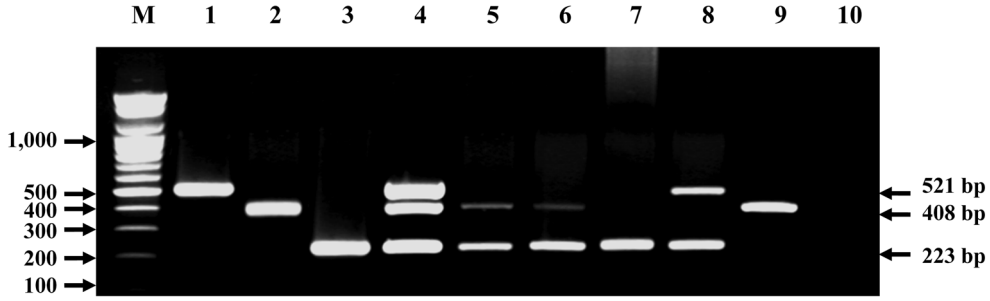

Fig. 2. Agarose gel electrophoresis of DNA fragments generated by multiplex PCR directly from tissue samples. Lanes 1~3: *S.* Enteritidis, *L.* Pomona and *B. abortus*, respectively; Lane 4: combination of *Salmonella*, *Leptospira* and *Brucella*; Lanes 5, 6 and 9: DNA template isolated from kidney of rodent and stray cat; Lane 7: spleen of a stray cat; Lane 8: liver of a rodent; Lane 10: negative control; M: 100 bp DNA ladder.

Application of mPCR for tissue samples from rodents and cats

This mPCR was developed for detection of *Salmonella*, *Leptospira* and *Brucella* in tissue samples of rodents and stray cats. The DNA templates were extracted directly from spleen, kidney and liver using a commercial kit and subjected to the mPCR using three specific primer sets (Table 2). As shown in Fig. 2, a clear amplification product was obtained for all tested tissue samples; these results also reveal that our mPCR was able to simultaneously detect all three pathogens. The PCR amplicons produced by mPCR were confirmed as regions of the *Brucella* and *Leptospira* genome by automated sequencing and subsequent entry of the sequence into the BLAST search engine (data not shown).

Discussion

The diagnosis of *Brucella, Leptospira* and *Salmonella* is typically based on isolation from clinical specimens or serologic evidence of antibodies. Culturing takes days to weeks but has the advantage of detecting the organism directly. For rapid testing of clinical samples or for field surveys, immunologic methods are used. However, antigen-antibody interactions can be complicated by nonspecific interactions, and false positives from vaccinated animals with high levels of circulating antibodies can be misdiagnosed as active infections.

PCR is a promising option for the diagnosis of various pathogens, and it is a potentially useful method for the detection of *Brucella, Leptospira* and *Salmonella* species from isolated bacteria, blood, semen or highly contaminated tissues [1, 2, 5, 11, 13, 14, 16, 17, 20]. Previous studies have also demonstrated the successful application of single PCR using specific primer pairs for the detection of *Brucella* species in bacterial cultures or blood samples [1, 16], for the detection of *Leptospira* species in clinical samples [14, 27] and for the detection of *Salmonella* species [26]. However, single PCR only allows the detection of nucleic acid from one specific pathogen at a time. Our recent attempts to use individual PCR for the detection of those three pathogens in various tissues of wild rodents and cats demonstrated that this technique is relatively costly and time-consuming. Therefore, in this study, we improved the diagnostic value of these PCR methods by taking a multiplex approach. The mPCR has the advantage of simultaneous detection of multiple pathogens and has been proven to be sensitive, specific and cost-effective. It can be useful in diagnosis, screening and surveillance of flocks [9].

The sensitivity comparison of this developed mPCR with single PCRs for individual bacteria (data not shown) revealed that the sensitivity of the mPCR was comparable to that of a single PCR with the same limit of detection: *Brucella,* 100 fg; *Leptospira,* 1 pg; and *Salmonella* species, 1 pg. The detection limits of both single PCRs and mPCR for *Leptospira* and *Salmonella* species, but not *Brucella,* are similar to those previously reported in the literature [16, 24, 26]. The excellent sensitivity of PCR reported by Navarro *et al.* [16] using the primers B4/B5 (*BCSP31*) in the diagnosis of *Brucella* species in bacterial culture and blood samples showed that the primers B4/B5 amplified 5 pg of purified genomic DNA [16], compared with 100 fg in this study. The concentrations of primers, dNTPs, $MgCl_2$

and *Taq* polymerase in this PCR assay were different from those in the report of Navarro *et al*. [16], which may account for the difference in sensitivity.

According to previous publications [1, 7, 13, 16, 26, 27], the primers used in mPCR were specific for *Brucella, Leptospira* and *Salmonella* and were applicable to all species and biovars among those 3 bacterial genera. Previous publications on single PCR showed that each primer pair was highly specific for each pathogen. In examining the specificity of primers for *Brucella* genus, Da Costa *et al.* [7] also surveyed 98 non-*Brucella* bacteria and found that all organisms were negative for amplification of the *BCSP31* gene. For the genus *Leptospira*, successful amplification of the predicted 408 bp amplicon from all *Leptospira interrogans* was described; an assessment of specificity showed that the gene could not be amplified in a panel of 15 non-*Leptospira* species [27]. For the genus *Salmonella*, the *invA* primer pair is specific [26], but no studies have evaluated the specificity of these primers for all species in the genus. In this study, to further examine the specificity of each primer pair used in mPCR, all 4 strains of *Brucella*, 3 strains of *Leptospira* and 4 strains of *Salmonella* were tested and gave specific amplification of the correct predicted product size. In addition, the specificity of the primers used for mPCR has also been tested against 15 closely related bacterial species and a variety of other common pathogens, and the absence of amplification of the DNA from these bacterial species indicates that this assay is highly specific for *Brucella, Leptospira* and *Salmonella*.

Several studies have indicated that the inhibition of PCR can be a confounding factor because unknown inhibitors may be released from tissues in DNA extraction. The presence of inhibitors could affect the sensitivity of PCR, especially for various tissue samples such as milk, blood, semen and urine [2, 4, 5, 8, 14, 16, 18, 22]. The sensitivity of mPCR could also be affected by modification of the original PCR protocol [13, 21]. Interestingly, the mPCR described in this paper is the first successful application of mPCR using the B4/B5 (*BCSP31*) primer pair. These primers were used to amplify a 223 bp region encoding a 31-kDa immunogenic *BCSP31* of *Brucella* spp. from kidney, liver and spleen samples of rodents and cats. The use of tissue samples did not reduce the ability of the PCR or mPCR to detect specific target genes or different combinations of the three infectious pathogens. This result suggests that other primer pairs might also be used for the direct detection of *Brucella* species in tissue samples. The positive PCR results for *Salmonella* were confirmed by isolation of the pathogen from the tissue (data not show), but no further sequencing was performed on those samples. As the isolation of *Leptospira* and *Brucella* are normally difficult, time-consuming and laborious, the positive PCR products for *Leptospira* and *Brucella* were confirmed by sequencing. To confirm the identity of the PCR product, the nucleotide sequence was determined and compared with the *Brucella* and *Leptospira* sequences published on NCBI-BLAST. We conducted a sequence analysis of 18 fragments (10 samples of *Brucella* and 8 samples of *Leptospira*) amplified with *BCSP31* and *LipL41* primers. Sequence analysis of the 233 bp fragment using *BCSP31* and the 408 bp fragment using *LipL41* yielded perfect matches in the BLAST search.

In summary, the mPCR described in this study provides an improved capacity to detect *Salmonella*, *Leptospira* and *Brucella* simultaneously in a single reaction. The assay appears to be very specific and highly sensitive, with a limit of 100 fg for *Brucella* and 1 pg for both *Salmonella* and *Leptospira* strains. mPCR can be used to test clinical samples such as blood and tissue samples as well as bacterial culture. This method is not a substitute for single PCR, but it can be used to reduce the number of required tests and deliver results more rapidly and cheaply.

Acknowledgments

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