Molecular characterization and seroprevalence of *Coxiella burnetii* from healthy cattle in the Republic of Korea

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Abstract

This study was conducted to investigate the prevalence of *Coxiella burnetii* infection according to cattle breeds and growth types. A total of 491 cattle [cattle breed: 216 dairy cattle and 275 beef cattle; according to growth type: indoor housing (*n* = 294) and grazing (*n* = 197)] were tested for the presence of *C. burnetii* DNA and antibodies against *C. burnetii* using a commercial enzyme-linked immunosorbent assay (ELISA). Overall, 10.8% and 8.8% of the cattle were positive by PCR and for *C. burnetii* antibodies, respectively. The prevalence of *C. burnetii* was significantly higher in beef cattle than in dairy cattle using PCR (13.6% vs 7.4%; *P* = 0.032) and ELISA (14.6% vs 1.4%; *P* = 0.000), respectively. The overall infection rate of *C. burnetii* was significantly high in grazing cattle (PCR: 24.9%, ELISA: 21.3%; *P* = 0.000) compared with housing cattle (PCR: 1.4%, ELISA: 0.3%). The results indicate that beef cattle have a significantly higher risk of contracting *C. burnetii* infection compared with dairy cattle (21.5% vs. 7.9%, $\chi^2 = 5.82$, *P* = 0.000, odds ratio = 3.197, 95% CI: 1.80–5.67). In addition, the infection of *C. burnetii* was significantly associated with grazing (*P* = 0.000). Moreover, a risk of contracting *C. burnetii* infection in grazing cattle was increased by 32.57-fold (95% CI: 12.84–82.60, *P* = 0.000) compared with indoor housed cattle. The phylogenetic analysis based on the *IS111* gene revealed that our isolates were grouped together with humans, ticks, goats, and cattle isolates found in several countries. *C. burnetii* isolates circulating in the Republic of Korea exhibit genetic variations. Consequently, our results suggest that cattle are potential reservoirs for *C. burnetii* infection and most importantly, grazing acts as a high risk factor for the occurrence and transmission of this infection.
Keywords: *Coxiella burnetii*, dairy cattle, beef cattle, grazing, ELISA, IS1111
Introduction

*Coxiella burnetii* is known as the causative agent for Q fever in humans and coxiellosis in animals worldwide. *C. burnetii* is a highly infectious zoonotic intracellular bacterium which can infect a wide range of hosts including wild and domestic animals, birds, and arthropods [1-3]. Of these, domestic ruminants, such as cattle, goats, and sheep, are considered the primary sources of human infection. Transmission to humans occurs mainly through inhalation of contaminated aerosols or dust in nature or from direct contact with infected animal products [4]. Infected animals are often asymptomatic; however, *C. burnetii* infection is associated with abortion and stillbirth in sheep and goats and infertility and endometritis in cattle, respectively [5, 6]. The bacteria in infected animals can be shed in vaginal discharges, urine, feces, semen, milk, and birth products (placenta and birth fluids) [7, 8]. Most importantly, the shedding of *C. burnetii* in milk poses a potentially significant threat to public health, because raw milk and unpasteurized milk products are still being consumed and this could be the source of human infections [9, 10]. Q fever in humans is underestimated due to its difficulty to diagnose and its relatively asymptomatic nature to be noticed. Nevertheless, Q fever can lead to public health concerns because it is ranked as one of the top 13 global priority zoonoses. In addition, it has been considered a potential biological weapon due to widespread availability, aerosolized use, and environmental stability [11, 12].

The diagnosis of coxiellosis in fields is very difficult because of non-specific clinical symptoms [2]. The exposure to *C. burnetii* and the zoonotic risk in cattle have generally been assessed by serological surveys in most countries [13-16]. However, seropositivity to *C. burnetii* is not strongly correlated with the shedding of the bacterium. Although serologic analysis cannot be used to estimate the actual contamination rate in herds, it is a valuable tool
for the screening of *C. burnetii* infection within herds. Recent studies performed in the Republic of Korea (ROK) revealed that the overall seroprevalence was 10.5% in cattle and 19.1% in Korean native goats (*Capra hircus coreanae*) [14, 17], which indicates that the prevalence of *C. burnetii* is of significance in domestic ruminants. The number of Korean cattle breeding heads ranks 65th in the world and the size has been gradually increasing. In addition, meat consumption is increasing due to the influence of westernization; Koreans in particular tend to eat raw meat of beef. Despite its zoonotic potential, there is not much known about the importance and risk factors of *C. burnetii* in cattle in the ROK. Therefore, the objective of this study was to evaluate the prevalence of *C. burnetii* infection according to cattle breeds and growth types and to characterize the genetic diversity of the isolates circulating in the ROK.

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Results

Of the 491 cattle examined, 53 (10.8%) and 43 (8.8%) cattle were considered positive by PCR analysis and for C. burnetii antibodies, respectively (Table 1). Interestingly, no seropositivity was observed in beef cattle that were housed indoors. As shown in Table 1, the prevalence of C. burnetii was significantly higher in beef cattle than in dairy cattle using PCR (13.5% vs. 7.4%; \( P = 0.032 \)) and ELISA (14.5% vs. 1.4%; \( P = 0.000 \)), respectively. No significance was observed between two groups in the prevalence of C. burnetii by PCR. According to the growth type, the prevalence of C. burnetii was significantly higher in grazing cattle (PCR: 24.9%, 95% CI: 18.8–30.9%; ELISA: 21.3%, 95% CI: 15.6–27.0%) than in housing cattle (PCR: 1.4%, 95% CI: 0–2.7%; ELISA: 0.3%, 95% CI: −0.3–1.0%) using both molecular (\( P = 0.000 \)) and serologic (\( P = 0.000 \)) methods. Overall, the infection rate of C. burnetii was significantly higher in grazing cattle (\( P = 0.000 \)) than in housing cattle (Table 1).

The prevalence of C. burnetii according to growth type was compared using multinomial logistic regression analysis. The infection of C. burnetii was significantly associated with grazing (\( P = 0.000 \); Table 2). In grazing cattle, C. burnetii was detected at 33.33-fold higher in the Ag test (95% CI: 11.76–94.79, \( P = 0.000 \)) and 114.50-fold higher in the Ab test (95% CI: 15.56.90–842.40, \( P = 0.000 \)), respectively, compared with the housed cattle. Based on Ag or Ab positivity for C. burnetii, the possible risk factors for coxiellosis in cattle breed and growth type are shown in Table 3. The results indicated that beef cattle (OR = 3.197, 95% CI: 1.80–5.67, \( P = 0.000 \)) had a significantly higher risk of contracting C. burnetii infection compared with dairy cattle. Most importantly, when cattle were permitted
to graze in pasture, the risk of contracting a *C. burnetii* infection was increased by 32.57-fold (95% CI: 12.84–82.60, *P* = 0.000) compared with cattle housed indoors.

To investigate the genetic relationship among *C. burnetii* detected in dairy and beef cattle, a total of 53 positive samples were sequenced. Of these, 13 different sequences were included in a phylogenetic tree and compared with reference sequences published previously. The *C. burnetii* isolates exhibited 95.6%–99.5% homology to one another. A phylogenetic tree constructed from the partial 202 bp gene sequences revealed that *C. burnetii* isolates found in beef and dairy cattle were clustered with several strains of *C. burnetii* isolated from ticks, human, goats, and cattle from other countries (Fig. 1). Interestingly, cattle isolates obtained in this study shared 93.7%–97.1% similarity with Korean water deer (*Hydropotes inermis argyropus*) isolate recently found by our group. These results demonstrate that genetic variation exists within *C. burnetii* isolates collected in the ROK.
Discussion

In the present study, the overall prevalence of *C. burnetii* in cattle was determined to be 10.8% by PCR and 8.8% by ELISA. The seroprevalence of this result was low when compared with another study performed in the ROK [14]. Seroprevalence for bovine coxiellosis varies in many countries and it has been reported to range from 11% to 31.3% [18-23]. In addition, the prevalence of *C. burnetii* infection by PCR analysis was much higher in this study compared with the result obtained in the ROK. The difference between the two groups in infection rate may be explained by the number of cattle sampled, the management of the selected farms, and variations in the target gene used for detection. The *IS1111* PCR assay conducted in this study has been known to be highly specific and sensitive for the direct detection of *C. burnetii* in various clinical samples [24, 25]. Our PCR results were similar to those obtained in Iran (7.5%) and Zambia (7.7%) [26, 27].

The seroprevalence of infection with *C. burnetii* was 1.4% in dairy cattle and 14.6% in beef cattle. The results demonstrated that beef cattle were significantly more likely to be seropositive compared with dairy cattle. To date, the study of *C. burnetii* has been mostly conducted in dairy cattle [13, 28-31]. It is believed that the risk of *C. burnetii* transmission through milk consumption in humans is a significant public health issue. In this study, a higher seroprevalence of *C. burnetii* was observed in beef cattle, showing a difference of at least 10-fold (Table 1). A previous study reported that crossbred cattle were more likely to be seropositive [16]; however, this was opposite to our findings. According to our results, local breeds (Korean native cattle) were much more likely to be seropositive compared with crossbred cattle. Although we cannot make a precise conclusion at this point, the reason that seroprevalence in beef cattle was high is likely due to the difference in farm management systems, rather than cattle breed.
Coxiella burnetii DNA was detected by PCR in blood samples from beef and dairy cattle. In dairy cattle, the presence of C. burnetii DNA was much higher compared with seropositivity, whereas in beef cattle, the prevalence of C. burnetii was significantly high using both PCR ($P = 0.032$) and ELISA ($P = 0.000$) methods. PCR analysis has the advantage of detecting bacteremia and ongoing infection. Since all cattle examined in this study were healthy and exhibited no adverse clinical signs, we did not expect that these animals would be infected with C. burnetii. Nevertheless, the PCR results support the possibility that these cattle may shed the bacterium through milk, urine, and feces, indicating that the cattle are a source for human infection. Additionally, because people in the ROK have a tendency to consume raw meat from beef cattle, this represents an important public health concern. Thus, our results highlight the importance of a control and surveillance program.

We found that the prevalence of C. burnetii infection was significantly associated with grazing. The infection of C. burnetii was much higher in pastured cattle compared with that of housed cattle. In this study, we estimated that the odds of testing positive for C. burnetii were related to grazing. We also confirmed that the likelihood of being positive for Ag or Abs against C. burnetii in pastured cattle was significantly increased (Table 2). Grazing systems have many advantages including animal welfare, but there is a higher risk of contracting tick-borne diseases because of increased exposure to ticks. Because of global warming, the climate of Korea has become subtropical, and tick species are expanding their territory. As a result, they are likely to be a growing concern for humans and animal health. It is easy to conclude that ticks were infesting grazing cattle, but C. burnetii infection was not investigated in these ticks, thus the route of transmission in these cattle remains uncertain. Haemaphysalis longicornis is a predominant tick species widespread in the ROK. According
to a recent report, *Coxiella*-like endosymbionts were found in *H. longicornis* on horses [32]. In addition, ticks shed significant loads of *C. burnetii* in their feces and saliva, and may be another potential source of bacterial transmission [33]. Consequently, this suggests that the possibility of transmission by ticks cannot be excluded. Therefore, additional epidemiological studies of ticks are needed.

Our results revealed that grazing beef cattle were at significantly high risk for infection with *C. burnetii*. Generally, dairy cattle are less likely to graze than beef cattle in the ROK. Although the sample number in this study was small, the prevalence of *C. burnetii* infection in grazing dairy cattle was relatively high. According to our results, grazing represents a significant factor for *C. burnetii* infection in cattle. One possibility is that infected animals that are grazing can shed bacteria into the environment. The shedding of bacteria is a potential hazard to humans and animals because the bacteria remain in the environment and may be aerosolized [26]. *C. burnetii* spores can spread several kilometers away from the primary infection source via wind, raising the latter as a potential player for bacterial dispersal [34]. When cattle graze in a pasture, *C. burnetii* can be transmitted through the inhalation of contaminated aerosols or dust, rather than ticks, leading to infection. Another possibility is that grazing cattle may come in contact with wild animals, so they could be infected in this manner. A recent study performed by our group reported that *C. burnetii* infection was identified in Korean water deer [35]. Korean water deer may be potential reservoirs for this bacterium and play an important role in the transmission to humans, animals, and livestock. Overall, our results suggest that cattle grazing in pastures are at risk for the transmission and spread of infection because of multiple factors.
In this study, we exploited the genetic characterization of *C. burnetii* isolates identified in beef and dairy cattle using *IS1111*. The isolates from these cattle exhibited a slightly different sequence homology with Korean water deer isolate previously reported by our group. Our findings indicate that genetic variation exists in *C. burnetii* isolates circulating in the ROK. The cattle isolates shared 95.6–100% similarity with pathogenic *C. burnetii* strains isolated from *Hyalomma dromedarii* in Tunisia, humans in Greece, and cattle in France [36]. These findings suggest that *C. burnetii* isolates detected in the ROK are probably zoonotic and pathogenic. Therefore, the results represent the nature of *C. burnetii* isolates circulating in the ROK and additional molecular epidemiological studies are needed to investigate the genetic diversity of this bacterium in human and animals.

**Conclusions**

The present study demonstrates that cattle are potential reservoirs for *C. burnetii* infection as determined by molecular and serological analyses and grazing represents a higher risk factor in the transmission of the infection to animals. *C. burnetii* is a public health concern and poses a significant risk to humans that come in close contact with animals. Our findings increase awareness of the importance of *C. burnetii* as a potential zoonotic pathogen of grazing cattle in the ROK. These results provide useful information for better understanding the occurrence of *C. burnetii* infection and also for designing control strategies for cattle. Further studies should be done to evaluate potential transmission risks and the pathogenicity of *C. burnetii* circulating in the ROK.
Materials and methods

All animal procedures were performed according to the ethics guidelines for the use of animal samples as permitted by Chonbuk National University (Institutional Animal Care and Use Committee decision No. CBU 2014-00026). All procedures and possible consequences were explained to farm owners/managers associated with the surveyed farms. Written informed consent was obtained for the collection of blood samples from the owners of the cattle.

Blood sample collection

About 10 mL blood samples were collected from the jugular veins of 491 cattle (216 dairy cattle and 275 beef cattle) from different regions of the ROK (Table 1). The cattle were divided into two groups: grazing and indoor housing without pasturing. Blood was equally divided into an anti-coagulated collection tube (BD Vacutainer®, Franklin Lakes, NJ, USA) and an SST blood tube (BD Vacutainer®), and then delivered to the laboratory. The serum was separated and collected by centrifugation at 3,000 g for 20 min and then stored at −20 °C until use. Whole blood was used for DNA extraction and serum was used for serology. All animals were clinically healthy.

DNA extraction and PCR

DNA was extracted from 200 μL of each blood sample using the DNeasy Blood Kit (Qiagen, Valencia, California, USA) according to the manufacturer’s instructions and stored at −80°C. The detection of C. burnetii was screened using the IS1111 (transposase insertion
element) [37]. PCR conditions included 93°C for 3 min, followed by 30 cycles of 93°C for 30 s, annealing at 54°C for 30 s, and 72°C for 1 min. For each PCR run, negative and positive controls were included. The size of the amplified fragment was 202 bp. Secondary PCR products were separated by electrophoresis on 1.5% agarose gels and visualized after staining with ethidium bromide.

**Serological screening of serum samples**

Serum samples from 491 cattle were tested for antibodies against *C. burnetii* using a commercial enzyme-linked immunosorbent assay (ELISA) kit (ID Screen® Q fever Indirect Multi-species kit; ID.vet, Gabriels, France) according to the manufacturer’s instructions. According to the internal validation report, to normalize the optical density (OD) results, the sample/positive control (S/P) ratio was calculated for each sample as follows: Value (%) = (OD sample – OD negative control) / (OD positive control – OD negative control) × 100. Samples with an S/P% greater than 50% were considered positive; between 40% and 50%, doubtful; and less than 40%, negative. In this study, doubtful results were considered negative.

**Phylogenetic analysis**

All secondary PCR products were purified using the AccuPower PCR Purification Kit (Bioneer, Daejeon, ROK) and used for direct sequencing (Macrogen, Daejeon, ROK). The nucleotide sequences obtained in this study were aligned using ClustalX and compared with the reference sequences from the GenBank database. A phylogenetic tree was constructed based on the *IS1111* fragments using the maximum-likelihood method in the MEGA 7 software [38]. The reliabilities of the tree were assessed using bootstrap analysis with 1000 replicates.
Statistical analysis

Statistical analysis was performed using the SPSS Statistics 25 software package for Windows (SPSS Inc, Chicago, IL, USA). Chi-square test was used to compare the prevalence of *C. burnetii* according to cattle breeds and growth types. A 95% confidence interval (CI) was estimated. In addition, the detection rate of *C. burnetii* in dairy and beef cattle depending on the growth type was determined using multinomial logistic regression analysis. The analysis of risk factors associated with *C. burnetii* infection was performed using multivariable logistic regression models. The odds ratio (OR) and 95% CI were calculated to determine the probability of association. A *P*-value of ≤0.05 was considered to be statistically significant.
Author Contributions

SWH: Conceptualization, Investigation, Methodology, HCC: Conceptualization, Analysis and Interpretation of data, SUS: Conceptualization, Analysis and Interpretation of data, HYK: Resources, Methodology, YJP: Investigation, DHJ: Investigation, EMK: Investigation, JWK: Resources, JHP: Resources, KSC: Conceptualization, Resources, Writing, Supervision, Funding acquisition.

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Conflicts of Interest

The authors declare that they have no conflict of interest.
References


Figure legend

Fig. 1. Phylogenetic analyses based on the IS1111 sequences of Coxiella burnetii from beef and dairy cattle identified in the ROK. The tree was constructed using the MEGA7 software by employing the maximum-likelihood method. The numbers at the nodes of the tree indicate bootstrap values as a percentage of 1000 replicates that support each phylogenetic branch. The isolates identified in this study are marked in bold type as a circle symbol.

Table 1 Prevalence of C. burnetii according to cattle breeds and growth types in the Republic of Korea

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No. of samples</th>
<th>No. of PCR positive</th>
<th>95% CI</th>
<th>P-value</th>
<th>No. of ELISA positive</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef cattle</td>
<td>275</td>
<td>37 (13.5%)</td>
<td>9.4–17.5%</td>
<td>0.032</td>
<td>40 (14.6%)</td>
<td>10.4–18.6%</td>
</tr>
<tr>
<td>Dairy cattle</td>
<td>216</td>
<td>16 (7.4%)</td>
<td>3.9–10.9%</td>
<td></td>
<td>3 (1.4%)</td>
<td>–0.2–1.2%</td>
</tr>
<tr>
<td>Grazing</td>
<td>197</td>
<td>49 (24.9%)</td>
<td>18.8–30.9%</td>
<td>0.000</td>
<td>42 (21.3%)</td>
<td>15.6–27.1%</td>
</tr>
<tr>
<td>Housing</td>
<td>294</td>
<td>4 (1.4%)</td>
<td>0.0–2.7%</td>
<td></td>
<td>1 (0.3%)</td>
<td>–0.3–1.6%</td>
</tr>
<tr>
<td>Total</td>
<td>491</td>
<td>53 (10.8%)</td>
<td></td>
<td></td>
<td>43 (8.8%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2 Multinomial logistic regression analysis for the detection of *C. burnetii* in cattle depending on growth type

<table>
<thead>
<tr>
<th>Detection of C. burnetii</th>
<th>Growth type</th>
<th>OR</th>
<th>P-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag positive</td>
<td>Housing</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grazing</td>
<td>33.339</td>
<td>0.000</td>
<td>11.767–94.796</td>
</tr>
<tr>
<td>Ab positive</td>
<td>Housing</td>
<td>–</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Grazing</td>
<td>114.509</td>
<td>0.000</td>
<td>15.565–42.409</td>
</tr>
</tbody>
</table>

depending on growth type
**Table 3** Risk factors associated with *C. burnetii* infection

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Variables</th>
<th>No. of <em>C. burnetii</em> positive*</th>
<th><em>P</em>-value</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breed</td>
<td>Dairy cattle</td>
<td>17/216</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Beef cattle</td>
<td>59/275</td>
<td>0.000</td>
<td>3.197</td>
<td>1.803–5.670</td>
</tr>
<tr>
<td>Growth</td>
<td>Housing</td>
<td>5/294</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>types</td>
<td>Grazing</td>
<td>71/197</td>
<td>0.000</td>
<td>32.570</td>
<td>12.842–82.605</td>
</tr>
</tbody>
</table>

*PCR or ELISA positive