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 to 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 leptospirosis have 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 critical organisms, Brucella spp. and L. interrogans based on multiplex nested PCR in the canine blood from asymptomatic dogs.

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 Chunecheon 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
Fig 1. Specific 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 positive for *Leptospira interrogans* (170 bp). Lane 6 and 8 are positive 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-sequence regent 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 performed on both strands, using the ABI Dye Terminator Cycle Sequencing kit (Amersham Biosciences, 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 $\chi^2$ 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

<table>
<thead>
<tr>
<th></th>
<th>Brucella spp.</th>
<th>Leptospira interrogans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of examined</td>
<td>No. of positives</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Females</td>
<td>186</td>
<td>2</td>
</tr>
<tr>
<td>Males</td>
<td>174</td>
<td>2</td>
</tr>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 3</td>
<td>112</td>
<td>1</td>
</tr>
<tr>
<td>3-6</td>
<td>148</td>
<td>3</td>
</tr>
<tr>
<td>&gt; 6</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>360</td>
<td>4</td>
</tr>
</tbody>
</table>

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, brucellosis usually 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 hygieneric status, 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.

References

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

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요약 : 본 연구는 특이적인 박상증상이 없는 360두의 개들 대상으로 multiplex nested PCR 기법을 이용하여 Brucella spp. 및 Leptospira interrogans을 검출하였다. Brucella spp.는 총 4두 (1.1%, 앞 Ferd 2두, 수컷 2두)가 검출되었고, Leptospira interrogans는 59두 (16.4%, 앞 Ferd 31두, 수컷 28두)에서 검출되었다. 결론적으로 Brucella spp.의 검출률은 낮은 반면 Leptospira interrogans는 높았다. 또한 multiplex nested PCR기법은 무증상의 개 혈액에서 Brucella spp. 및 Leptospira interrogans을 초기에 검출하는데 빠르고 편리한 기법으로 판단된다.

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