

# Prevalence of *Coxiella burnetii* Infection in Domestic and Wild-animals in Korea Detected by Real-time Loop-mediated Isothermal Amplification (LAMP)

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**ABSTRACT:** Background: *Coxiella burnetii* (*C. burnetii*) is an obligate intracellular bacterium and causative agent of Q fever that has been considered as a zoonotic disease worldwide, animals and humans can be infected by this bacteria. Rapid detection of the infection by this microorganism is difficult due to its two phase's infection as acute and chronic. Real-time loop-mediated isothermal amplification (LAMP) is an appropriate diagnostic tool due to its rapidity, sensitivity and specificity.

**Materials and methods:** A total of one hundred ninety ticks of different stages were collected from seven wild animals like Korean squirrels, raccoons, Korean water deer, otters, martens, owls, and buzzards from various regions in Korea and prepared in pool one hundred two samples. Their extracted genomic DNA were tested by LAMP for the molecular detection of *C. burnetii* in ticks by which animals can be infected where ticks play as vector. Transposon gene of *C. burnetii* IS1111a insertion sequence was used as a detection marker to assure the presence of *C. burnetii* infection in wild animals by LAMP. LAMP assay was evaluated in fluorescence detector Genie II (Optigen, United Kingdom) at 65-67°C for 30-37 min under isothermal conditions.

**Results:** After amplification of DNA, seven samples out of 102 (6.86%) samples showed their positivity in LAMP. Overall results in wild animals showed that the Korean water deer and raccoon were mostly infected by *C. burnetii* among other wild animals. None of infection by *C. burnetii* was detected in otter, marten and birds.

**Conclusion:** The presence of *C. burnetii* in wild animals indicates that Q fever can be considered as a threatening disease but is not currently considered as a significant threat for wild animals in Korea.

## INTRODUCTION

*C. burnetii* is a gram negative, obligate intracellular bacterium and responsible for Q fever that has been considered as a zoonotic disease worldwide. This bacterium causes Q fever in human, and domestic and wild animals such as sheep, goats, cats, dogs; and owls, buzzards, Korean squirrels, raccoons, Korean water deer, otters, martens. Q fever has been described as two stages: an acute stage and chronic stage. Most commonly Q fever is transmitted by inhalation of contaminated aerosol, contact with contaminated milk, meat, wool and particularly birthing products. Q fever is well recognized cause of abortions in ruminants and in pets, When infected animals give birth, the environment becomes contaminated by the infected amniotic fluid and placenta, and the organism can survive and present in soil and dust for a long period in the environment surfaces up to 60 days and can spread the disease long distances by the air. This

pathogen is exceedingly sustainable and virulent, and highly resistant to environmental surfaces such as high temperature, osmotic pressure, chemicals and ultraviolet rays, and for those reasons is exceedingly difficult to eradicate from the environmental components (Kagawa et al., 2003; Kazár 2005; Tissot-Dupont et al., 2004). *C. burnetii* infection has also been observed in ticks, are the most important vectors for playing an important role to transmit Q fever through their almost painless bites or exposure. Ticks are considered to be the natural primary reservoirs of *C. burnetii* responsible for the transmit of the infection in wildlife and domestic animals (Parisi et al., 2006).

Real-time loop-mediated isothermal amplification (LAMP) assay is highly sensitive diagnostic method that has several advantages, including rapidity, high sensitivity, specificity, ease of application and low cost with effective outcome in less than under isothermal conditions (60-65)°C. LAMP is the most effective diagnostic tool for the detection of Q fever because it can amplify the DNA in 30 min. LAMP assay was developed by Notomi et al (2000), with high sensitivity, rapidity, efficiency and specificity under isothermal condition. This method applies a DNA polymerase and a set of four specially designed primers that recognizes a total of six distinct regions on the target DNA on the basis of autocycling strand displacement DNA synthesis. An important nature of this method is that no denaturation step of the DNA template is demanded (Nagamine et al., 2001). A set of two inner primers, two outer primers and loop primers has been used for the detection of the insertion sequence IS1111a transposes gene of *C. burnetii*. In this study, prevalence of *Coxiella burnetii* infection was detected by LAMP in domestic and wild-animals in Korea.

## **MATERIALS AND METHODS**

A total of one hundred ninety ticks were collected from various regions in Korea, that are shown in (Table 1). Collected ticks of different stages were prepared in pool turned 102 samples. These ticks were collected from seven wild animals like raccoon (n = 37), Korean water deer (n = 54), owl (n = 3), Buzzard (n = 4), otter (n = 1), marten (n = 1) and Korean squirrel (n = 2) between 2012 and 2013 from the different provinces in South Korea. Ticks were identified as two species e.g. *Ixodes ricinus* and *I. scapularis*.

Collected feeding ticks were removed from mentioned wild life with forceps and placed in a 1.5 ml Eppendorf tube filled with 70% ethanol and stored at -20°C until DNA extraction.

### ***DNA extraction from ticks***

DNA extraction from ticks (*Ixodid*) is exceedingly difficult due to their hard exoskeleton dermis. A total of 190 ticks were collected and pooled in 102 samples and disruption of ticks must be needed prior to DNA extraction. Total genomic DNA was extracted from frozen individual 102 tick samples. Frozen ticks were prepared in pools of (2-6) nymphs, (1-5) larvae, and 49 adult ticks were used individually and various quantities of mixed adults and nymphs also used for extraction. Ticks were washed three times in 1ml 1x Dulbecco's phosphate buffered saline (DPBS) and finally added 500  $\mu$ l DPBS. Individual tick samples were dissected into pieces with a sterilized scissor then those were placed in a separate 1.5 ml eppendorf tube to avoid cross contamination. A mixture of SSB14B 0.9-2.0 mm (NEXTADVANCED) sterilized stainless steel beads were used to homogenate for all stages of ticks. Then tick specimens were set into a Bullet Blender® Homogenizer in 2 times for 3 and 2 min with 30s intervals. The homogenate was centrifuged at room temperature and the supernatant was stored at  $-80^{\circ}\text{C}$  for further processed. DNA was extracted from supernatant of ticks using MagMax™Viral RNA Isolation kit (ambion® by life technologies™, USA) according to manufacturer's instructions. This kit can isolate total nucleic acid, DNA and RNA. DNA and RNA were eluted in a final volume of 50  $\mu$ l each and stored at  $-80^{\circ}\text{C}$  for further use. The DNA concentrations of ticks were measured by spectrophotometer.

### ***Design of primer set for real-time LAMP assay***

A set of primers was designed using Primer Explorer V4 software [<http://primerexplorer.jp>, Eiken chemical Co., Ltd., Tokyo, Japan] against the multicopy 198 bp sequence IS1111a of *C. burnetii* RSA 493 (AE016828.2) based on conserved sequences. Sequences and positions of the repetitive sequence IS1111a, are shown in Figure 1. Designed LAMP primer set was defined as CB\_01 are shown in Table 2. 5 primers were designed (F3, B3, FIP, BIP and LF) for each set and that were forward outer primer (F3), backward outer primer (B3), forward inner primer (FIP) contains the complementary sequence of the F1 region and the sense sequence of the F2 region (F1c + F2) and backward inner primer (BIP) constitutes the complementary sequence of the B1 region and the sense sequence of the B2 region (B1c + B2),

forward loop primer (LF), applied those for the detection of IS1111a gene in LAMP assay for accomplishing molecular screening of ticks DNA samples.

### ***Reaction of LAMP***

The LAMP assay was carried out in a total 25 µl of the final reaction mixture. The mixture contained 15 µl of 1x ISO-001 isothermal Master Mix (Optigen, United Kingdom) including *GspSSD* large fragment DNA polymerase isolated from *Geobacillus* species, thermostable organic pyrophosphatase, optimized reaction buffer, MgCl<sub>2</sub>, deoxynucleoside triphosphatase and a double stranded DNA binding dye (Optigen), 1 µl each of two outer primers F3 and B3, two forward and backward inner primers FIP and BIP respectively, and one loop primer LF, and 4 µl of ticks DNA used as a template, and 1 µl of deionized sterile distilled water ( BIONEER), was used as a negative control as well and *Coxiella burnetii* DNA control (Amplirun®) was used as a positive control in each reaction. The LAMP reaction mixture was run in a real-time fluorometer Genie II (Optigen, UK) that is easy to use and determines the optimal temperature with the shortest amplification time including highest speed fluorescence reading and followed at the temperatures of 65°C to 67°C. All the LAMP assays were run for the time between 30 min and 37 min for the presence of *C. burnetii* in ticks DNA followed by heating and cooling steps of 98°C to 80°C at 0.05°C/s to allow reannealing of amplified DNA and Genie II displays annealing curve with annealing temperature values for each reaction. After amplification, the LAMP products were also run by electrophoresis on a 2% agarose gels in TBE (Trizma base, Boric acid, EDTA ) buffer (BIONEER), stained with Redsafe™ Nucleic Acid staining solution and visual inspection with under UV light.

### **RESULTS**

Genomic DNA from 102 tick samples was determined by LAMP for the detection of *C. burnetii* and the prevalence of this pathogen indicates that few positive samples LAMP (Figure 2). Only seven *C. burnetii* infected tick samples of *I. scapularis* of two nymph samples and one adult samples which were obtained from raccoon, and one nymph and one adult samples of *I. ricinus* and one nymph samples of *I. scapularis*

from Korean water deer, and one adult unfed *I. ricinus* tick from Korean squirrel showed positivity according to LAMP assay (Table 3). The results presented that 6.86% (7/102) of the samples were positive to LAMP and shown in Table 4.

**Sensitivity of the LAMP assay**

The sensitivity of LAMP assay was determined by testing ten-fold serial dilutions of positive ticks DNA samples containing the IS1111a transposon gene of *C. burnetii* and the detection limits were (10<sup>9</sup> to 10<sup>1</sup> copies/μl) (Figure 3).



**Table 1. Host, host species and the vector tick species including their life stages.**

| Family      | Host common Name  | Host species                        | Tick species         | Larvae | Nymphs | Adults | Total |
|-------------|-------------------|-------------------------------------|----------------------|--------|--------|--------|-------|
| Procyonidae | Raccoon           | <i>Procyon lotor</i>                | <i>I. scapularis</i> | 7      | 33     | 12     | 52    |
|             |                   |                                     | <i>I. ricinus</i>    | 7      | 16     | 2      | 25    |
| Cervidae    | Korean water deer | <i>Hydropotes inermis argyropus</i> | <i>I. scapularis</i> | 0      | 21     | 0      | 21    |
|             |                   |                                     | <i>I. ricinus</i>    | 0      | 24     | 43     | 67    |
| Mustelidae  | Otter             | <i>Lontra felina</i>                | <i>I. ricinus</i>    | 0      | 0      | 1      | 1     |
| Mustelidae  | Marten            | <i>Martes martes</i>                | <i>I. scapularis</i> | 0      | 3      | 0      | 3     |
| Sciuridae   | Korean squirrel   | <i>Eutamias sibiricus</i>           | <i>I. ricinus</i>    | 0      | 0      | 2      | 2     |
| Strigidae   | Owl               | <i>Athene cunicularia</i>           | <i>I. scapularis</i> | 0      | 5      | 0      | 5     |
|             |                   |                                     | <i>I. ricinus</i>    | 0      | 0      | 1      | 1     |

|              |         |                    |                      |                      |                       |                       |            |
|--------------|---------|--------------------|----------------------|----------------------|-----------------------|-----------------------|------------|
| Accipitridae | Buzzard | <i>Buteo buteo</i> | <i>I. scapularis</i> | 6                    | 0                     | 0                     | 6          |
|              |         |                    | <i>I. ricinus</i>    | 7                    | 0                     | 0                     | 7          |
| <b>Total</b> |         |                    |                      | <b>28</b><br>(14.2%) | <b>107</b><br>(54.5%) | <b>61</b><br>(31.12%) | <b>190</b> |

**Table 2. A primer set CB\_01 for amplification of IS1111a gene in LAMP.** LAMP primers; OF, OR, IF, IR and LF indicate outer forward, outer reverse, inner forward, inner reverse and loop forward, respectively.

| Primer Name  | Type | Length (nt) | Amplicon size | Sequence(5'-3')                                      |
|--------------|------|-------------|---------------|--|
| F3           | OF   | 18          | 198 bp        | AGCCACCTTAAGACTGG                                    |
| B3           | OR   | 18          |               | CGTCATAATGCGCCAACA                                   |
| FIP (F1c-F2) | IF   | 45          |               | GATGAGTGGGGTAAAGTGATCTAC-<br>CTACGGTGGATACATACTGAG   |
| BIP (B1c-B2) | IR   | 47          |               | TTCCACACAGTTGAAAAACATCTTT-<br>AAAAAAGGAGAAGGTCCATGAA |
| LF           | LF   | 19          |               | ACGAGACGGGTTAAGCGTG                                  |

**Table 3. *C. burnetii* infected tick samples including 2 species showed positivity in real-time LAMP and conventional PCR.**

| Species                                  | Life stages   | Real-time LAMP       |                    |
|--|---------------|----------------------|--------------------|
|  |               | <i>I. scapularis</i> | <i>I. ricinus</i>  |
| <b>Raccoon</b><br>(37, 36.27%)           | <b>Adults</b> | <b>11% (1/9)</b>     | <b>0(0/2)</b>      |
|  | <b>Nymphs</b> | <b>14.2 % (2/14)</b> | <b>0(0/3)</b>      |
|  | <b>Larvae</b> | <b>0(0/2)</b>        | <b>0(0/2)</b>      |
|  | <b>Mixed</b>  | <b>0(0/2)</b>        | <b>0(0/3)</b>      |
| <b>Korean water deer</b><br>(54, 52.94%) | <b>Adults</b> | <b>0</b>             | <b>2.9% (1/34)</b> |
|  | <b>Nymphs</b> | <b>16.6% (1/6)</b>   | <b>12.5% (1/8)</b> |
|  | <b>Mixed</b>  | <b>0</b>             | <b>0(0/6)</b>      |
| <b>Otter (1, 0.98%)</b>                  | <b>Adult</b>  | <b>0</b>             | <b>0(0/1)</b>      |
| <b>Marten (1, 0.98%)</b>                 | <b>Nymph</b>  | <b>0(0/1)</b>        | <b>0</b>           |

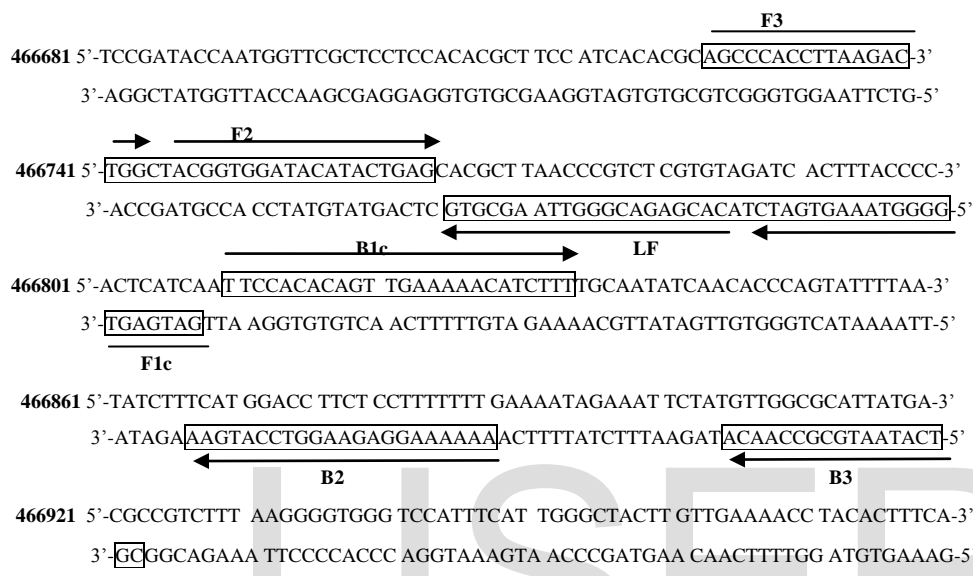
|                                   |                     |                    |                    |
|-----------------------------------|---------------------|--------------------|--------------------|
| <b>Korean squirrel (2, 1.96%)</b> | <b>Unfed adults</b> | <b>0</b>           | <b>50%(1/2)</b>    |
| <b>Owl (3, 2.94%)</b>             | <b>Adult</b>        | <b>0</b>           | <b>0(0/2)</b>      |
|                                   | <b>Nymphs</b>       | <b>0(0/1)</b>      | <b>0</b>           |
| <b>Buzzard (4, 3.92%)</b>         | <b>Larvae</b>       | <b>0(0/2)</b>      | <b>0(0/2)</b>      |
| <b>Total of samples</b>           | <b>102</b>          | <b>10.5%(4/38)</b> | <b>4.68%(3/64)</b> |

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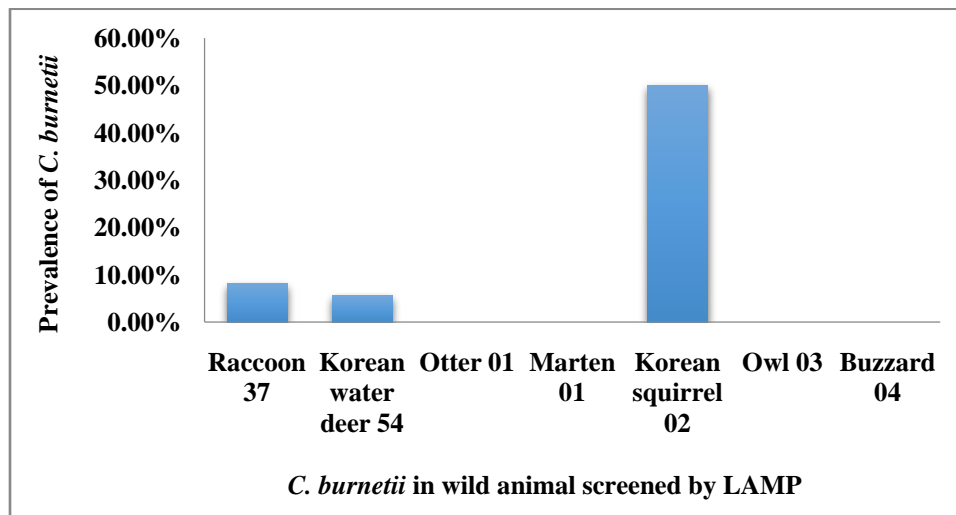
**Table 4. Results of LAMP. A total of 190 ticks prepared in pooled in 102 samples.**

| Collected feeding ticks  |           | Real-time LAMP  |                  |
|--------------------------|-----------|-----------------|------------------|
|                          |           | Positive        | Negative         |
| <b>Raccoon</b>           | <b>37</b> | <b>3(8.1%)</b>  | <b>34(91.8%)</b> |
| <b>Korean water deer</b> | <b>54</b> | <b>3(5.55%)</b> | <b>51(94.4%)</b> |
| <b>Otter</b>             | <b>01</b> | <b>0</b>        | <b>01</b>        |
| <b>Marten</b>            | <b>01</b> | <b>0</b>        | <b>01</b>        |
| <b>Korean squirrel</b>   | <b>02</b> | <b>01(50%)</b>  | <b>01(50%)</b>   |

|                      |            |                  |                   |
|----------------------|------------|------------------|-------------------|
| <b>Owl</b>           | <b>03</b>  | <b>0</b>         | <b>03</b>         |
| <b>Buzzard</b>       | <b>04</b>  | <b>0</b>         | <b>04</b>         |
| <b>Total samples</b> | <b>102</b> | <b>07(6.86%)</b> | <b>95(93.13%)</b> |

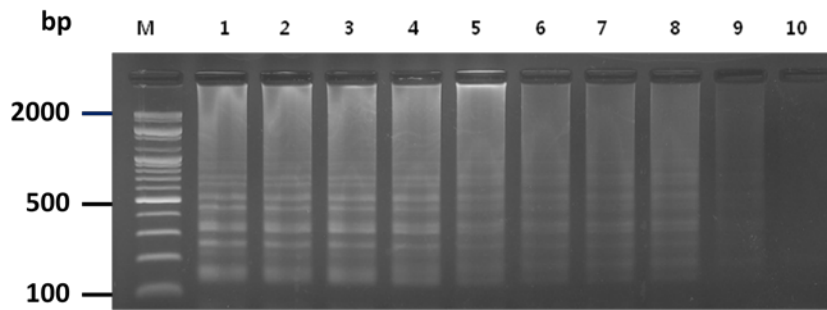


**Figure 1. Location and sequences of LAMP primer (CB\_01):** Primer targets for IS1111a gene of *Coxiella burnetii*. 5 LAMP primers: F3, B3, FIP (F1c-F2), BIP (B1c-B2) and LF, arrows indicate including box the direction of the pathway. GenBank accession number AE016828.2.



**Figure 2. Prevalence of *C. burnetii* infection in wild animal.**





**Figure 3. Sensitivity detection of LAMP including primer CB\_01:** LAMP was performed using 10-fold serial dilutions of positive tick sample DNA containing IS1111a gene of *C. burnetii*. Lanes: 1-9 represent  $10^9$ ,  $10^8$ ,  $10^7$ ,  $10^6$ ,  $10^5$ ,  $10^4$ ,  $10^3$ ,  $10^2$ ,  $10^1$  copies/ $\mu$ l per reaction. Lane M 100bp DNA Ladder and lane 10, negative control (DW).

## DISCUSSION

The objective of this study was to determine the prevalence of *C. burnetii* infection in wild animal by LAMP in Korea. There is a difference between outcomes of real-time LAMP (7/102) In this study, it was found few positive *C. burnetii* infections in wild animals like raccoon, Korean water deer and Korean squirrel using LAMP assay, and other animals were not infected by *C. burnetii*. 11% (1/9) individual adult and 14.2% (2/14) nymph samples of *I. scapularis*; 16.6% (1/6) nymph samples of *I. scapularis*, 2.9% (1/34) individual adult and 12.5% (1/8) nymph samples of *I. ricinus*; and one adult (1/2) *I. ricinus* ticks were found to be *C. burnetii* positive which were ectoparasites on raccoon, Korean water deer and Korean squirrel respectively, according to real-time LAMP assay.

In order to demonstrate the mechanism, the sensitivity, the specificity and the easy handle to use of real-time LAMP, for this purpose, all of the tick samples were screened in LAMP which was carried out with the temperature of 65-67°C for 30-37 min and observed with portable device Genie II (Optigen.Co.UK). The use of four primers (FIP, BIP, F3 and B3) which recognized of six distinct regions in the initial steps of LAMP and two primers that recognized of four distinct sequences during the subsequent steps ensure high specificity for target amplification. Moreover, in LAMP four primers are simultaneously used to

initiate DNA synthesis from the original unamplified DNA to generate A stem-loop DNA for subsequent LAMP cycling, during which the target is recognized by four sequence (Notomi et al., 2000). But for this present study we used one forward primer as a Loop primer instead of two Loop primers for the target gene. Tick borne *C. burnetii* species causes Q fever in both humans and animals to minimize cross contamination and false-positive results, positive and negative controls were included in each reaction for LAMP run. In addition, DNA extraction, reaction mix preparation, sample addition, and LAMP analysis were performed in separated.

Present study showed, obtained results that indicates Korean water deer is more affected with *C. burnetii* than other mammals and raccoon has also been more infected but lower than Korean water deer. But tested among wild-animals, all the mammals are infected by *C. burnetii* except birds. The positivity in wild-animal that demands more protection and attention for healthy animals, and can be affected by infected animals, also requires the eradication of *C. burnetii* for saving wildlife's lives.

In dairy cattle, sheep and goats are considered to be the reservoirs of *C. burnetii* responsible for the infection in humans and animals, and the presence of *C. burnetii* in wild animals in Korea premises, wild animals can be considered as the reservoir as well. Infected wild animals and cattle shed numerous numbers of the organisms in their amniotic fluid and infected placenta which are the potential sources of the infection in humans and animals via inhalation of contaminated aerosols or airborne dust (Aitken et al., 1989; Babudieri et al., 1959; Baca et al., 1983; Biberstein et al., 1974; Lang et al., 1990). In addition, several reports of isolation of *C. burnetii* from humans, cattle and ticks have been published (Nagaoka et al., 1996; Oda et al., 1989; To et al., 1995; To et al., 1996). The purpose of this work was to investigate the prevalence of *C. burnetii* infection in wild animals in Korea.

*C. burnetii* infection is highly associated with reproductive disorders (infertility, metritis and mastitis) in cattle in Japan. The high prevalence of *Coxiella* infection in dairy cattle with reproductive problems showed that these infected cattle play an important role in maintaining the infection and in dispersing the pathogenic agent into the environment, where the resistant organism can remain viable over long periods of time through excretion, i.e., milk, colostrums, urine and amniotic fluid. Thus excretions are considered

to be potential sources of the infection in humans and animals via inhalation of infectious aerosols or airborne dust (To et al., 1996; Yanase et al., Yasumoto et al., 1997).

This study has assured the presence of *C. burnetii* infection in wild animals in Korea, this pathogen was considered to occur with a low prevalence and there has been unknown outbreak of Q fever in Korea. The outcomes of Q fever in Korea is much lower than that in France, Australia and Taiwan (Frankel et al., 2011; NNDSS, 2012; CDC, Taiwan, 2011). Approximately 70% of Korean landscape is mountainous. As a result of a national tree planting policy instituted in the 1960s, young to mature planted groves and volunteer trees now cover mountains and hillsides. These forests provide harborage for wild and feral mammals (i.e, water deer (*Hydropotes inermis*), Siberian roe deer (*Capreolus pygargus*), wild boar (*Sus scrofa*) raccoon dog (*Nyctereutes procyonoides*) leopard cat (*Prionailurus bengalensis*), Eurasian badger, (*Meles leucurus*), weasel (*Mustela sibirica*), rodent, soricomorph; feral cat, (*Felis catus*) and feral dog (*Canis lupus*)) and forest dwelling birds like ring-necked pheasants (*Phasianus colchicus*)) that are host to a number of ectoparasites and pathogens they harbor. Comprehensive tick-borne diseases surveillance program that include host vector pathogen relationship provide a better understanding of the diversity of host and host-ectoparasites-zoonotic pathogen relationships that impact on the health of domestic animal, birds, and humans.

Q fever is likely to be under-diagnosed and under-surveyed in Korea due to complications in diagnose as asymptomatic nature. Q fever is not currently considered as a risk factor for public health in Korea. But on the basis of our study, ticks can be thought to be the natural reservoir of *C. burnetii* and wild-animal also can act as a reservoir in Korea. Therefore, increased awareness among physicians for better detection and treatment of Q fever, development of the diagnostic method, and more observations for under-diagnose syndromes, are required.

## CONCLUSION

The presence of *C. burnetii* in wild animals indicates that Q fever can be considered as a reservoir and little threatening disease because it is under-diagnosed and less-observed in Korea. Q fever is not

currently considered as a significant threat for wild animals in Korea but infected animals can spread the disease to healthy animals and this pathogen can survive in long periods of time in wild animals. Therefore, it requires further diagnosis and more observation with effective diagnostic tools because complications in diagnose as it is asymptomatic.

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