Epidemiological Survey for *Brucella* in Wildlife and Stray Dogs, a Cat and Rodents Captured on Farms

Lam Quang TRUONG1), Jung Taek KIM1), Byung-II YOON1), Moon HER2), Suk Chan JUNG2) and Tae-Wook HAHN3)*

1)College of Veterinary Medicine, Kangwon National University, Chuncheon, Gangwon 200–701 and 2)National Veterinary Research and Quarantine Service, Anyang, Gyeonggi 430–757, South Korea

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**ABSTRACT.** *Brucella* infections in wildlife originate either from contact with infected livestock or from a natural sustainable reservoir in wildlife populations. As South Korea has set a goal of brucellosis eradication by 2013, it is necessary to determine the prevalence of *Brucella* in wildlife and wild rodents. This information will play an important role in the control of brucellosis. Because of the absence of prominent clinical signs, direct and indirect laboratory tests are essential for diagnosing brucellosis. In this study, tissue and blood samples were taken from wild animals, abandoned dogs, a cat and wild rodents, and they were tested for *Brucella* or *Brucella*-specific antibodies by isolation, PCR and serology. Results showed that 18.6% (33/177) of blood samples were positive by PCR, and 5.7% (11/194) were positive by C-ELISA. However, none of these samples yielded culturable bacteria. Of the tissue samples, 9.7% (8/82) were positive by PCR.

**KEY WORDS:** *Brucella abortus*, isolation, prevalence, serology, wildlife.

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*Brucella* is a genus of Gram-negative, aerobic, facultative intracellular bacteria that can be pathogenic in humans, livestock and wildlife. *Brucella* species are generally host-specific: *B. melitensis* is found in sheep and goats, *B. abortus* in cattle, *B. canis* in dogs, *B. ovis* in rams, *B. suis* in pigs, reindeer and rodents, *B. neotomae* in desert wood rats, *B. certi* and *B. pinnipedialis* in marine mammals, *B. microti* in common voles and *B. inopinata* in humans [12]. There is a lack of molecular, serological and bacteriological data on the prevalence of *Brucella* spp. in wildlife such as deer, gorals or rodents. Recent evidence, however, indicates that *Brucella* infection has been reported in wildlife populations. Wildlife reservoirs, including white-tailed deer, boars, feral pigs, bison, elk and European hares, complicate eradication efforts for *B. abortus* and *B. suis* [7, 12, 18, 24, 26, 29]. Although *Brucellosis* has been largely eradicated, it is a continuing and apparently increasing global problem with severe economic consequences.

According to a review of the literature, the first outbreak of bovine brucellosis in livestock in South Korea was reported in 1955 [21]. Increasing numbers of brucellosis outbreaks in livestock have been reported over the past 50 years, and the disease is still recognized as a major animal health problem in South Korea [4, 12, 15, 23, 27]. There has also been an associated rise in human cases of brucellosis. The first suspected case of human brucellosis in South Korea was recorded in a livestock worker in 2002 [22], and the number of human cases has risen continually since then [15].

The impact of mutual transmission of pathogens between livestock and wildlife is still unknown [10]. Only long-term surveillance of wildlife will help to identify the natural reservoir of diseases in endemic areas, creating opportunities for preventive measures that can be taken before hot spots become outbreaks. For example, in 2008, *B. abortus* biovar 1 was isolated for the first time from domestic elk in South Korea [12]. Epidemiological studies have shown that there is no difference between livestock and wild animals in the pathogenicity and transmission rates of *Brucella*, suggesting the possibility of spillover between livestock and wildlife [10]. Other reports suggest that rodents act as reservoirs in the epidemiology of *Brucella* in wildlife [10, 17, 26]. Consequently, an understanding of *Brucella* prevalence in wildlife and rodents is crucial to the control of livestock brucellosis in South Korea.

The aim of our investigation was to establish the prevalence of brucellosis in wild animals, abandoned dogs, cats and wild rodents in and around the areas of farms in some of the provinces in South Korea using serological, bacteriological and molecular methods. To our knowledge, this study is the first comprehensive survey of brucellosis in wildlife and wild rodents in South Korea.

**MATERIALS AND METHODS**

Sample collection: We collected 257 blood and 138 tissue samples (spleen=138 and lymph node=138) from wild animals, stray dogs, a cat and rodents captured in the Gangwon
and Chungcheong provinces of South Korea from 2008 to 2010. At autopsy, blood, spleen and lymph node samples were aseptically prepared for isolation and detection of brucellosis using the polymerase chain reaction (PCR), serological testing and bacterial isolation.

**Serological tests:** Serological tests were performed by competitive ELISA (approved to the OIE-prescribed tests for international trade) using the Svanovir *Brucella*-Ab C-ELISA kit (Svanova Biotech AB, Uppsala, Sweden). All steps were performed according to the manufacturer’s protocol.

**Bacterial isolation:** Bacteria were cultured for isolation according to National Veterinary Research and Quarantine Service (NVRQS) guidelines [19]. Briefly, 1–5 mL of each blood sample was centrifuged at 4,000 rpm for 10 min. The pellets were then inoculated in tryptic soy broth (TSB-BD Bacto) containing 5% bovine serum. Tissue samples were homogenized in sterile phosphate-buffered saline (PBS) using a Tissue Lyzer (QIAGEN, TX, U.S.A.). Tissue homogenates were inoculated directly onto tryptic soy agar (TSA) and into TSB supplemented with 5% bovine serum and antibiotic mixtures (25 µM bacitracin, 20 µg/ml vancomycin, 5 µg/ml nalidixic acid, 5 µ/ml polymyxin B, 100 µg/ml cyclohexamide and 100 µg/ml nystatin). Plates were incubated under air supplemented with 5% CO₂ at 37°C for 5–10 days and examined daily for the presence of colonies. Colonies were selected, inoculated onto TSA containing 5% bovine serum and cultivated for 3–4 days at 37°C under air supplemented with 5% CO₂. To confirm the presence of *Brucella* spp., the organisms were identified by colony morphology, Gram staining, oxidase and catalase production, urease activity, H₂S production, CO₂ requirement and sensitivity to nalidixic acid, bacitracin, polymyxin, cyclohexamide, nystatin, gentamicin, streptomycin, kanamycin, and rifampicin. Plates were incubated at 20°C for 4 days. Tissue samples were incubated at 20°C for 4 days.

**DNA isolation and amplification:** Genomic DNA from peripheral blood and tissue samples was prepared for PCR using a commercial kit (DNAeasy Blood & Tissue, QIAGEN) following the manufacturer’s instructions. For bacterial cells, the genomic DNA of isolated colonies was obtained using the HiYield Genomic DNA kit (RBC, Taiwan).

For the detection of *Brucella* in blood and tissue samples, the amplifications were performed using previously described primers [25], which produce 905-bp amplicons. Primers were synthesized by Bioneer Ltd. (Daejeon, South Korea). Each reaction tube contained the following: 50 mM KCl, 10 mM Tris HCl (pH 9.0), 0.1% Triton X-100 (Promega Corp. U.S.A.), 200 µM each deoxynucleoside triphosphate (dNTPs), 1 mM MgCl₂, 0.5 µM each primer, 0.5 U of *Taq* DNA polymerase (Promega Corp.) and 5 µl of template DNA in a total volume of 25 µl. The reaction was performed in a Programmable Thermal Controller PTC100 (MJ Research, Watertown, MA, U.S.A.) under the following conditions: initial denaturation at 95°C for 5 min followed by 35 cycles of 30 sec of denaturation at 95°C, 90 sec of primer annealing at 54°C and 90 sec of primer extension at 72°C with a final extension at 72°C for 6 min. To identify and differentiate between *Brucella* species, the advanced Bruce-ladder PCR was performed as described previously [9, 12, 14]. Amplifications were prepared in a 20 µl reaction mixture containing 2 µl of template DNA, 2X PCR premix (Cosmo Genetech, South Korea) and 10 pmol of each primer. Thermal cycling was performed with a GeneAmp PCR System 2700 (Applied Biosystems, CA, U.S.A.). After initial denaturation at 95°C for 5 min, the PCR performed as was follows: 30 sec of denaturation at 95°C, 2 min of primer annealing at 60°C and 1 min of primer extension at 72°C for 30 cycles with a final extension at 72°C for 5 min. Negative controls containing all PCR reagents except template DNA were used to monitor cross contamination. The positive controls were genomic DNA isolated from *B. abortus* biovar (bv) 1–544, *B. canis* RM6/66, *B. suis* bv1 1330, *B. ovis* 63/290, *B. neotomae* 5K33 and *B. melitensis* bv1 16M reference strains, which were kindly supplied by the OIE Reference Laboratory for Brucellosis in National Veterinary Research and Quarantine Service in Korea. To check the reliability of the results and to detect any external contamination, all samples were processed in duplicate. The size of the amplified DNA was determined by electrophoresis on 1.5% agarose gels and comparison with DNA molecular-weight standards (Bioneer, Daejeon, Korea). The presence of a well-defined band was considered as a positive result.

**RESULTS**

The prevalence of brucellosis in the 257 blood samples examined during the study period is shown in Table 1. Seropositivity by C-ELISA was found in 5.7% (11/194) of the samples, including 8.2% (4/49) of the Chinese water deer and 10.2% (7/68) of the rodents. Thirty-three samples were PCR-positive for *Brucella*, yielding an overall prevalence of 18.6% (33/177), including 33.3% (9/27) in gorals, 11.1% (1/9) in raccoons, 16.6% (7/42) in Chinese water deer and 34.7% (16/46) in dogs. There were, however, inconsistent results; some samples were positive by PCR but negative by serological testing and vice versa. In addition, in this study, the number of samples used for each method of testing differed because the volume of blood and tissue received was sometimes small, or the blood was clotted and hemolyzed. Some specimens were received as tissue and serum only, so other tests could not be applied. Although sera samples were examined both by RBT (Rose Bengal Test) and C-ELISA assays, RBT yielded high numbers of false positive results due to hemolysis. Additionally, positive results were poorly correlated between RBT and C-ELISA tests (data not shown). As seen in Table 1, all blood samples were culture-negative but positive by both PCR and C-ELISA.

In Table 2, we demonstrate that 8 out of 82 tissue samples (9.7%) originating from wildlife were *Brucella*-positive by 16S rRNA PCR. Of these samples, 10.8% (4/37) and 40% (2/5) were obtained from Chinese water deer and raccoons,
respectively, while samples from badger and cat were found to be positive for the amplified target DNA of *Brucella*. By contrast, *Brucella* was isolated by tissue culture from only one sample out of 138 (0.7%). Interestingly, this isolate was obtained from Chinese water deer tissue (1/37).

The characterization and identification of pathogenic *Brucella* isolates clearly indicate that the etiological pathogen in Chinese water deer was *B. abortus* biovar 1, which had the following characteristics: small Gram-negative, non-motile and non-fermentative coccobacilli requiring 10% CO2 on primary isolation and no serum necessary for growth; catalase (+), oxidase (+), urease (+), gelatin (–), H2S (+), thionin (–), basic fuchsin (+); agglutination in A (+), M (–) and R (–); phage sensitivity to Tbilisi (+), Webridge (+); non-smooth cultures were lysed by *brucella*-phage R at RTD. Specific 905-bp PCR products indicated that the isolate belonged to the genus *Brucella* (Fig. 1A). In addition, the Bruce ladder PCR used for the identification and differentiation of *Brucella* species amplified four fragments that were 1,682, 587, 450 and 152 bp in size, proving that the isolate was *B. abortus* (Fig. 1B).

**DISCUSSION**

It was unknown whether the animals were in the early or chronic stages of infection when sampled. The possibility remains that some of the animals may have cleared the

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**Table 1.** *Brucella* prevalence in blood samples from wild animals collected from 2008–2010

<table>
<thead>
<tr>
<th>Species</th>
<th>C-ELISAa) Positive (%)</th>
<th>Direct PCRb) Positive (%)</th>
<th>Isolation Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goral</td>
<td>0/27 (0)</td>
<td>9/27 (33.3)</td>
<td>0/27</td>
</tr>
<tr>
<td>Raccoon</td>
<td>0/32 (0)</td>
<td>1/9 (11.1)</td>
<td>0/9</td>
</tr>
<tr>
<td>Chinese water deer</td>
<td>4/49 (8.2)</td>
<td>7/42 (16.6)</td>
<td>0/83</td>
</tr>
<tr>
<td>Roe deer</td>
<td>0/3 (0)</td>
<td>0/3 (0)</td>
<td>0/3</td>
</tr>
<tr>
<td>Dog</td>
<td>0/14 (0)</td>
<td>16/46 (34.7)</td>
<td>0/46</td>
</tr>
<tr>
<td>Weasel</td>
<td>0/1 (0)</td>
<td>0/1 (0)</td>
<td>0/1</td>
</tr>
<tr>
<td>Rodent</td>
<td>7/68 (10.2)</td>
<td>0/49 (0)</td>
<td>0/88</td>
</tr>
<tr>
<td>Total</td>
<td>11/194 (5.7)</td>
<td>33/177 (18.6)</td>
<td>0/257</td>
</tr>
</tbody>
</table>

a, b) The number of samples obtained by each method differed because the volume of blood received was sometimes small, or the blood was clotted and hemolyzed. Some specimens were received as serum only, so other tests could not be applied.

**Table 2.** *Brucella* prevalence in tissue samples from wild animals collected from 2008–2010

<table>
<thead>
<tr>
<th>Species</th>
<th>Direct PCR Positive (%)</th>
<th>Isolation Positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goral</td>
<td>0/2 (0)</td>
<td>0/2 (0)</td>
</tr>
<tr>
<td>Raccoon</td>
<td>2/5 (40)</td>
<td>0/5 (0)</td>
</tr>
<tr>
<td>Chinese water deer</td>
<td>4/37 (10.8)</td>
<td>1/37 (2.7)</td>
</tr>
<tr>
<td>Roe deer</td>
<td>0/4 (0)</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Badger</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Cat</td>
<td>1/1 (100)</td>
<td>0/1 (0)</td>
</tr>
<tr>
<td>Rodent</td>
<td>0/32a) (0)</td>
<td>0/88 (0)</td>
</tr>
<tr>
<td>Total</td>
<td>8/82 (9.7)</td>
<td>1/138 (0.7)</td>
</tr>
</tbody>
</table>

a) The tissue samples obtained from rodents were small. Hence, these samples were used for isolation only.
infection prior to sampling. It is also possible that the bacteria were present in very low numbers, which would be consistent with the small number of colony-forming units detected in blood samples by bacteriological methods. Previous studies [12, 13, 16] have demonstrated that antibodies are not present at detectable levels in the first 12–16 days after infection in artificially inoculated animals. Hence, the stage of infection at which a sample is collected could have a major impact on Brucella diagnosis. Therefore, C-ELISA and bacterial isolation may be inappropriate diagnostic methods in the early stages of infection. In addition, some tested animals may yield false seropositive results because of infection by other Gram-negative microorganisms, particularly Yersinia enterocolitica O:9 and Escherichia coli O157:H7. Low isolation rates could also be attributed to the fact that recovery of bacteria from blood cultures is not sufficiently sensitive [11, 13, 16]. For these reasons, PCR could be especially useful for the detection of Brucella during early infection.

In the lymphoid tissue samples, culture and the molecular method yielded conflicting results. We found Brucella DNA in 8 samples, but these samples yielded only one isolate. This difference is likely a result of the higher sensitivity of PCR compared with traditional culture methods [3, 28]. On the other hand, PCR can yield positive results when only bacterial DNA is present and there are no live bacteria in a tissue sample [28] or when bacteria closely related to the target species, such as Ochrobactrum spp., are present [25]. Differences in isolation techniques, sample types and the viability of the organism in the sample may also have affected the Brucella isolation rates [2, 13, 28]. Additionally, negative results from tissue samples may be attributable to contamination, a difficulty that previously proved to be a major factor in the rate of B. abortus isolation [1, 8].

Brucella infections have been documented world-wide and in a great variety of terrestrial wildlife species. B. abortus in particular has been reported to have a wide host range in wild mammals. An important consideration with regard to terrestrial brucellosis in wildlife is to distinguish between a spillover of infection from domestic animals and a sustainable infection within a susceptible wildlife population. The impact of mutual transmission of pathogens between livestock and wildlife is still unknown [10], but several species of deer have been shown to be either naturally or experimentally susceptible to Brucella infection. White-tailed deer, mule deer, sika deer, fallow deer and axis deer are all experimentally susceptible to B. abortus infection [5, 6], and serologic evidence has shown that their antibody responses are similar to those of cattle [6, 12, 24]. Correspondingly, it appears that Brucella in general and B. abortus in particular can be transferred to deer from cattle either directly or indirectly, as B. abortus biovar 1 is the main Brucella species isolated from domestic elk and cattle in South Korea [12].

In conclusion, our results demonstrated the presence of Brucella spp. infections in wildlife, abandoned dogs, a cat and wild rodents in areas near farms in the Gangwon and Chungcheong provinces of South Korea. This is the first report of brucellosis caused by B. abortus biovar 1 in wild Chinese water deer, and we suggest that the Chinese water deer is a potential reservoir of infection in wildlife. Hence, to effectively control and prevent brucellosis in Korea, wildlife should be included in the routine Brucella surveillance program, as they may be important intermediaries in the transfer of Brucella species to livestock and may also pose an serious public health risk [12]. More studies are recommended to isolate the bacterium for characterization and comparison with the strains circulating in livestock.

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REFERENCES


