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Detection of *Brucella* spp. and *Leptospira interrogans* in the Canine Blood by Multiplex Nested PCR

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Abstract : This study examined the prevalence of *Brucella* spp. and *Leptospira interrogans* in 360 clinically healthy dogs using multiplex nested PCR. Four dogs (1.1%, 2 females and 2 males) tested positive to *Brucella* spp. by multiplex nested PCR. Fifty nine (16.4%, 31 females and 28 males) of 360 dogs tested positive *L. interrogans*. In 1 and 2 of the samples that tested positive to *Brucella* spp. and *L. interrogans*, the partial sequences of the virB1 and 16S rRNA genes were identified by direct sequence analysis, respectively. In conclusion, prevalence of *Brucella* spp. and *L. interrogans* by multiplex nested PCR revealed low and high, respectively. Multiplex nested PCR is can be useful for early detection of *Brucella* spp. and *L. interrogans* in the canine blood from asymptomatic dogs.

Key words **:** Multiplex, nested PCR, *Brucella* spp., *Leptospira interrogans*, dog.

Introduction

Canine brucellosis and leptospirosishave a worldwide distribution around the world and can cause significant economic losses in animal production and considerable risks to human health (1). Reproductive disorders such as abortion and premature births are the clinical signs of these bacterial diseases in pregnant dogs. Both diseases can be diagnosed by the detection of the serum specific antibodies, however, these serological methods are presumptive because many factors can cause false positive and negative results. Bacteriological isolation is normally employed, but the methodology is difficult, time consuming and dangerous (4,5).

Multiplex nested PCR analysis used in this study was developed for the detection of *Brucella* spp. and *Leptospira interrogans* in canine semen supported for use in artificial reproduction. Kim *et al.*(3)reported this method to be easier to perform and produce results more rapidly than other methods. In this study, multiplex nested PCR was used for the detection of *Brucella* spp. and *L. interrogans* in DNA isolated from blood samples. Unfortunately, there was no reliable data for canine brucellosis and leptospirosis in Korea. The aim of this study was to survey the prevalence of two critical organisms, *Brucella* spp. and *L. interrogans* based on multiplex nested PCR in the canine blood from asymptomatic dogs.

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Materials and Methods

Animals and blood collections

Three hundreds and sixty dogs (186 females and 174 males) in the 2 training centers for hunting (one Chuncheon area and one Namyangju area) and stray dogs in the Daejeon area were examined from 2004 to 2006 in Korea. Blood samples were collected from the jugular to a tube containing EDTA and collected samples were then stored at −20°C until DNA isolation.

DNA isolation

For DNA isolation, 300 μl of whole blood was lysed in 0.1 M Tris-Hcl (pH 8.0) containing 1% SDS, 0.1 M NaCl and 10 mM EDTA. The samples were then treated with proteinase K, for 2 hr, at 55°C The DNA was extracted with phenol/chloroform, precipitated by ethanol, and then dissolved in 50 μl of a TE buffer. The isolated DNA was stored at −20°C for the PCR assay.

PCR amplification

Multiplex nested PCR was preformed using the previously described primers (Fig 1). These specific primers were designed to detect the virB2and 16S rRNA genes for a *Brucella* spp. and *L. interrogans*, respectively (2,3,8). For the first-round amplification, the PCR reaction was performed in a 20 μl reaction mixtures, containing 50ng template DNA, 20 pmol of each primer, 10 mM of dNTP mixtures and 1 units of prime Taq DNA polymerase (Genet Bio, Ltd., Korea). The cycling conditions were 95°C or 5 min, 40 cycles of 95°C for

A

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      460        470        480        490        500        510        520        530        540        550        560        570        580        590        600
atgatggtac ctgcctaaag caccggctna ctacctntgt gtctggagtt tgggagaggc nagtggaatt ccaggtgtag cggtgaaatg cgtagatatc tggaggaaca ccagtggcga aggcgacttg ctggcctaaa actgacgctg
                                                                                                          Lep-F primer                    LepN-F priemr
      610        620        630        640        650        660        670        680        690        700        710        720        730        740        750
aggcacgaaa gcgtgggtag tgaacgggat tagatacccc ggtaatccac gccctaaacg ttgtctacca gttgttgggg gttttnaccc tcagtaacga acctnacgga ttnagtagac cgcctgggga ctatgctcgc aagagtgaaa
                                                                                                                                                LepN-R primer
      760        770        780        790        800        810        820        830        840        850        860        870        880        890        900
ctcnaaggaa ttgacggggg tccgcacaag cggtggagca tgtggtttaa ttcgatgata cgcgaaaaac ctcacctagg cttgacatgg agtggaatca tgtagagata catgagcctt cgggccgctt cacaggtgct gcatggttgt
                          Lep-R primer
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B

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      1510       1520       1530       1540       1550       1560       1570       1580       1590       1600       1610       1620       1630       1640       1650
tgcagtcagc aagacaaatc tttggaggaa ccaagatgaa tagacattga gcaagcagac cgtgcagaaa tccatgaata cacttcatcg acataaggaa taaagatcat gaaaaccgct tcccccagca agaagtcgct gtcgcggatt
                                                                                                                    B2F primer                    B2N-F primer
      1660       1670       1680       1690       1700       1710       1720       1730       1740       1750       1760       1770       1780       1790       1800
ctacctcacc tactgctggc cctcattgtc tccatcgctg caatcgagcc taacctggcg cacgccaacg gtggcctcga taaggtaaat acaagcatgc aaaaagtgct ggacttgcta agcggcgtat cgatcaccat cgttaccata

      1810       1820       1830       1840       1850       1860       1870       1880       1890       1900       1910       1920       1930       1940       1950
gccatcatct ggtccggtta caagatggca ttccggcacg cccgcttcat ggatgtagtg ccggtgctgg gcggcgccct ggtggttggc gctgccgccg aaattgcctc ttacctgctt aggtaaaggg acacagatca tgacaacggc
                                                                                                          B2N-R primer                    B2R primer
      1960       1970       1980       1990
accacaggaa tccaacgcac gaagcgcagg ttatcgcggc
```

Fig 1. Specifc primer sequences for the 16S rRNA gene of *Leptospira interrogans*. (A) and the virB2 gene of *Brucella* spp. (B).

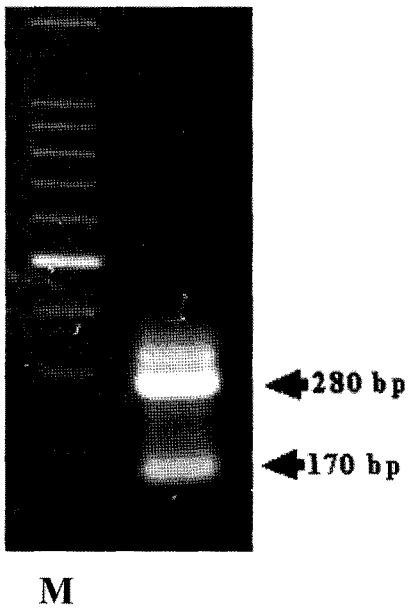

Fig 2. Positive control of *Brucella* spp. (280 bp) and *Leptospira interrogans* (170 bp).

30 sec, 52°C for 1 min, 72°C for 1 min and a final extension of 72°C for 1 min. Nested PCR was performed in 50ng first-round PCR product as template DNA and 20 pmol of the inner primers. The cycling conditions were 95°C for 5 min, 40 cycles of 95°C for 30 sec, 52°C for 1 min, 72°C for 1 min and a final extension of 72°C for 1 min. PCR products were analyzed by electrophoresis using 2% TBE agarose gel and visualized using ethidium bromide staining and UV radiation (Fig 2,3).

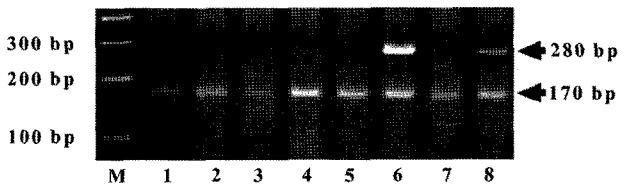

Fig 3. Nested PCR results. All lanes are positve for *Leptospira interrogans* (170 bp). Lane 6 and 8 are positve for *Brucella* spp. (280 bp). M; marker, Lane 1 to 8; examined samples.

Sequencing analysis

For each sample found PCR-positive for *Brucella* spp. and *L. interrogans*, another 5ul sample of the PCR product pre-sequencing regeant pack (Amersham Pharmacia Biotech, NJ) according to the manufacturer's instruction, before being used as the template for sequencing. Sequencing of the PCR products was performedon both strands, using the ABI Dye Terminator Cycle Sequencing kit (Amersham Biosciemces, Little Chalfont, UK). The sequencing analysis was performed using version 1.2.6 of the Cartographer software package (MJ research).

Statistical analysis

Prevalence according to the age groups (< 3 years, 3-6 years and > 6 years) and gender were compared using a^2 test with version 10.0 of the SPSS for Windows software package (SPSS Inc., Chicago, IL).

Results and discussion

Four samples (1.1%, 2 females and 2 males) tested positive for *Brucella* spp. in the nested PCR. (Table 1), and the

Table 1. Nested PCR-based detection of *Brucella* spp. and *Leptospira interrogans* in the canine blood

	Brucella spp.			*Leptospira interrogans*	
	No. of examined	No. of positives	Positive rate(%)	No. of positives	Positive rate(%)
Gender					
Females	186	2	1.1	31	16.7
Males	174	2	1.1	28	16.1
Age (yr)					
< 3	112	1	0.9	18	16.1
3~6	148	3	2. 0	29	19.6
> 6	100	0	0. 0	12	12. 0
Total	360	4	1.1	59	16.4

partial sequences of the virB1 gene were identified in one positive sample. Positive detection rate of 1.1% might be considered to be low. On the other hand, it is possible that, in Korea, Brucellainfections are rare in clinically healthy dogs. However, brucellosisusually does not show evident symptoms, and the range of clinical signs varies from asymptomatic to mild despite there being an ongoing systemic infection. In addition, bitches may conceive and give birth even without antibiotics treatment but the puppies are born infected, which can spread through the kennel. Male dogs can excrete the bacteria into the semen and urine. For the prevention of brucellosis, it is necessary to exclude subclinical or asymptomatic patients from clinically normal dogs.

For *L. interrogans* 59 samples (16.4%, 31 females and 28 males) tested positive to in nested PCR (Table 1). All positive samples were collected from a training center in the Chuncheon area, therefore, they were may be infected with each other. In 2 of those positive samples, the 16S rRNA gene of *L. interrogans* was identified by direct sequence analysis. Although female dogs showed a slightly higher positive rate than male dogs, there was no significant difference between the genders. In terms of age, there was also no significant difference (Table 1). In Korea, the serological prevalence of *Leptospira infections* have been reported (6,7), however, there was no report about the prevalence of *Leptospira infections* in dogs using nested PCR.

In this study, the prevalence by using multiplex nested PCR was similar to that determined by other seroprevalence results. Considering these results and the hygienicstatus, the incidence of *L. interrogans* could be high in dogs in Korea.

Conclusion

Multiplex nested PCR revealed prevalence of *Brucella* spp. and *L. interrogans* were low and high, respectively. This method is expected to be useful for early detection of *Brucella* spp. and *L. interrogans* from asymptomatic dogs.

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개 혈액에서 Multiplex Nested PCR기법을 이용한 *Brucella* spp. 및 *Leptospira interrogans* 검출

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요 약 : 본 연구는 특이적인 임상증상이 없는 360두의 개를 대상으로 multiplex nested PCR 기법을 이용하여 *Brucella* spp. 및 *Leptospira interrogans*를 검출하였다. *Brucella* spp.는 총4두 (1.1%, 암컷 2두, 수컷 2두)가 검출되었고, *Leptospira interrogans*는59두 (16.4%, 암컷31두, 수컷28두)에서 검출되었다. 결론적으로 *Brucella* spp.의 검출률은 낮은 반면 *Leptospira interrogans*는 높았다. 또한 multiplex nested PCR기법은 무증상의 개 혈액에서 *Brucella* spp. 및 *Leptospira interrogans*를 조기에 검출하는데 빠르고 편리한 기법으로 판단된다.

주요어 : multiplex, nested PCR, *Brucella* spp., *Leptospira interrogans*, 개.