Detection of Brucella sp. and Leptospira sp. in dogs using conventional polymerase chain reaction

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Abstract

The study was conducted to detect Brucella sp. and Leptospira sp. in blood samples of dogs in Isfahan and Shahrekord province in Iran. A total of 94 blood samples were collected from dogs of different breed, age, sex, and dogs’ type (stray or non-stray). The samples were examined using conventional polymerase chain reaction (PCR). Fourteen (14.89%) dogs were positive for Brucella sp. and 18 (19.15%) dogs for Leptospira sp. There were no significant differences between the prevalence of the pathogens, provinces, sex, and age groups (P > 0.05). However, there was a statistically significant difference in prevalence of Brucella sp. and Leptospira sp. between stray and non-stray dogs (P < 0.0001; χ2 = 30.3767). The study also demonstrated that PCR was successfully used for the first time in Iran for the detection of Brucella sp. and Leptospira sp. in blood samples of dogs. Therefore, we recommend the PCR as a supplementary method with other commonly recognised methods (e.g. serological methods) for the diagnosis of subclinical infections with the microorganisms. Strict measures for the control of stray dogs are also highly recommended.

Keywords: dogs, leptospirosis, brucellosis, PCR, epidemiology, Iran.

Introduction

Brucella and Leptospira are pathogenic bacteria that cause brucellosis and leptospirosis in many animal species including cattle, sheep, goat, camel, buffalo, dog, and horse worldwide (5, 6, 13, 14). Both bacteria are responsible for abortions in infected animals. Canine brucellosis and leptospirosis are important causes of abortion in dogs. The importance of these infectious diseases causes not only economic losses in the dog breeding business, but also poses a potential risk to human health especially to dog breeders and children who come in close contact with the dogs (10, 18). The transmission of canine brucellosis occurs among animals by sexual contact or by exposure to an environment contaminated with secretions and/or infected placental membranes (4), while transmission of canine leptospirosis occurs by a direct exposure to urine or organs of infected animals, or indirectly when there is a self-exposure to an environment contaminated with the bacteria, such as standing water, wet soils, vegetation or fomites (9).

Different studies have revealed sero-prevalence of either or both canine brucellosis and leptospirosis in dogs (3, 7, 15). Although, both diseases can be serologically diagnosed, there are many factors that may cause false positive and negative results. Direct bacteriological isolations can be also conducted, but they are difficult, time consuming and risky. In order to improve the direct diagnosis and minimise the aforementioned problems, molecular diagnosis based on polymerase chain reaction (PCR) has been successfully used for the detection of Leptospira and Brucella in clinical samples (2, 14, 17).

In Iran, studies on the use of PCR in the detection of Leptospira and Brucella in ruminant, especially in abortion cases, were performed (8, 11, 12, 16). However, there is little or no information on the detection of these pathogenic bacteria in blood samples of dogs using PCR method. Thus, the aim of this study was to detect the
DNA of *Leptospira* sp. and *Brucella* sp. in the blood samples of dogs in Iran using conventional PCR. This stays with an agreement that the molecular epidemiological studies on canine brucellosis and leptospirosis are vital in understanding of distribution of both diseases and associated public health risk.

**Material and Methods**

**Animals.** The study was conducted in Isfahan and Shahrekord Provinces of Iran between May and December 2013. For the purpose of the study, a cross sectional sampling was adopted regardless of age, breed, and sex of dogs. Companion dogs used for the study were apparently healthy and were regularly vaccinated at the veterinary hospital of Islamic Azad University of Shahrekord. Information about the dogs was obtained from their owners including their age. The common breeds of dogs sampled were German shepherd, Doberman Pincher, Terrier, Pekingese, and Spitz. Some dogs were of mixed breed. Stray dogs were sampled within the streets of the province. The age of the dogs was estimated using dental formula. A total of 64 companion dogs and 30 stray dogs were sampled. The dogs were classified based on province, sex, and age (below one year, one to six years, and over six years).

**Sample collection.** Five millilitres of blood was collected from each dog, via cephalic venipuncture into vacuum tubes containing anticoagulant (BD Vacutainer EDTA Tube). The samples were then transported to the Laboratory of Islamic Azad University of Shahrekord.

**DNA extraction.** Genomic DNA was extracted from whole blood specimens using DNA extraction and purification kit (CinnaGen, Iran) following manufacturer’s recommendation. The extracted DNA was quantified via spectrophotometric measurement at 260 nm optical density according to the method described by Sambrook and Russell (19). Extracted DNA of each sample was kept frozen at -20°C until used, and then delivered to Biotechnology Research Center of Islamic Azad University of Shahrekord.

**Conventional PCR.** The conventional PCR was performed using specific primers previously published (13). Species-specific oligonucleotide primers for *Leptospira* sp.: Lp-F: 5'-GCCGCTCCTAAAACATGC AAG-3' and Lp-R: 5'-CTTACTGCTGCCTCCCCG TAG-3' designed from 16S ribosomal RNA gene of *Leptospira* (accession number: JF460977.1) were used for gene amplification. PCR amplification for *Leptospira* sp. was performed in a volume of 50 μL in 0.5 mL microtubes containing 2 μg of template genomic DNA, 1 μM of each primer, 2 mM MgCl₂, 200 μM dNTP, 5 μL of 10 × PCR buffer and one unit of Taq DNA polymerase (Roche Applied Science, Germany). The following conditions of PCR were used for gene amplification: initial denaturation at 95°C for 5 min, followed by 32 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1 min; and the last extension step at 72°C for 5 min. A negative control (sterile water) and a positive control (DNA from *Leptospira* ATCC 43642 strain) were used in each gel electrophoresis run.

The PCR for *Brucella* sp. was performed in 25 μL of reaction mixture containing: 2.5 μL of 10 × PCR buffer, 2 mM MgCl₂, 200 μM dNTP, one unit of Taq DNA polymerase (Roche Applied Science, Germany), 20 pmol of each primer (Bru-F: 5'- CTA TTA CCC GAT TGG TGG TCT G-3' and Bru-R: 5'- GGT AAA GCG TCG CCA GAA GG-3'), and 2 μL of template DNA. The thermal profile for *Brucella* sp. involved an initial denaturation step at 95°C for 3 min followed by 32 cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 40 s, and extension at 72°C for 1 min. The cycling was followed by a final extension step at 72°C for 7 min. A negative control (sterile water), and a positive control (DNA) were included in each amplification run.

**Gel electrophoresis.** The products amplified in PCR (15 μL) were subjected to electrophoresis in a 1% agarose gel in 1X TBE buffer at 80V for 30 min, stained with solution of ethidium bromide, and examined under ultra violet illumination (Uvitec, U.K.). The PCR products were identified by 100 bp DNA size marker (Fermentas, Germany). The expected size of amplicons for *Brucella* was 243 bp (Fig. 1), while 307 bp size of amplicons was expected for *Leptospira* (Fig. 2).

**Statistical analysis.** Data was analysed using Statistical Package for Social Sciences version 16 (SPSS 16.0 statistical software). Prevalence statistics were presented using simple frequency. Comparison between age, sex, and province were performed using chi-square ($\chi^2$) test and interpreted at the 5% level of significance.

**Results**

Out of 94 blood samples collected from the dogs in both provinces, 14 (14.89%) and 18 (19.15%) were positive for *Brucella* sp. and *Leptospira* sp. respectively. There was no significant difference between the prevalence of *Brucella* sp. and *Leptospira* sp. and between the two provinces ($P > 0.05$), even though the number of samples positive for *Leptospira* sp. was higher than that of *Brucella* sp. The prevalence of *Brucella* sp. and *Leptospira* sp. in dogs with respect to province, sex, age groups, and dog types is presented in Table 1. Higher prevalence of *Brucella* sp. and *Leptospira* sp. was observed in Isfahan province, in dogs 1-6 years old, and in stray dogs. There was no significant difference in prevalence of the bacteria between provinces, sex, and age groups ($P > 0.05$); while there was a statistically significant difference in the prevalence of *Brucella* sp. and *Leptospira* sp. between dog types ($P < 0.0001$; $\chi^2 = 30.3767$).
Table 1. Prevalence of Brucella sp. and Leptospira sp. in dogs in regard to province, sex, age groups, and dogs’ types using conventional PCR

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Number of samples</th>
<th>Brucella sp.</th>
<th>Leptospira sp.</th>
<th>( \chi^2 ) test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Province</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shahrekord</td>
<td>34</td>
<td>5 (14.71)</td>
<td>7 (20.59)</td>
<td>( \chi^2 = 0.0715; P &gt; 0.05 )</td>
</tr>
<tr>
<td>Isfahan</td>
<td>60</td>
<td>9 (18.00)</td>
<td>11 (18.33)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>41</td>
<td>5 (12.20)</td>
<td>10 (24.39)</td>
<td>( \chi^2 = 1.4700; P &gt; 0.05 )</td>
</tr>
<tr>
<td>Male</td>
<td>53</td>
<td>9 (16.98)</td>
<td>8 (15.10)</td>
<td></td>
</tr>
<tr>
<td>Age groups</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1 year</td>
<td>29</td>
<td>2 (6.90)</td>
<td>3 (10.34)</td>
<td>( \chi^2 = 7.5456; P &gt; 0.05 )</td>
</tr>
<tr>
<td>1-6 years</td>
<td>51</td>
<td>9 (17.64)</td>
<td>10 (19.60)</td>
<td></td>
</tr>
<tr>
<td>&gt;6 years</td>
<td>14</td>
<td>3 (21.4)</td>
<td>5 (35.71)</td>
<td></td>
</tr>
<tr>
<td>Dog types</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stray</td>
<td>30</td>
<td>10 (33.33)</td>
<td>12 (40.00)</td>
<td>( \chi^2 = 30.3767; P &lt; 0.0001 )</td>
</tr>
<tr>
<td>Non-stray</td>
<td>64</td>
<td>4 (6.25)</td>
<td>6 (9.38)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

Dogs as human companions may also pose risk for human health. The possibility of asymptomatic carrier in dogs with some zoonotic infections is one of such risks. Dogs play an important role in the maintenance of different infectious and parasitic agents in the environment and their possible transmission to humans is of a public health concern. Different studies on seroprevalence of Brucella sp. and Leptospira sp. in dogs, and detection of the bacteria in abortion cases in ruminants using PCR in Iran have been conducted (8, 11-14), but in this study conventional PCR was used for the first time for the detection of these pathogens in dogs in Iran.

The prevalence of Brucella sp. (14.85%) in this study was higher than that reported by Mosallanejad et al. (15) and Behzadi and Mogheisheh (3), who reported 4.9% and 10.62% in a sero-epidemiological survey in Ahvaz and Fars provinces of Iran respectively. In this study, the prevalence of Brucella sp. is higher than 1.75% and 2.85% sero-prevalences reported from Beijing, China (21) and Brazil (7), respectively. The higher prevalence may be due to higher sensitivity and specificity of PCR compared to serological methods. The prevalence of Leptospira sp. in this study was higher than 5.4% sero-prevalence reported by Avizeh et al. (1) in Ahvaz, Iran, and slightly lower than 20% sero-prevalence reported by de Paula Dreer et al. (7). The detection of both pathogens in the blood samples not only revealed that these pathogens are endemic within these provinces, it also shows how dogs play an important role in maintenance of these pathogens in the environment, and how they can serve as reservoir of these infections not only to other dogs but also to other domestic animals and humans being in contact with them.

Although, there is no significant difference in the prevalence of Brucella spp. between the sexes, this finding is different from the report of Behzadi and Mogheisheh, (3) who report a significant difference.
between sexes among dogs in Fars province of Iran. The detection of Brucella in males more than females may be related to the habit of sniffing the genital among male dogs (20). This may also suggest the important venereal role the male dogs can play in the dissemination of the pathogen. There is also no significant difference in prevalence of Leptospira between the sexes, the finding which have also been reported by Avizheh et al. (1). However, this may show the equal chances of transmission of this pathogen by both sexes. Brucella spp. and Leptospira spp. were detected in dogs of all age groups although detected more among the adult dogs. This is not surprising, since these pathogens could be transmitted vertically in-utero and intra-partum from pregnant bitches to puppies (transplacental transmission) and horizontally between dogs.

The role of stray dogs in maintenance and transmission of infectious diseases cannot be emphasized, since these dogs not only wander the streets scavenging garbage, drinking pools of water on the streets and possibly hunting natural reservoir of diseases, such as rodents to feed themselves, but also, they may be exposed to an environment contaminated with infective pathogens. A significant higher prevalence of Brucella spp. and Leptospira spp. in stray dogs compare to companion dogs (P < 0.0001) observed in this study revealed that these dogs can travel long distances, and allows dissemination of these pathogens within the province, thereby provides chances of infection to other dogs, animals and to humans through environmental contamination. This finding supported the report of de Paula Dreer et al. (7) who demonstrated Brucella and Leptospira antibodies in stray dogs in Brazil. In previous study in Iran, using serological tests, the prevalence of Brucella canis antibodies was not evaluated in stray dogs compared with pure breeds (15). While in the report of Behzadi and Mogheiseh (3), all stray and mixed breeds were seronegative for Brucella canis antibodies in Fars province. This finding is opposite to our results. In another study in Ahvaz province, Ahvez et al. (1) demonstrated 7% sero-prevalence of Leptospira sp. in rural dogs, which are mostly stray dogs compared to 2.04% prevalence in urban dogs. These reports demonstrated the role which can be played by stray dogs in epidemiology of these pathogens in Iran.

Although, the majority of the work on survey of Brucella sp. and Leptospira sp. in dogs in Iran is based on serological tests such as microscopic agglutination test (MAT) and enzyme linked immunosorbent assay (ELISA), serological methods may only provide estimation of the exposure rate in dog population, but they do not provide information on proportion of dogs actively shedding these pathogens (1). However, the use of PCR has been effective in the detection of these active carriers and detection of subclinical infections. PCR has been used for the detection of various microorganisms. Sensitivity and specificity of the method are so high, that they are often used to compare other methods such as isolation and culture. PCR technique has been increasingly used for the detection of Brucella and Leptospira in clinical and research applications (2, 8). This work demonstrated the presence of DNA of the two studied bacteria, and that means a subclinical acute infection. This suggests the importance of the detection of pathogens in the blood, since the microorganisms are circulating in the blood stream and the infected animals seem to be healthy.

In conclusion, this study has confirmed that Brucella sp. and Leptospira sp. occur among the dogs in Isfahan and Shahrekord province, especially among stray dogs. This study has also demonstrated, for the first time in Iran, the usefulness of PCR in the detection Brucella sp. and Leptospira sp. in blood samples of dogs, and indicates that PCR can be used in the diagnosis of brucellosis and leptospirosis in dogs. However, in spite of PCR high sensitivity, the method is not routinely used in the diagnosis of leptospirosis and brucellosis. It can be used as a supplementary method with other, universally recognised methods (e.g. serological), especially valuable for diagnosis of bacteraemia or subclinical infections. Strict measures for the control of stray dogs are also highly recommended.

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References