

RESEARCH ARTICLE

Detection and Genotyping of *Coxiella burnetii* and *Coxiella*-Like Bacteria in Horses in South Korea

Min-Goo Seo^{1,2}, Seung-Hun Lee¹, Dorene VanBik¹, In-Ohk Ouh², Sun-Hee Yun³, Eunsang Choi⁴, Yong-Soo Park⁵, Sang-Eun Lee⁶, Jong Wan Kim², Gil-Jae Cho¹, Oh-Deog Kwon¹, Dongmi Kwak^{1,7*}

1 College of Veterinary Medicine, Kyungpook National University, Daegu, South Korea, **2** Animal and Plant Quarantine Agency, Gimcheon, South Korea, **3** Division of Veterinary Service Lab, Institute of Public Health & Environment, Incheon, South Korea, **4** Smile Equine Clinic, Busan, South Korea, **5** Dept. of Horse Industry, Korea National College of Agriculture and Fisheries, Jeonju, South Korea, **6** Division of Malaria & Parasitic Disease, Korea National Institute of Health, Cheongju, South Korea, **7** Cardiovascular Research Institute, Kyungpook National University, Daegu, South Korea

* dmkwak@knu.ac.kr



OPEN ACCESS

Citation: Seo M-G, Lee S-H, VanBik D, Ouh I-O, Yun S-H, Choi E, et al. (2016) Detection and Genotyping of *Coxiella burnetii* and *Coxiella*-Like Bacteria in Horses in South Korea. PLoS ONE 11(5): e0156710. doi:10.1371/journal.pone.0156710

Editor: James E Samuel, Texas A&M Health Science Center, UNITED STATES

Received: February 1, 2016

Accepted: May 18, 2016

Published: May 31, 2016

Copyright: © 2016 Seo et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: This research was supported by a grant from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (Grant No. NRF-2013R1A1A2013102). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The Smile Equine Clinic provided support in the form of salary for author EC, but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Coxiella burnetii and *Coxiella*-like bacteria (CLB) are genetically and ecologically distinct despite some genetic similarities. Furthermore, CLB are exceptionally diverse and widespread in ticks, but rarely detected in domestic animals. Since *Coxiella* bacteria can be transmitted from infected horses by inhalation or by coming in contact with ticks during activities such as horseback riding, it is necessary to study their prevalence. To the best of our knowledge, this is the first large-scale nationwide investigation of the prevalence of *C. burnetii* and CLB among horses reared in South Korea. Of 816 blood samples collected between 2007 and 2013, 11 (1.3%) were identified as *C. burnetii* by ELISA, and six (0.7%) as CLB by 16S rRNA sequencing. While a sequence from Jeju Island was similar (97.9–100%) to those within clade B, five sequences obtained from the northern region were categorized into a new clade, indicating the sequence diversity of the genus *Coxiella*. Studies until date had detected CLB only in ticks; here, we describe their detection in mammals. Given their zoonotic potential, strategic monitoring and appropriate control programs for *Coxiella* species need to be established.

Introduction

Coxiella burnetii, an obligate intracellular gram-negative bacterium, is a zoonotic pathogen that causes Q fever or coxiellosis. *C. burnetii* has been detected in species across the animal kingdom, including many domestic and wild mammals, birds, and arthropods such as ticks [1]. *C. burnetii* was originally named as *Rickettsia burnetii* on the basis of its similarity to *Rickettsia* species, but 16S rRNA analysis-based phylogeny placed this species in the genus *Coxiella*,

The specific roles of this author are articulated in the "author contributions" section.

Competing Interests: Author EC is employed by the Smile Equine Clinic, which has links with commercial sponsors, however this does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

which belongs to the gamma subdivision of the phylum Proteobacteria [2]. *C. burnetii* is currently the only species in this genus [3]; however, another species, *C. cheraxi*, presumed to belong to this genus, has been detected in crayfish [4]. Moreover, the fact that ticks transmit both *C. burnetii* and *Coxiella*-like bacteria (CLB) emphasizes the need to accurately discriminate between them [5]. In addition, questions about these bacteria remain, including the potential role of CLB in the population dynamics of ticks, and the possibility of CLB conversion leading to the emergence of Q fever [6].

Infertility and abortion caused by *C. burnetii* have been reported in numerous animals, but it is often difficult to identify the infection owing to its asymptomatic nature [7]. Stillbirth, abortion, and neonatal death caused by *C. burnetii* lead to economic loss in the horse industry [8]. To date, the role of horses as a reservoir for *C. burnetii* has not been extensively studied. *C. burnetii* is a well-known cause of abortion in ruminants; however, several recent studies have examined this characteristic in horses, too. *C. burnetii* DNA was detected in the aborted fetuses of horses, indicating the abortogenic nature of *C. burnetii* in horses [9,10]. *C. burnetii* DNA was also detected in the placenta of horses without any known abortion history in the Netherlands. Seven recent studies had detected *C. burnetii* in horse samples, in particular, in aborted fetuses, while another 34 studies determined the seroprevalence of *C. burnetii* in horses [7]. Therefore, horses should be considered as a reservoir for *C. burnetii* [11].

Although several studies have investigated *C. burnetii* infection in dairy cattle, goats, and water deer in South Korea [12–14], no studies have examined the occurrence of *C. burnetii* in horses. Recently, there has been a boom in the horse industry in South Korea, as the international trade of horses has increased. The potential risk of transmission of *Coxiella* species to humans may increase after exposure to infected horses or ticks during horseback riding. Therefore, the objective of this study was to detect and survey the current epidemiological prevalence and distribution of *C. burnetii* in horses reared in South Korea, by using ELISA and PCR.

Materials and Methods

Ethics statement

This study did not receive approval from the Institutional Animal Care and Use Committee (IACUC) at Kyungpook National University (KNU) in 2007, as the IACUC at KNU evaluates laboratory animals maintained in indoor facilities, not outdoor animals. Equine veterinarians collected blood samples at horse farms after receiving consent from the horse owners.

Sample size determination and sample collection

The total number of horses reared in South Korea in 2014 was recorded at 25,819 [15]. The sample size for this study was determined using the following formula, with an expected disease prevalence of 50%, accepted absolute error of 5%, and a confidence level of 99% by using a simple random sampling design [16]:

$$n = \frac{1.96^2 p_{exp} (1 - p_{exp})}{d^2}$$

where n = required sample size, p_{exp} = expected prevalence, and d = desired absolute precision

According to the formula, a minimum of 664 samples was required. In this study, 816 horses were randomly selected from multiple regions in South Korea, between 2007 and 2013 (Fig 1). Following blood collection from the jugular vein, whole blood was used for PCR, and serum samples were used for serology. Age, sex, breed, and region were recorded for data



Fig 1. A map of South Korea showing the four different study regions where blood samples were collected from horses to detect *Coxiella* species.

doi:10.1371/journal.pone.0156710.g001

analysis, and missing information was recorded as “unknown” (Table 1). The mean age of the study animals was 7.2 years, with a standard deviation of 4.6 years.

Serology

Serum samples were checked for the presence of antibodies against *C. burnetii* by ELISA with the ID Screen Q Fever Indirect Multi-species Kit (IDvet, Montpellier, France), in accordance with the manufacturer’s instructions. The microwells were coated with *C. burnetii* phases I and II. The optical density ratio of the sample and the positive control (S/P) was calculated for each sample as follows:

$$\text{Value (\%)} = \frac{OD_{\text{sample}} - OD_{\text{negative control}}}{OD_{\text{positive control}} - OD_{\text{negative control}}} \times 100$$

Samples with an S/P value greater than 50% were considered positive; values between 40% and 50% were deemed doubtful, and those less than 40% were determined to be negative. Doubtful results were considered negative.

DNA extraction and PCR

Genomic DNA was extracted from whole blood, using the commercial DNeasy Blood and Tissue Kit (Qiagen, Melbourne, Australia) according to the manufacturer’s instructions. The extracted DNA was stored at -20°C until use. The commercial AccuPower HotStart PCR Premix Kit (Bioneer, Daejeon, South Korea) was used for PCR amplification. Multiple primer sets

Table 1. Detection of *Coxiella* infection among horses raised in Korea between 2007 and 2013.

Group		No. tested	No. (%) of horses			
			ELISA		PCR	
			Positive	95% CI†	Positive	95% CI†
Region	Northern	295	7 (2.4)	0.6–4.1	5 (1.7)	0.2–3.2
	Central	184	2 (1.1)	0–2.6	0	0
	Southern	243	2 (0.8)	0–2.0	0	0
	Jeju Island	94	0	0	1 (1.1)	0–3.1
Breed	Thoroughbred	566	11 (1.9)	0.8–3.1	5 (0.9)	0.1–1.7
	Native Korean pony	109	0	0	1 (0.9)	0–2.7
	Warm blood	61	0	0	0	0
	Mixed	80	0	0	0	0
Sex	Male	159	3 (1.9)	0–4.0	1 (0.6)	0–1.9
	Female	283	5 (1.8)	0.2–3.3	3 (1.1)	0–2.3
	Castrated	280	3 (1.1)	0–2.3	1 (0.4)	0–1.1
	Unknown	94	0	0	1 (1.1)	0–3.1
Age	<5	271	5 (1.8)	0.2–3.5	5 (1.8)*	0.2–3.5
	5–10	249	4 (1.6)	0.1–3.2	0	0
	>10	202	2 (1)	0–2.4	0	0
	Unknown	94	0	0	1 (1.1)	0–3.1
Total		816	11 (1.3)	0.6–2.1	6 (0.7)	0.2–1.3

*Significantly different, $p < 0.05$

†CI = confidence interval.

doi:10.1371/journal.pone.0156710.t001

were used to amplify 16S rRNA of the genus *Coxiella*. *Coxiella* was first screened by nested PCR (nPCR), as previously described [6,17]. First-round PCR was performed with the primers Cox16SF1 (5'-CGTAGGAATCTACCTTRTAGWGG-3') and Cox16SR2 (5'-GCCTACCCGC TTCTGGTACAATT-3'), which produced amplicons with 1,321–1,429 bp. Then, nPCR was performed using the primers Cox16SF2 (5'-TGAGAACTAGCTGTTGGRRAGT-3') and Cox16SR2, which produced amplicons with 624–627 bp. Samples yielding amplicons of the expected size were sequenced using the primers Cox16SF1 and Cox16SR1 (5'-ACTYYCCAACAGCTAGTTC TCA-3'), which produced amplicons with 719–826 bp. All PCR amplifications were performed using the Mastercycler Pro (Eppendorf, Hamburg, Germany), with a pre-denaturation cycle at 93°C for 3 min, followed by 30 cycles of denaturation at 93°C for 30 s, annealing at 56°C for 30 s, and polymerization at 72°C for 1 min, with a final post-polymerization cycle at 72°C for 5 min. PCR products of the second round of amplification were evaluated by electrophoresis, using 10 µl of the reaction mixture and a 100 bp DNA ladder (Bioneer) in 1.5% agarose gel for 30 min at 100 V, and visualized using UV transillumination, after ethidium bromide staining.

DNA sequencing and phylogenetic analysis

Purified amplicons, obtained from nPCR using the primers Cox16SF1 and Cox16SR1, were sent to Solgent (Daejeon, South Korea) for nucleotide sequencing. The sequences were analyzed using the multiple sequence alignment program CLUSTAL Omega (ver. 1.2.1). Alignment results were corrected using BioEdit (ver. 7.2.5). Phylogenetic analysis was performed using MEGA (ver. 6.0) and the aligned sequences of *Coxiella* 16S rRNA were compared to determine homology. Stability of the trees obtained was estimated by bootstrap analysis with 1,000 replicates.

Statistical analysis

Chi-square test was used to analyze significant differences among the groups. Data for the “unknown” group were disregarded in the chi-square test. A *p* value of < 0.05 was considered statistically significant. The analytical software package GraphPad Prism version 5.04 (GraphPad Software Inc., La Jolla, CA, USA) was used for statistical analysis. A confidence interval (CI) of 95% was calculated for all estimates.

Results

Serological and molecular analyses

As shown in [Table 1](#), the sera of 11 horses (1.3%, 95% CI: 0.6–2.1) tested positive for *C. burnetii* by ELISA. In addition, the sera of six horses (0.7%, 95% CI: 0.2–1.3) tested positive for CLB by 16S rRNA sequencing. With respect to region, sex, and breed, no statistically significant differences were observed. However, prevalence was relatively high (2.4% by ELISA and 1.7% by PCR) in the northern region compared to other regions. In Jeju Island, none of the 94 tested samples were positive for *C. burnetii*, but one (1.1%, 95% CI: 0–3.1), a native Korean pony, tested positive for CLB by PCR. Although some thoroughbreds were positive by either ELISA or PCR, none tested positive by both assays. When PCR data were analyzed by age, prevalence was observed to be significantly higher (*p* < 0.05) in horses less than 5 years of age (1.8%, 95% CI: 0.2–3.5).

Prevalence based on ELISA and PCR data

None of the samples tested positive by both assays. Six (0.7%) samples were PCR+/ELISA–, 11 (1.3%) samples were PCR–/ELISA+, and 799 (97.9%) samples were PCR–/ELISA– ([Table 2](#)).

DNA sequencing and phylogenetic analysis

Among the six samples producing an amplicon of the expected size from *Coxiella* 16S rRNA, one sequence (H-JJ-10) from Jeju Island and five sequences (H-GG-169, 171, 175, 178, and 194) from the northern region of South Korea were studied. This analysis revealed that these sequences shared 92.7–100% similarity. The sequences of *Coxiella* 16S rRNA obtained from six horses were deposited in GenBank (accession nos. KT835658–KT835661, KU324470–KU324471). Comparative analysis of the 16S rRNA nucleotide sequences from the Korean samples with the 22 *Coxiella* isolates included in the GenBank database is shown in [Fig 2](#).

Coxiella 16S rRNA sequences were compared to published sequences (available in GenBank). This phylogenetic analysis (Kimura/neighbor joining) showed that one sequence (H-JJ-10) from Jeju Island shared high similarity (97.9–100%) with those of nine CLB strains, within clade B, isolated from *Haemaphysalis* ticks, in South Korea (AY342035, AY342036), Japan (AB001519), China (KC776318, JN866564), and Thailand (JQ764626, KC170758, KC170759, and HQ287535) ([Figs 2 and 3](#)). In contrast, five sequences (H-GG-169, 171, 175, 178, and 194)

Table 2. Comparison of *Coxiella* detection by ELISA and PCR.

		PCR		Total
		No. positive	No. negative	
ELISA	No. positive	0	11	11
	No. negative	6	799	805
Total		6	810	816

doi:10.1371/journal.pone.0156710.t002

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
H-JJ-10	1	92.9	92.7	92.9	92.9	92.7	100	100	100	100	99.9	99.6	99.7	98.4	97.9	95.0	95.7	95.6	95.7	93.5	85.6	86.1	86.8	86.4	86.2	85.6	85.7	76.0
H-GG-169	2	48	99.9	100	100	99.9	92.9	92.9	92.9	92.7	92.7	92.9	92.9	93.0	93.5	92.9	93.6	93.5	93.6	94.4	83.9	85.3	85.0	84.6	85.6	84.9	85.7	76.2
H-GG-171	3	49	1	99.9	99.9	99.9	92.7	92.7	92.7	92.6	92.6	92.7	92.7	92.9	93.3	92.7	93.5	93.3	93.5	94.2	83.9	85.3	85.0	84.6	85.6	84.9	85.5	76.0
H-GG-175	4	48	0	1	100	99.9	92.9	92.9	92.9	92.7	92.7	92.9	92.9	93.0	93.5	92.9	93.6	93.5	93.6	94.4	83.9	85.3	85.0	84.6	85.6	84.9	85.7	76.2
H-GG-178	5	48	0	1	0	99.9	92.9	92.9	92.9	92.7	92.7	92.9	92.9	93.0	93.5	92.9	93.6	93.5	93.6	94.4	83.9	85.3	85.0	84.6	85.6	84.9	85.7	76.2
H-GG-194	6	49	1	1	1	92.7	92.7	92.7	92.6	92.6	92.7	92.7	92.9	93.3	92.7	93.5	93.3	93.5	94.2	84.0	85.5	85.2	84.7	85.8	85.0	85.5	76.0	
AB001519	7	0	48	49	48	49	100	100	99.9	99.9	99.6	99.7	98.4	97.9	95.0	95.7	95.6	95.7	93.5	85.6	86.1	86.8	86.4	86.2	85.6	85.7	76.0	
AY342035	8	0	48	49	48	49	0	100	99.9	99.6	99.7	98.4	97.9	95.0	95.7	95.6	95.7	93.5	85.6	86.1	86.8	86.4	86.2	85.6	85.7	76.0		
KC776318	9	0	48	49	48	48	0	0	100	99.9	99.6	99.7	98.4	97.9	95.0	95.7	95.6	95.7	93.5	85.6	86.1	86.8	86.4	86.2	85.6	85.7	76.0	
JN866564	10	1	49	50	49	49	50	1	1	1	99.7	99.4	99.6	98.2	97.8	94.8	95.6	95.4	95.6	93.6	85.5	85.9	86.7	86.2	86.4	85.8	85.8	75.8
AY342036	11	1	49	50	49	49	50	1	1	1	2	99.4	99.6	98.2	97.8	94.8	95.6	95.4	95.6	93.3	85.5	85.9	86.7	86.2	86.1	85.5	85.5	75.8
JQ764626	12	3	48	49	48	48	49	3	3	3	4	4	99.9	97.9	97.8	94.5	95.3	95.1	95.3	93.0	85.6	85.8	86.8	86.1	85.9	85.3	85.4	75.8
KC170758	13	2	48	49	48	48	49	2	2	2	3	3	1	98.1	97.9	94.7	95.4	95.3	95.4	93.2	85.6	85.8	86.8	86.1	85.9	85.3	85.4	75.8
KC170759	14	11	47	48	47	47	48	11	11	11	12	12	14	13	97.8	95.1	95.9	95.7	95.9	93.6	86.4	86.8	86.9	86.5	86.7	86.2	85.2	76.1
HQ287535	15	14	44	45	44	44	45	14	14	14	15	15	14	15	95.0	95.7	95.6	95.7	94.1	85.9	86.4	87.1	86.7	86.5	85.8	85.2	76.2	
D89791	16	34	48	49	48	48	49	34	34	34	35	35	37	36	33	34	99.3	99.1	99.3	94.2	86.2	88.0	88.7	88.3	86.4	86.5	86.4	76.1
KT894209	17	29	43	44	43	43	44	29	29	29	30	30	32	31	28	29	5	99.9	100	95.0	86.8	88.6	89.3	88.9	86.9	87.1	86.8	76.4
FJ787329	18	30	44	45	44	44	45	30	30	30	31	31	33	32	29	30	6	1	99.9	94.8	86.8	88.6	89.3	88.9	86.9	87.1	86.7	76.6
D89800	19	29	43	44	43	43	44	29	29	29	30	30	32	31	28	29	5	0	1	95.0	86.8	88.6	89.3	88.9	86.9	87.1	86.8	76.4
LC000780	20	44	38	39	38	38	39	44	44	44	43	45	47	46	43	40	39	34	35	34	84.3	85.5	85.0	84.9	86.2	85.5	85.1	76.0
KP994835	21	97	109	109	109	109	108	97	97	97	98	98	97	97	92	95	93	89	89	89	106	97.4	95.6	95.1	96.2	96.4	77.2	71.3
KP994777	22	94	99	99	99	99	98	94	94	94	95	95	96	96	89	92	81	77	77	77	98	16	97.2	96.7	97.2	97.4	78.4	72.4
KP994781	23	89	101	101	101	101	100	89	89	89	90	90	89	88	87	76	72	72	72	101	27	17	98.9	95.7	95.9	78.4	72.1	
KP994770	24	92	104	104	104	104	103	92	92	92	93	93	94	94	91	90	79	75	75	102	30	20	7	95.2	95.4	78.3	72.5	
KP994811	25	93	97	97	97	97	96	93	93	93	92	94	95	95	90	91	92	88	88	88	93	23	17	26	29	99.2	78.3	72.7
KP994813	26	97	102	102	102	102	101	97	97	97	96	98	99	99	93	96	91	87	87	87	98	22	16	25	28	5	78.4	72.2
NR041742	27	97	97	98	97	97	98	97	97	97	96	98	99	99	100	100	92	89	90	89	101	154	146	146	147	147	146	76.6
U11021	28	162	161	162	161	161	162	162	162	162	163	163	163	163	161	161	161	159	158	159	162	190	183	185	182	181	184	158

Fig 2. Comparison of *Coxiella* 16S rRNA nucleotide sequences. The upper matrix shows percent identity between the partial sequences of the *Coxiella* 16S rRNA gene. The lower matrix presents the number of differences in nucleotide bases.

doi:10.1371/journal.pone.0156710.g002

from the northern region were clustered into a new clade, showing 94.2–94.4% similarity to *Coxiella* sp. S40 from Japan (LC000780), again within the same new clade (Figs 2 and 3).

Discussion

In 1997, the development of 16S rRNA sequencing led to the first instance of identification of CLB in three species of ticks [18]. The sequences of CLB 16S rRNA were closely related to those of *C. burnetii*, which indicated diversity within the genus *Coxiella*, a previously overlooked aspect [19]. *C. burnetii* and CLB are genetically and ecologically distinct, despite genetic similarities. Furthermore, CLB are exceptionally diverse and widespread in ticks, but rarely described in domestic animals; however, CLB have recently been reported to be a leading cause of lethal systematic infections in domestic birds [20–22]. There is an important risk of misidentification, given that the current protocols for detecting *C. burnetii* in ticks depend on PCR-based detection of a single gene, without subsequent confirmation by sequencing [23].

An increase in the seroprevalence of *C. burnetii* in ruminant herds is a useful index for studying their occurrence in humans [24]. This could also be extrapolated to *C. burnetii* infections in horses. The primary route of transmission to humans is via nasal inhalation, and the rate of tick-borne transmission of Q fever in humans is considered low. However, some cases of possible tick-borne transmission have been reported [25, 26]. Those studies described patients with serological and clinical evidence of a tick-borne disease and subsequent or concomitant Q fever after tick bites. Furthermore, a case report indicates that *C. burnetii* could be spread by various ticks during horseback riding [27]. This might be a different approach of transmission involving horses. Our study confirms the results of a previous study indicating that humans can be exposed to *C. burnetii* from infected horses through contact with ticks during horseback riding; contaminated stable materials could also expose humans to polluted air or dust from the environment. In South Korea, the popularity of horseback riding has surpassed that of horseracing.

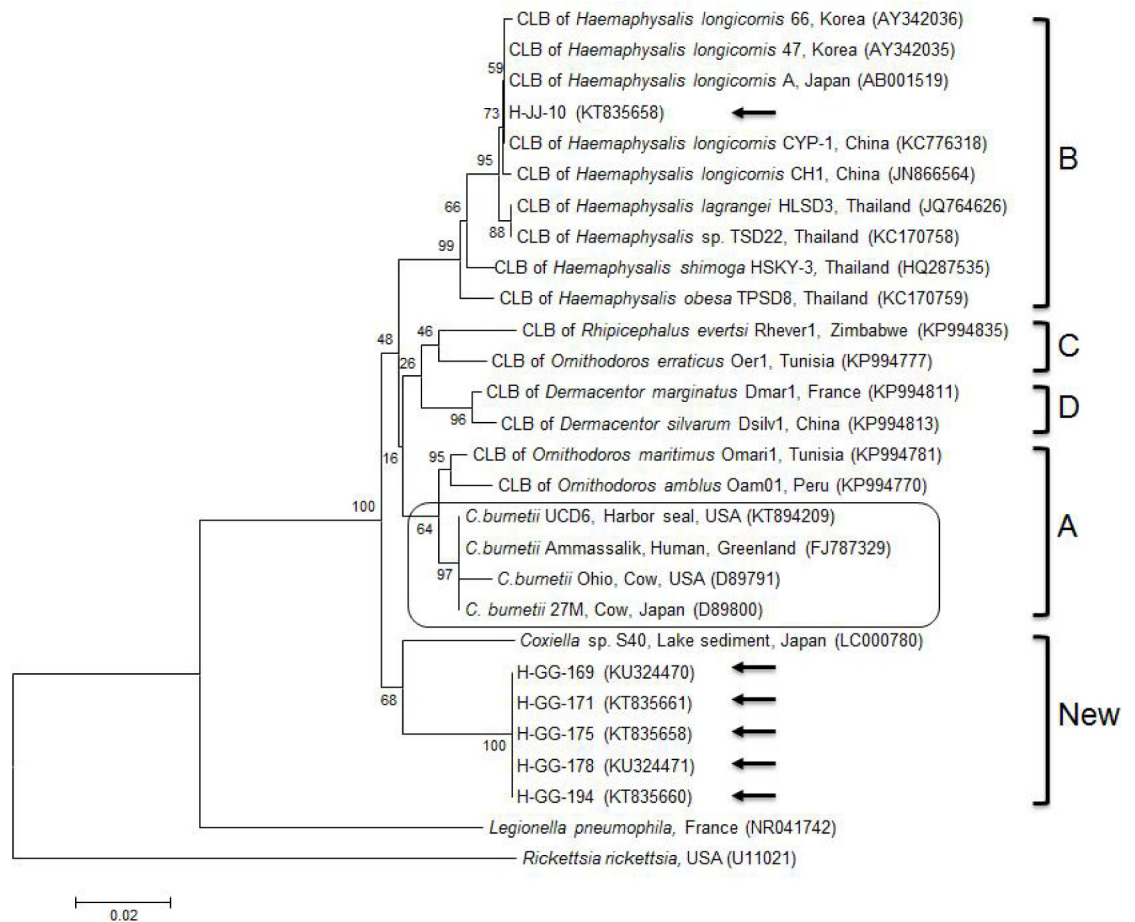


Fig 3. Phylogenetic tree constructed using Kimura/neighbor-joining methods based on 16S rRNA sequences of *Coxiella*. The four clades (A–D) of *Coxiella* are categorized. The sequences of *Coxiella*-like bacteria obtained in this study are marked using arrows. The *C. burnetii* group is found within clade A. The accession numbers of sequences obtained from GenBank are shown with the sequence names and countries of origin. Numbers on the branches indicate bootstrap support (1,000 replicates). Scale bar indicates a phylogenetic distance of 0.02 nucleotide substitution per position. CLB = *Coxiella*-like-bacteria.

doi:10.1371/journal.pone.0156710.g003

Recently, a meta-analysis of studies performed over an extended period across countries showed that the mean seroprevalence of *C. burnetii* in horses was expected to be 15.8% (95% CI: 9.6–23%) [7]. However, seroprevalence rates differ with the geographical area, test design, population, cut-off value used, year, diagnostic method, and sensitivity and specificity of the assay [7]. In the present study, the prevalence of *C. burnetii* was found to be 1.3% by ELISA and 0.7% by PCR among the 816 horses tested. Although we initially expected to detect *C. burnetii* in horses by PCR, the sequencing data indicate that only CLB were detected in horses. Because CLB are known to exist in the salivary glands of ticks, they could be transmitted to humans and vertebrates during blood sucking. Potential tick-to-vertebrate transmission of CLB is likely because ticks occur worldwide and feed on various hosts [5]. Because CLB detection has been restricted to ticks, CLB might pose a much lesser threat to vertebrates than *C. burnetii* [23]. However, the risk of vertebrate infection by CLB is unknown, as these bacteria have not been detected in vertebrates or associated with clinical symptoms [6]. Thus, to the best of our knowledge, the present study is the first to report the detection of CLB in a mammalian species, namely, the horse. Further studies should be performed to determine whether CLB infection manifests the clinical signs of the disease in mammals, including humans.

This study found relatively low positive yield of *Coxiella*, compared to other studies on ruminants in South Korea. A previous study using ELISA showed a detection rate of 24.2% (119/492) in dairy cattle and 54% (175/324) in bulk milk tanks in the southern regions [12]. Other studies also showed high infection rates in native Korean goats by ELISA (19.1%; 114/597) and PCR (9.5%; 57/597) in the central and southern regions [13], and wild Korean water deer by ELISA (9.2%; 18/196) and real-time PCR (6.6%; 13/196) in the northern, central, and southern regions [14]. Horse studies performed in other countries showed relatively low positive rates in aborted fetuses (0%; 0/122) by using PCR in Italy [7], blood (0%; 0/105) by complement fixation test in Denmark [28], aborted fetuses (1.5%; 6/407) by real-time PCR in France [10], and aborted fetuses (4.3%; 1/23) by real-time PCR in Germany [9]. However, much higher positivity was reported in aborted pregnancies (42.2%; 19/45) by PCR in Croatia [29], blood (22.2%; 4/18) by loop-mediated isothermal amplification in China [30], aborted or non-aborted placenta (7.7%; 3/39) by real-time PCR in the Netherlands [11], and blood (12.5%; 14/112) and urine (7.1%; 1/14) by real-time PCR in Australia [31].

The health status of horses that yielded positive results for *C. burnetii* could provide better insight into the pathogenesis of *C. burnetii*; however, this was not recorded in this study. While the data indicated no regional variations, the northern region did have a higher prevalence, as only one horse from Jeju Island was determined to be positive by PCR. This result was unexpected, as infectivity is likely to be observed in regions with a warm and wet climate, such as Jeju Island. However, the one native Korean pony infected with CLB was raised on Jeju Island. All horses of Jeju Island used in this experiment were native Korean ponies. Therefore, additional investigation is required for other breeds of horses reared on Jeju Island. The recent climate change has probably contributed to the widespread distribution of ticks [32] and an increase in the period of activity. With respect to breed, prevalence was mostly detected in thoroughbred horses, except one native Korean pony. Higher prevalence of *Coxiella* in thoroughbreds was expected based on the geographical distribution of thoroughbreds (46.7%, 12,066 horses were raised in South Korea) [15]. No significant difference was observed with respect to sex. Younger horses (< 5 years) showed significantly higher prevalence of CLB by PCR. This result was also unexpected because older animals are likely to have had more opportunities for exposure than younger animals [12,13].

In this study, 11 (1.3%) and six (0.7%) of the 816 horse blood samples tested positive for *Coxiella* by ELISA and PCR, respectively. None of the samples tested positive by both assays. The PCR assay employed detects genomic DNA common to all *Coxiella* species, including *C. burnetii* and CLB, while the ELISA is based on the specific detection of serum antibodies against only *C. burnetii*. Hence, one plausible explanation for the lack of consistency observed is that only CLB, but not *C. burnetii*, were present in the PCR-positive samples. Another possible explanation for this inconsistency may be the detection limit of ELISA, and the fact that this assay can detect antibodies from both active and prior infections. PCR can solely detect active infections [33]. The ID screen ELISA kit is adapted to mammalian IgG antibodies, and it was originally developed to react with cattle, goats, and sheep. However, it has also been used to react with antibodies against *C. burnetii* in blood samples from other mammalian species, including cats, foxes, and rodents [34].

Genotypically and phenotypically different features of CLB and *C. burnetii* could also conceivably lead to different rates of positivity. The widespread genetic variability in CLB strains compared to *C. burnetii* strains has led to a clear sub-classification of this genus into four largely divergent clades (A–D) [5]. The clustering of all *C. burnetii* strains within clade A indicates that the progenitor of *C. burnetii* was a tick-related bacterium that succeeded in infecting vertebrates. Based on the phylogenetic analysis, H-JJ-10 on Jeju Island is closely related to the CLB in *Haemaphysalis* ticks belonging to clade B. Therefore, further studies on CLB in ticks

associated with horses are required, especially on Jeju Island. The H-JJ-10 clustered together with the CLB in *Haemaphysalis* ticks from South Korea, China, Japan, and Thailand, which implies a close epidemiological connection between these isolates. The five isolates from the northern region were placed into a new distinct clade. Because of the geographical differences between the northern region of the mainland and Jeju Island, the origin of *Coxiella* may differ, contributing to the diversity of this species in South Korea. Further molecular studies are needed to fully understand the diversity of the genus *Coxiella*.

To the best of our knowledge, this is the first report of a large-scale nationwide study to report the serological and molecular detection of the genus *Coxiella* in mammals, namely, horses. Nevertheless, further studies will be required to describe these CLB isolates, characterize their genetic relationship, and evaluate their potential to cause infections in vertebrates [5]. Considering the zoonotic potential of the genus *Coxiella* and climate change, which affects the widespread distribution of ticks and increases their period of activity, it is necessary to establish strategic monitoring, epidemiological insight, and appropriate control programs for *Coxiella* and other tick-borne diseases. Future research on possible cross-reactivity between *C. burnetii* and CLB will be essential to better evaluate the specificity of diagnostic assays and screening tools now used in vertebrates [5]. In addition, further epidemiological studies on *Coxiella* species in healthy horse herds, epizootics in horses, and positive cases of horse abortion are significant for better understanding *C. burnetii* or CLB infections.

Conclusions

C. burnetii can be transmitted to humans following exposure to ticks during horseback riding. Global warming is expected to cause an increase in the number and distribution of ticks. To the best of our knowledge, this is the first large-scale nationwide investigation to study the prevalence of *C. burnetii* and CLB among 816 horses in South Korea. Eleven (1.3%) and six (0.7%) samples tested positive by ELISA and PCR, respectively. This study describes the first instance of CLB detection (previously restricted to ticks) in mammals, thereby providing evidence about the potential for transmission of Q fever-causing bacteria to humans from infected horses during activities such as horseback riding.

Author Contributions

Conceived and designed the experiments: MGS DK. Performed the experiments: MGS SHL DV IOO. Analyzed the data: MGS SHL ODK DK. Contributed reagents/materials/analysis tools: SHY EC YSP SEL JWK GJC. Wrote the paper: MGS ODK DK.

References

1. Babudieri B. Q fever: a zoonosis. *Adv Vet Sci.* 1959; 5:81–181.
2. Maurin M, Raoult D. Q fever. *Clin Microbiol Rev.* 1999; 12:518–553. PMID: [10515901](#)
3. Skerman VBD, McGowan V, Sneath PHA. Approved lists of bacterial names. *Int J Syst Bacteriol.* 1980; 30:225–420.
4. Tan CK, Owens L. Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. *Dis Aquat Organ.* 2000; 41:115–122. PMID: [10918979](#)
5. Duron O, Sidi-Boumedine K, Rousset E, Moutailler S, Jourdain E. The importance of ticks in Q fever transmission: What has (and has not) been demonstrated? *Trends Parasitol.* 2015; 31:536–552. doi: [10.1016/j.pt.2015.06.014](#) PMID: [26458781](#)
6. Duron O, Noël V, McCoy KD, Bonazzi M, Sidi-Boumedine K, Morel O, et al. The recent evolution of a maternally-inherited endosymbiont of ticks led to the emergence of the Q fever pathogen, *Coxiella burnetii*. *PLoS Pathog.* 2015; 11:e1004892. doi: [10.1371/journal.ppat.1004892](#) PMID: [25978383](#)

7. Marenzoni ML, Stefanetti V, Papa P, Casagrande Proietti P, Bietta A, Coletti M, et al. Is the horse a reservoir or an indicator of *Coxiella burnetii* infection? Systematic review and biomolecular investigation. *Vet Microbiol.* 2013; 167:662–669. doi: [10.1016/j.vetmic.2013.09.027](https://doi.org/10.1016/j.vetmic.2013.09.027) PMID: [24144862](https://pubmed.ncbi.nlm.nih.gov/24144862/)
8. Acland HM. Abortion in mares. In: McKinnon AO, Voss JL. *Equine, reproduction*. Philadelphia: Lea & Febiger; 1993. pp. 554–562.
9. Runge M, Hilbert A, Henning K. Contribution to the occurrence of *Coxiella burnetii*-infection in horses. *Praktische Tierarzt.* 2012; 93:220–222.
10. Leon A, Richard E, Fortier C, Laugier C, Fortier G, Pronost S. Molecular detection of *Coxiella burnetii* and *Neospora caninum* in equine aborted foetuses and neonates. *Prev Vet Med.* 2012; 104:179–183. doi: [10.1016/j.prevetmed.2011.11.001](https://doi.org/10.1016/j.prevetmed.2011.11.001) PMID: [22130310](https://pubmed.ncbi.nlm.nih.gov/22130310/)
11. Roest HI, van Solt CB, Tilburg JJ, Klaassen CH, Hovius EK, Roest FT, et al. Search for possible additional reservoirs for human Q fever, the Netherlands. *Emerg Infect Dis.* 2013; 19:834–835. doi: [10.3201/eid1905.121489](https://doi.org/10.3201/eid1905.121489) PMID: [23697680](https://pubmed.ncbi.nlm.nih.gov/23697680/)
12. Ouh IO, Seo MG, Do JC, Kim IK, Cho MH, Kwak DM. Seroprevalence of *Coxiella burnetii* in bulk-tank milk and dairy cattle in Gyeongbuk province, Korea. *Korean J Vet Serv.* 2013; 36:243–248.
13. Jung BY, Seo MG, Lee SH, Byun JW, Oem JK, Kwak D. Molecular and serologic detection of *Coxiella burnetii* in native Korean goats (*Capra hircus coreanae*). *Vet Microbiol.* 2014; 173:152–155. doi: [10.1016/j.vetmic.2014.06.029](https://doi.org/10.1016/j.vetmic.2014.06.029) PMID: [25061007](https://pubmed.ncbi.nlm.nih.gov/25061007/)
14. Shin GW, Kim EJ, Lee HB, Cho HS. The prevalence of *Coxiella burnetii* infection in wild Korean water deer, Korea. *J Vet Med Sci.* 2014; 76:1069–1071. PMID: [24717533](https://pubmed.ncbi.nlm.nih.gov/24717533/)
15. Ministry of Agriculture, Food and Rural Affairs, South Korea. Report of “A fact finding survey of horse industry in South Korea during 2014”. 2015.
16. Thrusfield M. *Veterinary epidemiology*, 3rd ed. Oxford: Wiley Blackwell; 2005. pp. 228–330.
17. Duron O, Jourdain E, McCoy KD. Diversity and global distribution of the *Coxiella* intracellular bacterium in seabird ticks. *Ticks Tick Borne Dis.* 2014; 5:557–563. doi: [10.1016/j.ttbdis.2014.04.003](https://doi.org/10.1016/j.ttbdis.2014.04.003) PMID: [24915875](https://pubmed.ncbi.nlm.nih.gov/24915875/)
18. Noda H, Munderloh UG, Kurtti TJ. Endosymbionts of ticks and their relationship to *Wolbachia* spp. and tick-borne pathogens of humans and animals. *Appl Environ Microbiol.* 1997; 63:3926–3932. PMID: [9327557](https://pubmed.ncbi.nlm.nih.gov/9327557/)
19. Zhong J. *Coxiella*-like endosymbionts. In: Toman R. et al. *Coxiella burnetii*: Recent advances and new perspectives in research of the Q fever bacterium. New York: Springer; 2012. pp. 365–379.
20. Shivaprasad HL, Cadenas MB, Diab SS, Nordhausen R, Bradway D, Crespo R, et al. *Coxiella*-like infection in psittacines and a toucan. *Avian Dis.* 2008; 52:426–432. PMID: [18939630](https://pubmed.ncbi.nlm.nih.gov/18939630/)
21. Vapniarsky N, Barr BC, Murphy B. Systemic *Coxiella*-like infection with myocarditis and hepatitis in an eclectus parrot (*Eclectus roratus*). *Vet Pathol.* 2012; 49:717–722. doi: [10.1177/0300985811409251](https://doi.org/10.1177/0300985811409251) PMID: [21712515](https://pubmed.ncbi.nlm.nih.gov/21712515/)
22. Woc-Colburn AM, Garner MM, Bradway D, West G, D'Agostino J, Trupkiewicz J, et al. Fatal coxiellosis in swainson's blue mountain rainbow lorikeets (*Trichoglossus haematodus moluccanus*). *Vet Pathol.* 2008; 45:247–254. doi: [10.1354/vp.45-2-247](https://doi.org/10.1354/vp.45-2-247) PMID: [18424842](https://pubmed.ncbi.nlm.nih.gov/18424842/)
23. Smith TA, Driscoll T, Gillespie JJ, Raghavan R. A *Coxiella*-like endosymbiont is a potential vitamin source for the Lone Star tick. *Genome Biol Evol.* 2015; 7:831–838. doi: [10.1093/gbe/evv016](https://doi.org/10.1093/gbe/evv016) PMID: [25618142](https://pubmed.ncbi.nlm.nih.gov/25618142/)
24. Georgiev M, Afonso A, Neubauer H, Needham H, Thiery R, Rodolakis A, et al. Q fever in humans and farm animals in four European countries, 1982 to 2010. *Euro Surveill.* 2013; 18:pii = 20407. PMID: [23449232](https://pubmed.ncbi.nlm.nih.gov/23449232/)
25. Eklund CM, Parker RR, Lackman DB. A case of Q fever probably contracted by exposure to ticks in nature. *Public Health Rep.* 1947; 62:1413–1416. PMID: [20344767](https://pubmed.ncbi.nlm.nih.gov/20344767/)
26. Rolain JM, Gouriet F, Brouqui P, Larrey D, Janbon F, Vene S, et al. Concomitant or consecutive infection with *Coxiella burnetii* and tickborne diseases. *Clin Infect Dis.* 2005; 40:82–88. PMID: [15614696](https://pubmed.ncbi.nlm.nih.gov/15614696/)
27. Nett RJ, Book E, Anderson AD. Q Fever with unusual exposure history: a classic presentation of a commonly misdiagnosed disease. *Case Rep Infect Dis.* 2012; 916142. doi: [10.1155/2012/916142](https://doi.org/10.1155/2012/916142) PMID: [22848855](https://pubmed.ncbi.nlm.nih.gov/22848855/)
28. Agerholm JS, Svejstrup CA, Christoffersen AB, Agger JFG. Apparent seroprevalence for *Coxiella burnetii* in the Danish horse population. *Dansk Veterinaertidsskrift.* 2015; 98:26–29.
29. Račić I, Spičić S, Galov A, Duvnjak S, Zdelar-Tuk M, Vujnović A, et al. Identification of *Coxiella burnetii* genotypes in Croatia using multi-locus VNTR analysis. *Vet Microbiol.* 2014; 173:340–347. doi: [10.1016/j.vetmic.2014.08.016](https://doi.org/10.1016/j.vetmic.2014.08.016) PMID: [25213233](https://pubmed.ncbi.nlm.nih.gov/25213233/)

30. Pan L, Zhang L, Fan D, Zhang X, Liu H, Lu Q, et al. Rapid, simple and sensitive detection of Q fever by loop-mediated isothermal amplification of the *htpAB* gene. *PLoS Negl Trop Dis*. 2013; 7:e2231. doi: [10.1371/journal.pntd.0002231](https://doi.org/10.1371/journal.pntd.0002231) PMID: [23696915](https://pubmed.ncbi.nlm.nih.gov/23696915/)
31. Tozer SJ, Lambert SB, Strong CL, Field HE, Sloots TP, Nissen MD. Potential animal and environmental sources of Q fever infection for humans in Queensland. *Zoonoses Public Health*. 2013; 61:105–112. doi: [10.1111/zph.12051](https://doi.org/10.1111/zph.12051) PMID: [23663407](https://pubmed.ncbi.nlm.nih.gov/23663407/)
32. Dantas-Torres F. Climate change, biodiversity, ticks and tick-borne diseases: The butterfly effect. *Int J Parasitol Parasites Wildl*. 2015; 4:452–461. doi: [10.1016/j.ijppaw.2015.07.001](https://doi.org/10.1016/j.ijppaw.2015.07.001) PMID: [26835253](https://pubmed.ncbi.nlm.nih.gov/26835253/)
33. Kirkan S, Kaya O, Tekbiyik S, Parin U. Detection of *Coxiella burnetii* in cattle by PCR. *Turk. J. Vet. Anim. Sci*. 2008; 32:215–220.
34. Meredith AL, Cleaveland SC, Denwood MJ, Brown JK, Shaw DJ. *Coxiella burnetii* (Q-fever) seroprevalence in prey and predators in the United Kingdom: evaluation of infection in wild rodents, foxes and domestic cats using a modified ELISA. *Transbound. Emerg. Dis*. 2015; 62:639–649. doi: [10.1111/tbed.12211](https://doi.org/10.1111/tbed.12211) PMID: [24479951](https://pubmed.ncbi.nlm.nih.gov/24479951/)