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

4

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30 **Abstract**

31 This study was conducted to investigate the prevalence of *Coxiella burnetii* infection
32 according to cattle breeds and growth types. A total of 491 cattle [cattle breed: 216 dairy
33 cattle and 275 beef cattle; according to growth type: indoor housing (n = 294) and grazing (n
34 = 197)] were tested for the presence of *C. burnetii* DNA and antibodies against *C. burnetii*
35 using a commercial enzyme-linked immunosorbent assay (ELISA). Overall, 10.8% and 8.8%
36 of the cattle were positive by PCR and for *C. burnetii* antibodies, respectively. The
37 prevalence of *C. burnetii* was significantly higher in beef cattle than in dairy cattle using PCR
38 (13.6% vs 7.4%; *P* = 0.032) and ELISA (14.6% vs 1.4%; *P* = 0.000), respectively. The
39 overall infection rate of *C. burnetii* was significantly high in grazing cattle (PCR: 24.9%,
40 ELSIA: 21.3%; *P* = 0.000) compared with housing cattle (PCR: 1.4%, ELISA: 0.3%). The
41 results indicate that beef cattle have a significantly higher risk of contracting *C. burnetii*
42 infection compared with dairy cattle (21.5% vs. 7.9%, χ^2 = 5.82, *P* = 0.000, odds ratio =
43 3.197, 95% CI: 1.80–5.67). In addition, the infection of *C. burnetii* was significantly
44 associated with grazing (*P* = 0.000). Moreover, a risk of contracting *C. burnetii* infection in
45 grazing cattle was increased by 32.57-fold (95% CI: 12.84–82.60, *P* = 0.000) compared with
46 indoor housed cattle. The phylogenetic analysis based on the *IS111* gene revealed that our
47 isolates were grouped together with humans, ticks, goats, and cattle isolates found in several
48 countries. *C. burnetii* isolates circulating in the Republic of Korea exhibit genetic variations.
49 Consequently, our results suggest that cattle are potential reservoirs for *C. burnetii* infection
50 and most importantly, grazing acts as a high risk factor for the occurrence and transmission of
51 this infection.

52

53 *Keywords: Coxiella burnetii*, dairy cattle, beef cattle, grazing, ELISA, IS1111

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55 **Introduction**

56 *Coxiella burnetii* is known as the causative agent for Q fever in humans and
57 coxiellosis in animals worldwide. *C. burnetii* is a highly infectious zoonotic intracellular
58 bacterium which can infect a wide range of hosts including wild and domestic animals, birds,
59 and arthropods [1-3]. Of these, domestic ruminants, such as cattle, goats, and sheep, are
60 considered the primary sources of human infection. Transmission to humans occurs mainly
61 through inhalation of contaminated aerosols or dust in nature or from direct contact with
62 infected animal products [4]. Infected animals are often asymptomatic; however, *C. burnetii*
63 infection is associated with abortion and stillbirth in sheep and goats and infertility and
64 endometritis in cattle, respectively [5, 6]. The bacteria in infected animals can be shed in
65 vaginal discharges, urine, feces, semen, milk, and birth products (placenta and birth fluids) [7,
66 8]. Most importantly, the shedding of *C. burnetii* in milk poses a potentially significant threat
67 to public health, because raw milk and unpasteurized milk products are still being consumed
68 and this could be the source of human infections [9, 10]. Q fever in humans is underestimated
69 due to its difficulty to diagnose and its relatively asymptomatic nature to be noticed.
70 Nevertheless, Q fever can lead to public health concerns because it is ranked as one of the top
71 13 global priority zoonoses. In addition, it has been considered a potential biological weapon
72 due to widespread availability, aerosolized use, and environmental stability [11, 12].

73 The diagnosis of coxiellosis in fields is very difficult because of non-specific clinical
74 symptoms [2]. The exposure to *C. burnetii* and the zoonotic risk in cattle have generally been
75 assessed by serological surveys in most countries [13-16]. However, seropositivity to *C.*
76 *burnetii* is not strongly correlated with the shedding of the bacterium. Although serologic
77 analysis cannot be used to estimate the actual contamination rate in herds, it is a valuable tool

78 for the screening of *C. burnetii* infection within herds. Recent studies performed in the
79 Republic of Korea (ROK) revealed that the overall seroprevalence was 10.5% in cattle and
80 19.1% in Korean native goats (*Capra hircus coreanae*) [14, 17], which indicates that the
81 prevalence of *C. burnetii* is of significance in domestic ruminants. The number of Korean
82 cattle breeding heads ranks 65th in the world and the size has been gradually increasing. In
83 addition, meat consumption is increasing due to the influence of westernization; Koreans in
84 particular tend to eat raw meat of beef. Despite its zoonotic potential, there is not much
85 known about the importance and risk factors of *C. burnetii* in cattle in the ROK. Therefore,
86 the objective of this study was to evaluate the prevalence of *C. burnetii* infection according to
87 cattle breeds and growth types and to characterize the genetic diversity of the isolates
88 circulating in the ROK.
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90 **Results**

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

103 The prevalence of *C. burnetii* according to growth type was compared using
104 multinomial logistic regression analysis. The infection of *C. burnetii* was significantly
105 associated with grazing (P = 0.000; Table 2). In grazing cattle, *C. burnetii* was detected at
106 33.33-fold higher in the Ag test (95% CI: 11.76–94.79, P = 0.000) and 114.50-fold higher in
107 the Ab test (95% CI: 15.56.90–842.40, P = 0.000), respectively, compared with the housed
108 cattle. Based on Ag or Ab positivity for *C. burnetii*, the possible risk factors for coxiellosis in
109 cattle breed and growth type are shown in Table 3. The results indicated that beef cattle (OR
110 = 3.197, 95% CI: 1.80–5.67, P = 0.000) had a significantly higher risk of contracting *C.*
111 *burnetii* infection compared with dairy cattle. Most importantly, when cattle were permitted

112 to graze in pasture, the risk of contracting a *C. burnetii* infection was increased by 32.57-fold
113 (95% CI: 12.84−82.60, $P = 0.000$) compared with cattle housed indoors.

114 To investigate the genetic relationship among *C. burnetii* detected in dairy and beef
115 cattle, a total of 53 positive samples were sequenced. Of these, 13 different sequences were
116 included in a phylogenetic tree and compared with reference sequences published previously.
117 The *C. burnetii* isolates exhibited 95.6%−99.5% homology to one another. A phylogenetic
118 tree constructed from the partial 202 bp gene sequences revealed that *C. burnetii* isolates
119 found in beef and dairy cattle were clustered with several strains of *C. burnetii* isolated from
120 ticks, human, goats, and cattle from other countries (Fig. 1). Interestingly, cattle isolates
121 obtained in this study shared 93.7%−97.1% similarity with Korean water deer (*Hydropotes*
122 *inermis argyropus*) isolate recently found by our group. These results demonstrate that
123 genetic variation exists within *C. burnetii* isolates collected in the ROK.
124

125 **Discussion**

126 In the present study, the overall prevalence of *C. burnetii* in cattle was determined to
127 be 10.8% by PCR and 8.8% by ELISA. The seroprevalence of this result was low when
128 compared with another study performed in the ROK [14]. Seroprevalence for bovine
129 coxiellosis varies in many countries and it has been reported to range from 11% to 31.3%
130 [18-23]. In addition, the prevalence of *C. burnetii* infection by PCR analysis was much higher
131 in this study compared with the result obtained in the ROK. The difference between the two
132 groups in infection rate may be explained by the number of cattle sampled, the management
133 of the selected farms, and variations in the target gene used for detection. The *IS1111* PCR
134 assay conducted in this study has been known to be highly specific and sensitive for the direct
135 detection of *C. burnetii* in various clinical samples [24, 25]. Our PCR results were similar to
136 those obtained in Iran (7.5%) and Zambia (7.7%) [26, 27].

137 The seroprevalence of infection with *C. burnetii* was 1.4% in dairy cattle and 14.6%
138 in beef cattle. The results demonstrated that beef cattle were significantly more likely to be
139 seropositive compared with dairy cattle. To date, the study of *C. burnetii* has been mostly
140 conducted in dairy cattle [13, 28-31]. It is believed that the risk of *C. burnetii* transmission
141 through milk consumption in humans is a significant public health issue. In this study, a
142 higher seroprevalence of *C. burnetii* was observed in beef cattle, showing a difference of at
143 least 10-fold (Table 1). A previous study reported that crossbred cattle were more likely to be
144 seropositive [16]; however, this was opposite to our findings. According to our results, local
145 breeds (Korean native cattle) were much more likely to be seropositive compared with
146 crossbred cattle. Although we cannot make a precise conclusion at this point, the reason that
147 seroprevalence in beef cattle was high is likely due to the difference in farm management
148 systems, rather than cattle breed.

149 *Coxiella burnetii* DNA was detected by PCR in blood samples from beef and dairy
150 cattle. In dairy cattle, the presence of *C. burnetii* DNA was much higher compared with
151 seropositivity, whereas in beef cattle, the prevalence of *C. burnetii* was significantly high
152 using both PCR ($P = 0.032$) and ELISA ($P = 0.000$) methods. PCR analysis has the advantage
153 of detecting bacteremia and ongoing infection. Since all cattle examined in this study were
154 healthy and exhibited no adverse clinical signs, we did not expect that these animals would be
155 infected with *C. burnetii*. Nevertheless, the PCR results support the possibility that these
156 cattle may shed the bacterium through milk, urine, and feces, indicating that the cattle are a
157 source for human infection. Additionally, because people in the ROK have a tendency to
158 consume raw meat from beef cattle, this represents an important public health concern. Thus,
159 our results highlight the importance of a control and surveillance program.

160 We found that the prevalence of *C. burnetii* infection was significantly associated
161 with grazing. The infection of *C. burnetii* was much higher in pastured cattle compared with
162 that of housed cattle. In this study, we estimated that the odds of testing positive for *C.*
163 *burnetii* were related to grazing. We also confirmed that the likelihood of being positive for
164 Ag or Abs against *C. burnetii* in pastured cattle was significantly increased (Table 2). Grazing
165 systems have many advantages including animal welfare, but there is a higher risk of
166 contracting tick-borne diseases because of increased exposure to ticks. Because of global
167 warming, the climate of Korea has become subtropical, and tick species are expanding their
168 territory. As a result, they are likely to be a growing concern for humans and animal health. It
169 is easy to conclude that ticks were infesting grazing cattle, but *C. burnetii* infection was not
170 investigated in these ticks, thus the route of transmission in these cattle remains uncertain.
171 *Haemaphysalis longicornis* is a predominant tick species widespread in the ROK. According

172 to a recent report, *Coxiella*-like endosymbionts were found in *H. longicornis* on horses [32].
173 In addition, ticks shed significant loads of *C. burnetii* in their feces and saliva, and may be
174 another potential source of bacterial transmission [33]. Consequently, this suggests that the
175 possibility of transmission by ticks cannot be excluded. Therefore, additional epidemiological
176 studies of ticks are needed.

177 Our results revealed that grazing beef cattle were at significantly high risk for
178 infection with *C. burnetii*. Generally, dairy cattle are less likely to graze than beef cattle in the
179 ROK. Although the sample number in this study was small, the prevalence of *C. burnetii*
180 infection in grazing dairy cattle was relatively high. According to our results, grazing
181 represents a significant factor for *C. burnetii* infection in cattle. One possibility is that
182 infected animals that are grazing can shed bacteria into the environment. The shedding of
183 bacteria is a potential hazard to humans and animals because the bacteria remain in the
184 environment and may be aerosolized [26]. *C. burnetii* spores can spread several kilometers
185 away from the primary infection source via wind, raising the latter as a potential player for
186 bacterial dispersal [34]. When cattle graze in a pasture, *C. burnetii* can be transmitted through
187 the inhalation of contaminated aerosols or dust, rather than ticks, leading to infection.
188 Another possibility is that grazing cattle may come in contact with wild animals, so they
189 could be infected in this manner. A recent study performed by our group reported that *C.*
190 *burnetii* infection was identified in Korean water deer [35]. Korean water deer may be
191 potential reservoirs for this bacterium and play an important role in the transmission to
192 humans, animals, and livestock. Overall, our results suggest that cattle grazing in pastures are
193 at risk for the transmission and spread of infection because of multiple factors.

194 In this study, we exploited the genetic characterization of *C. burnetii* isolates
195 identified in beef and dairy cattle using *IS1111*. The isolates from these cattle exhibited a
196 slightly different sequence homology with Korean water deer isolate previously reported by
197 our group. Our findings indicate that genetic variation exists in *C. burnetii* isolates circulating
198 in the ROK. The cattle isolates shared 95.6–100% similarity with pathogenic *C. burnetii*
199 strains isolated from *Hyalomma dromedarii* in Tunisia, humans in Greece, and cattle in
200 France [36]. These findings suggest that *C. burnetii* isolates detected in the ROK are probably
201 zoonotic and pathogenic. Therefore, the results represent the nature of *C. burnetii* isolates
202 circulating in the ROK and additional molecular epidemiological studies are needed to
203 investigate the genetic diversity of this bacterium in human and animals.
204
205 **Conclusions**
206 The present study demonstrates that cattle are potential reservoirs for *C. burnetii*
207 infection as determined by molecular and serological analyses and grazing represents a higher
208 risk factor in the transmission of the infection to animals. *C. burnetii* is a public health
209 concern and poses a significant risk to humans that come in close contact with animals. Our
210 findings increase awareness of the importance of *C. burnetii* as a potential zoonotic pathogen
211 of grazing cattle in the ROK. These results provide useful information for better
212 understanding the occurrence of *C. burnetii* infection and also for designing control strategies
213 for cattle. Further studies should be done to evaluate potential transmission risks and the
214 pathogenicity of *C. burnetii* circulating in the ROK.
215

216 ## Materials and methods

217 ### Ethical statement

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

224

225 ### Blood sample collection

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

234

235 ### DNA extraction and PCR

236 DNA was extracted from 200 μL of each blood sample using the DNeasy Blood Kit
237 (Qiagen, Valencia, California, USA) according to the manufacturer's instructions and stored
238 at −80°C. The detection of *C. burnetii* was screened using the *IS1111* (transposase insertion

239 element) [37]. PCR conditions included 93°C for 3 min, followed by 30 cycles of 93°C for 30
240 s, annealing at 54°C for 30 s, and 72°C for 1 min. For each PCR run, negative and positive
241 controls were included. The size of the amplified fragment was 202 bp. Secondary PCR
242 products were separated by electrophoresis on 1.5% agarose gels and visualized after staining
243 with ethidium bromide.
244

245 **Serological screening of serum samples**

246 Serum samples from 491 cattle were tested for antibodies against *C. burnetii* using a
247 commercial enzyme-linked immunosorbent assay (ELISA) kit (ID Screen® Q fever Indirect
248 Multi-species kit; ID.vet, Gabriels, France) according to the manufacturer's instructions.
249 According to the internal validation report, to normalize the optical density (OD) results, the
250 sample/positive control (S/P) ratio was calculated for each sample as follows: Value (%) =
251 (OD sample − OD negative control) / (OD positive control − OD negative control) × 100.
252 Samples with an S/P% greater than 50% were considered positive; between 40% and 50%,
253 doubtful; and less than 40%, negative. In this study, doubtful results were considered negative.
254

255 **Phylogenetic analysis**

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

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264 **Statistical analysis**

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

273

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.

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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

Parameters	No. of samples	No. of PCR positive	95% CI	*P*-value	No. of ELISA positive	95%
Beef cattle	275	37 (13.5%)	9.4–17.5%	0.032	40 (14.6%)	10.4
Dairy cattle	216	16 (7.4%)	3.9–10.9%		3 (1.4%)	−0.2
Grazing	197	49 (24.9%)	18.8–30.9%	0.000	42 (21.3%)	15.6
Housing	294	4 (1.4%)	0.0–2.7%		1 (0.3%)	−0.3
Total	491	53 (10.8%)			43 (8.8%)	

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409 **Table 2** Multinomial logistic regression analysis for the detection of *C. burnetii* in cattle

Detection of *C. burnetii*	**Growth type**	**OR**	***P*-value**	**95% CI**
Ag positive	Housing	–	-	-
	Grazing	33.339	0.000	11.767–94.796
Ab positive	Housing	–	-	-
	Grazing	114.509	0.000	15.565– 42.409

410 depending on growth type

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418 **Table 3** Risk factors associated with *C. burnetii* infection

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

419 *PCR or ELISA positive

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