Coxiella burnetii infection in a patient did not involve tick bite

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Research

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Abstract

Background: *Coxiella burnetii* that causes Q fever in humans is transmitted through contaminated aerosols or consumption of raw milk from infected animals. Ticks are known to be vectors of *C. burnetii*, but their role in human infection is still controversial. Method: In this study, an epidemiological investigation was conducted on a 60-year-old man hospitalized with fever. Polymerase chain reactions (PCRs) were performed on the blood sample of the patient and the four ticks collected from the patient to diagnose vector-borne infectious diseases. Indirect immunofluorescence assays (IFAs) were performed on the serum sample to detect IgG and IgM antibodies specific to Q fever, spotted fever, Lyme disease, and anaplasmosis. Results: The ticks collected were identified as adult female *Haemaphysalis longicornis*. All PCRs performed on blood specimens yielded negative results, except for the *Coxiella* sp.-specific 16S rRNA nested PCR (N-PCR). Coxiella 16S rRNA N-PCR and sequencing results confirmed the presence of *C. burnetii*. IFA results indicated that the serum sample showed ≥ 4-fold increase in both IgM and IgG antibody titers against Q fever. PCR results were positive only for three ticks and showed the presence of C. endosymbiont. The phylogenetic tree showed that ticks had Coxiella-like bacteria and the patient had *C. burnetii*. The ticks carrying Coxiella-like bacteria were *Haemaphysalis* symbionts. Conclusions: Even though the patient and ticks were all positive to *Coxiella* sp.-specific 16S rRNA N-PCR, since different bacterial species were isolated from the patient and the ticks, we concluded that he was not infected with *C. burnetii* through tick bites.

Introduction

Coxiella burnetii is an obligate intracellular, small, gram-negative bacterium that causes Q fever. It is a zoonotic bacterium whose most common reservoirs are livestock, such as cattle, sheep, and goats. However, a variety of other wild animals, birds, amphibians, and arthropods can also carry the bacterium (1). The Korea Centers for Disease Control and Prevention (KCDC) has reported that the number of Q fever infections has risen sharply since 2000 (Appendix Figure).

C. burnetii is highly infectious and survives environmental stresses for several weeks (2). The main mechanism of transmission is through contaminated aerosols or consumption of raw milk from infected animals (3). Other routes of transmission are minor or controversial (4). Hence, infection in humans is commonly associated with direct contact with animal reservoirs. People working in slaughterhouses, timber industries, or agricultural farm and veterinarians have a high risk for *C. burnetii* infection (5).

The best diagnostic tool is *C. burnetii* isolation. However, isolation is time-consuming, hazardous, and requires a biosafety level 3 facility. Hence, polymerase chain reaction (PCR) and indirect immunofluorescence assay (IFA) are preferred clinically, as diagnostic tools (6). If ticks are found on a patient with a tick bite, the causative agents could be detected through the PCR amplification of the hemolymph from these ticks (7). Ticks are *C. burnetii* vectors, but their role as a vector in human infection is controversial. Many studies have tried to find a relationship between tick bites and *C. burnetii* infection in humans. For instance, Eklund et al. reported a case of human *C. burnetii* infection that may have involved contact with ticks in a natural setting (8). However, the authors did not investigate the presence of the bacterium in the ticks.

Sequencing and phylogenetic analyses are necessary for the precise identification of *C. burnetii* (9). The present case involved a patient with Q fever. We explored whether tick bite was involved with the infection using PCR with a phylogenetic tree as well as IFA.

Materials And Methods

Case

On April 22, 2018, a 60-year-old man was hospitalized with fever. His vital signs were as follows: blood pressure as 140/90 mm Hg, heart rate of 88 beats per minute, respiratory rate of 18 times per minute, and a temperature of 39.2°C. He had no underlying disease. History-taking revealed that he had been mowing for three days, 13 days prior to hospitalization, and had manifested symptoms including headache, fatigue, nausea, and myalgia for six days before hospitalization. In an effort to alleviate the symptoms, he took medications acquired from a pharmacy. However, he had fever for three days before hospitalization and the symptoms had worsened, which prompted his hospital visit.

On admission, hematological and biochemical results revealed white blood cell count of 7780 cells/μL (normal range: 4800–10,800; neutrophils 83.3%), platelet count of 68,000 cells/μL (normal range: 130,000–450,000), hemoglobin level of 14.9 g/dL (normal range: 12–18), serum blood urea nitrogen level of 14.8 mg/dL (reference range: 8.0–20), and creatinine level of 0.83 mg/dL (reference range: 0.5–1.3).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were higher than the normal range at 144/183 IU/L (reference range: 5–40/5–40), but prothrombin time, total bilirubin, and C-reactive protein levels were within the normal range. PCR and antibody tests were performed to detect influenza A/B, *Leptospira*, Hantavirus, and *Orientia tsutsugamushi*, but none were detected. Blood was cultured and no bacterial growth was detected.

After hospitalization, the patient related that he had found ticks on his body and clothes while showering. However, he could not recall the moment of being bitten by ticks. Hence, we collected tick samples from the location of mowing. PCR targeting vector-borne infectious diseases was carried out on the four ticks collected. The results were compared with the PCR result of the blood from the patient.

DNA extraction, PCR detection, and molecular identification of vector-borne infectious diseases

The four ticks collected were first washed with 70% ethanol, then with sterile distilled water, and air dried. The life cycle stages and species of the ticks were morphologically classified using microscopy and standard taxonomic keys (10). The ticks were ground using a FastPrep®-24 Classic instrument (MP Biomedicals, Solon, OH, USA) and stored at −80 °C until used for DNA extraction.

Genomic DNAs were extracted from 300 µL of the patient's blood sample and from the ground tick lysate (150 µL) using the QIAamp Tissue & Blood Mini Kit (Qiagen, Hilden, Germany) by following the manufacturer's instructions. DNA was eluted into 50 µL TE (10 mM Tris·Cl, 0.5 mM EDTA, pH 9.0) buffer and stored at −20 °C until PCR amplification was carried out.

Nested-PCR (N-PCR) and real-time PCR were performed to diagnose Q fever, rickettsial disease, Lyme disease, and anaplasmosis in the blood specimen and the four ticks. The *htpAB*-associated repetitive element (*IS1111*) and 16S ribosomal RNA (16S rRNA) gene were targeted to detect *Coxiella* sp. (11, 12). For 16S rRNA N-PCR, we designed three additional primers (16s1s-tF0, 16s1st-R0, and Cox16SF3) based on the *C. burnetii* 16S rRNA sequence (GenBank Accession No. CP001019) to make one contig after DNA sequencing. To detect the presence of *Rickettsia* DNA, the outer membrane protein A gene (*ompA*) of the spotted fever group *Rickettsia* species and the 23S rRNA gene of *Rickettsia* (panrickettsia) were targeted (13-15). The CTP synthase (*pyrG*) gene was targeted to detect *Borrelia* species (16). The heat shock protein gene (*groEL*) and the ankyrin-related protein gene (*ankA*) were targeted to detect *A. phagocytophilum* (17, 18). All PCR primers and probes used for detecting bacterial pathogens, PCR conditions, and product sizes are given in Table 1.

Conventional PCR (C-PCR) was performed in 20 µL reaction volumes using the AccuPowerR PCR PreMix (Bioneer, Daejeon, Korea). Each PCR mixture consisted of 16 µL distilled water, 1 µL each primer (10 pmol/µL), and 2 µL genomic DNA as the template DNA. For N-PCR, the reaction mixture was identical to that used in C-PCR, except that the first PCR product was used as template DNA and the N-PCR primers were used. With each PCR run, a positive and a negative control (molecular grade water) were included.

All amplifications were performed in an AB thermal cycler (Applied Biosystem, Foster City, CA, USA). The amplified products were separated by 1.2% agarose gel electrophoresis and visualized by staining with ethidium bromide (Bioneer).

Real-time PCR was performed in 20 µL reaction volumes, which consisted of 5 µL genomic DNA, 4 µL LightCycler TaqMan Master mix (Roche Diagnostics, Indianapolis, IN, USA), 1 µL of each primer (10 pmol/µL), 1 µL probe (5 pmol/ µL), and 8 µL distilled water. The real-time PCR was carried out in an Exicycler^{TM} 96 Real-Time Quantitative Thermal Block (Bioneer).

Sequencing and phylogenetic analysis

The amplified PCR products were purified using QIAquick PCR purification kits (Qiagen) and directly sequenced with the PCR primers at Cosmogenetech Co, Ltd. (Daejeon, Korea). The resulting sequences obtained in this study were examined using the BlastN program from NCBI (Bethesda, MD, USA) to identify the bacteria present.

The concatenating 16S rRNA sequences were aligned and analyzed for homology comparisons using DNASTAR-Lasergene v6 software (DNASTAR, Madison, WI, USA) and the NCBI BlastN network service.

The phylogenetic tree was constructed using ClustalW of the MegAlign Program and the Tree Explorer program (DNASTAR) based on the alignments of positive gene sequences using the neighbor-joining method. Bootstrap analysis (1,000 replicates) was performed to improve the confidence level of the phylogenetic tree according to the Kimura 2-parameter method. Pairwise alignments were performed with an open-gap penalty of 10 and a gap extension penalty of 0.5.

IFA

To detect IgG and IgM antibodies specific to Q fever, spotted fever, Lyme disease, and anaplasmosis, IFAs were performed at the Korea Centers for Disease Control and Prevention (KCDC) as described previously (19).

Ethics approval and consent to participate

The study was approved by the Ethics in Human Research Committee of Chosun University Hospital (IRB No. 2013-10-001-018). The patient provided written informed consent to participate in the study.

Results

The four ticks collected were all identified as adult female *Haemaphysalis longicornis* (Figure 1) by morphological and molecular biological method targeting 16S rRNA (20).

N-PCR and real-time PCR analyses were carried out using the blood sample collected from the patient on admission to diagnose Q fever, rickettsial disease, Lyme disease, and anaplasmosis. All PCR procedures, except that for the *Coxiella* species-specific 16S rRNA N-PCR, were negative for vector-borne infectious diseases (Table 2). The 16S rRNA N-PCR employed two sets of PCR primers (Cox16SF1/ Cox16SR1 and Cox16SF3/ Cox16SR2) for N-PCR, yielding a final N-PCR product of 720 and 790 bp, respectively. The 16S rRNA PCR amplicons were purified and directly sequenced using PCR primers. The sequencing result was analyzed using the BlastN program and the presence of *C. burnetii* was confirmed. Homology testing showed that the 16S rRNA sequences of the sample from the *Coxiella*-positive patient had 99.9% similarity with the 16S rRNA partial sequence of *C. burnetii* MSU isolated from a goat in the United States.

The serum sample collected from the patient on admission was subjected to IFAs to detect IgG and IgM antibodies specific to Q fever, spotted fever, Lyme disease, and anaplasmosis. We also conducted a follow-up investigation to examine antibody titers in the patient. The results revealed that the patient had developed antibodies against Q fever, while having no antibodies against spotted fever, Lyme disease, and anaplasmosis. The IFA positive cut-off value for Q fever was ≥ 4-fold increase in the IgM or IgG antibody titers. The patient showed ≥ 4-fold increase in both IgM and IgG antibody titers against *C. burnetii*. The IgG phase II antibody titer specific to *C. burnetii* was negative on admission. In the follow-up at 7–8 weeks, the IgG phase II titer reached 1:1024. The IgM phase II antibody titer was also increased to 1:512 after 6 weeks. The IgG and IgM phase I antibody titers were negative and remained unchanged for over 2 months (Table 2).

We also performed N-PCR and real-time PCR using genomic DNA from the four ticks (2018-505-tick1, -tick2, -tick3, and -tick4) to diagnose Q fever, rickettsial disease, Lyme disease, and anaplasmosis. The *Coxiella* species-specific 16S rRNA N-PCR was positive only for three ticks (2018-505-tick1, -tick3, and -tick4) and the sequencing results confirmed the presence of *Coxiella* endosymbiont. *Coxiella* sp. harbored in the three ticks were identified to be *Coxiella*-like bacteria (CLB) and not *C. burnetii*. Homology testing showed that the 16S rRNA sequences of the three *Coxiella*-positive ticks (2018-505-tick1, -tick3, and -tick4) had 99.8%, 100%, and 100% similarity, respectively, with the 16S rRNA partial sequence of *H. longicornis* symbiont 47.

A phylogenetic tree was constructed using sequences of 16S rRNA gene fragments (1210 bp) from the patient blood sample and three *Coxiella*-positive tick samples, with the 16S rRNA gene sequences of *C. burnetii* and CLB from GenBank as reference sequences. The 16S rRNA gene sequences from the *Coxiella*-positive patient sample formed a cluster with *C. burnetii* sequences. All 16S rRNA gene sequences from *Coxiella*-positive tick samples formed a cluster with CLB from *H. longicornis* tick (Figure 2).

The *Rickettsia*-specific *ompA* N-PCR was positive for three ticks (2018-505-tick1, -tick2, and -tick3) and sequencing results confirmed the presence of *R. raoultii.* The pan-*rickettsial* real-time PCR targeting the 23S rRNA was positive in one tick (2018-505-tick1) and showed a Ct value of 36.09. The *Anaplasma* species-specific *groEL* N-PCR tested positive in one tick (2018-505-tick3) and the sequencing results confirmed the presence of *A. phagocytophilum.* The *Borrelia* species-specific *pyrG* gene N-PCR was negative in all four ticks. The patient showed symptoms of acute Q fever, which was caused by *C. burnetii* infection. If any of the four ticks harbored the same bacterium, it would indicate some chance of transmission between *C. burnetii* infection in the patient and tick bite. The phylogenetic tree test was performed to find whether *C. burnetii* from the patient blood was identical to the *Coxiella* bacterium identified in the ticks. However, according to the test result, the three ticks positive to 16S rRNA N-PCR turned out to have CLB and not *C. burnetii* that was found in the blood of the patient.

Discussion

C. burnetii infection in humans is usually asymptomatic or manifests as a mild disease with flu-like symptoms. However, in some patients, Q fever can occur, which causes serious complications or even death, especially to those who present meningoencephalitis, or myocarditis in the acute phase and endocarditis in the chronic phase. Rarely, pregnant women and immunocompromised hosts are also in danger of chronic Q fever (2). Although the present patient was asymptomatic, the result of the IFA showed that the patient had Q fever. Since 60% of people suffering from acute Q fever are asymptomatic, serological evidence is more important than clinical manifestations for diagnosis (21). The four ticks that were collected were *H. longicornis*. *C. burnetii* can be carried by only certain types of ticks. In Korea, *H. longicornisis* was reported as the first tick to harbor *Coxiella* spp. (22). However, CLB and *C. burnetii* share many genetic similarities, despite showing distinct ecological features (23). Even if *Coxiella* spp. is found in ticks and genetic and serological tests are performed, it is difficult to conclude whether it is *C. burnetii* or CLB before checking the phylogenetic tree.

To the best of our knowledge, there have been no reports of tick-borne *C. burnetii* infections in Korea. However, several cases of tick-borne *C. burnetii* infections have been reported worldwide. For instance, Eklund et al. published a case of Q fever probably contracted by exposure to ticks in a natural setting. In their study, a 24-year-old man showed symptoms of Q fever after crushing ticks from his clothes with his hands. After the manifestation of symptoms, a blood test was performed, and he was diagnosed with the Q fever infection. Epidemiologically, he had no other source of infection but ticks. With this epidemiological finding, the authors concluded that he was infected with *C. burnetii* by contact with ticks (8). Rolain et al. reported concomitant or consecutive *C. burnetii* infection and tick-borne diseases. They detected Q fever serologically and introduced a case of concomitant *C. burnetii* infection and other *ricketssial* infections by tick contact (24). However, they did not check the ticks or compare the *Coxiella* species identified in the patients and those in ticks. The patient in our study did not seem to be infected by *C. burnetii* transmitted by ticks. It was also noted that two of the ticks studied carried more than one bacterium in addition to CLB. 2018-505-tick 1 carried both *Rickettsia* and

Coxiella and 2018-505-tick 3 carried *Rickettsia, Anaplasma*, and *Coxiella.* Concomitant infection of *C. burnetii* and other *Rickettsia* bacteria is possible (24). If a patient is infected with *C. burnetii* by ticks, infection with other *Rickettsia* bacteria and *Anaplasma* bacteria as well is very likely. However, in our study, the patient was serologically positive only to Q fever. This fact supports the idea that he was not infected with *C. burnetii* through ticks, but from some other source. If the infection was due to tick contact, it is possible that the patient would have been infected with other bacteria as well.

Coxiella strains isolated from the patient and ticks were also different. We performed PCR on the four ticks collected and compared the results with the PCR result of the patient. Three ticks and the patient were positive for *Coxiella*-specific 16S rRNA N-PCR. As Eklund et al. concluded that *C. burnetii* infection was transmitted to the patient by tick contact, we could have concluded that the patient in our study was infected with *C. burnetii* by tick contact (8). However, after analyzing the phylogenetic data, we concluded that the present infection was not tick-borne. Even though the patient and the three ticks were positive in *Coxiella*-specific 16S rRNA N-PCR, the bacteria isolated from the patient and the three ticks were different. The bacterial isolate from the blood of the patient was *C. burnetii*, which causes Q fever. However, the three ticks merely harbored CLB. These findings do not support the idea that the patient was infected with *C. burnetii* by tick bite, even though ticks were found on his body.

Conclusion

We describe a case where a patient was infected with *C. burnetii* and manifested symptoms of Q fever. *Coxiella*-specific 16S rRNA N-PCR was performed on the patient and four ticks were collected from the suspected location of the tick bite. Three of the ticks and the patient were positive to *Coxiella*-specific 16S rRNA N-PCR. To ascertain whether the tick bite was the source of infection, phylogenetic testing was carried out. While the patient was infected with *C. burnetii*, the three ticks positive to *Coxiella*-specific 16S rRNA N-PCR merely harbored CLB. This suggests that the strains were different between *C. burnetii* isolated from the patient and the CLB in *Haemaphysalis*. We conclude that the patient with Q fever was not infected with *C. burnetii* by tick bites.

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics in Human Research Committee of Chosun University Hospital (IRB No. 2013-10-001-018). The patient provided written informed consent to participate in the study.

Consent for publication

Not applicable.

Availability of data and materials

Data and materials are available upon request to the corresponding author.

Competing interests

The authors declare that they have no competing interests.

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Bibliography sketch

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Tables

Table 1. Oligonucleotide primers and PCR conditions

Species	Target gene[a]	Primer name	Sequence (5′-3′)	Annealing temp (°C)	Size (bp)	Reference
Coxiella burnetii	*IS1111*	IS111F1	TACTGGGTGTTGATATTGC	52	485	(11)
		IS111R1	CCGTTTCATCCGCGGTG			
		IS111F2	GTAAAGTGATCTACACGA	56	260	
		IS111R2	TTAACAGCGCTTGAACGT			
	16S rRNA	16s1s-tF0	AAGAGTTTGATTCTGGCTCAG	56	1,440	This study
		16s1st-R0	AGGTTAGCCTACCCGCTTC			
		Cox16SF1	CGTAGGAATCTACCTTRTAGWGG	56	720	(12)
		Cox16SR1	ACTYYCCAACAGCTAGTTCTCA			
		Cox16SF3	GAGTATGGTAGAGGGAAGTGG	56	790	This study
		Cox16SR2	GCCTACCCGCTTCTGGTACAATT			(12)
Rickettsia species	*ompA*	RR190.70F	ATGGCGAATATTTCTCCAAAAA	50	634	(13, 14)
		RR190.701R	GTTCCGTTAATGGCAGCATCT			
		RR190.70F	ATGGCGAATATTTCTCCAAAAA	54	535	
		RR190.602R	AGTGCAGCATTCGCTCCCCCT			
	23S rRNA	PanR8_F	AGCTTGCTTTTGGATCATTTGG	55	110	(15)
		PanR8_R	TTCCTTGCCTTTTCATACATCTAGT			
		PanR8_P	[5FAM]CCTGCTTCTATTTGTCTTGCAGTAACACGCCA[3BHQ1]			
Borrelia species	*pyrG*	pyrG-1F	ATTGCAAGTTCTGAGAATA	45	801	(16)
		pyrG-1R	CAAACATTACGAGCAAATTC			
		pyrG-2F	GATATGGAAAATATTTTATTTATTG	49	707	
		pyrG-2R	AAACCAAGACAAATTCCAAG			
Anaplasma phagocytophilum	*groEL*	GRO607F	GAAGATGCWGTWGGWTGTACKGC	54	688	(17)
		GRO1294R	AGMGCTTCWCCTTCWACRTCYTC			
		GRO677F	ATTACTCAGAGTGCTTCTCARTG	57	445	
		GRO1121R	TGCATACCRTCAGTYTTTTCAAC			
	ankA	ANK-F1	GAAGAAATTACAACTCCTGAAG	53	705	(18)
		ANK-R1	CAGCCAGATGCAGTAACGTG			
		ANK-F2	TTGACCGCTGAAGCACTAAC	54	664	
		ANK-R2	ACCATTTGCTTCTTGAGGAG			

[a]*IS1111, htpAB*-associated repetitive element; 16S rRNA, 16S ribosomal RNA gene; *ompA,* outer membrane protein A gene; 23S rRNA, 23S ribosomal RNA gene; *pyrG*, CTP synthase gene; *groEL,* heat shock protein gene; *ankA,* ankyrin-related protein gene

Table 2. Follow-up IFA antibody titer assessment in the 60-year-old patient and PCR assays to diagnose Q fever, spotted fever, Lyme disease, and anaplasmosis in patient blood specimen and the four ticks.

Sample	Sampling day	Q fever				Spotted fever				Lyme disease			Anaplasmosis			
		16S rRNA N-PCR	*IS1111* N-PCR	IFA IgG Phase I/II	IFA IgM Phase I/II	PanR8 Q-PCR	*ompA* N-PCR	IFA IgG	IFA IgM	*pyrG* N-PCR	IFA IgG	IFA IgM	*groEL* N-PCR	*ankA* N-PCR	IFA IgG	IFA IgM
Patient	03/19	**P**	N	(−)/(−)	(−)/(−)	N	N	n/d	n/d	N	n/d	n/d	N	N	n/d	n/d
	04/23			n/d	n/d			n/d	n/d		(−)	(−)			(−)	(−)
	04/27			(−)/(−)	(−)/1:512			n/d	n/d		(−)	(−)			(−)	(−)
	05/09			(−)/1:1024	(−)/1:1024			n/d	n/d		(−)	(−)			(−)	(−)
	06/07			(−)/>1:2048	(−)/>1:2048			n/d	n/d		(−)	(−)			(−)	(−)
				Development stage				Microscopic identification			Molecular identification					
Tick1	05/05	**P**	N	Adult female		36.09	**P**	*H. longicornis*			*H. longicornis*		N	N		
Tick2	05/05	N	N	Adult female		n/d	**P**	*H. longicornis*			*H. longicornis*		N	N		
Tick3	05/05	**P**	N	Adult female		n/d	**P**	*H. longicornis*			*H. longicornis*		**P**	N		
Tick4	05/05	**P**	N	Adult female		n/d	N	*H. longicornis*			*H. longicornis*		N	N		

(−): <1:16, n/d: not determined, P: PCR positive, N: PCR negative

Figures

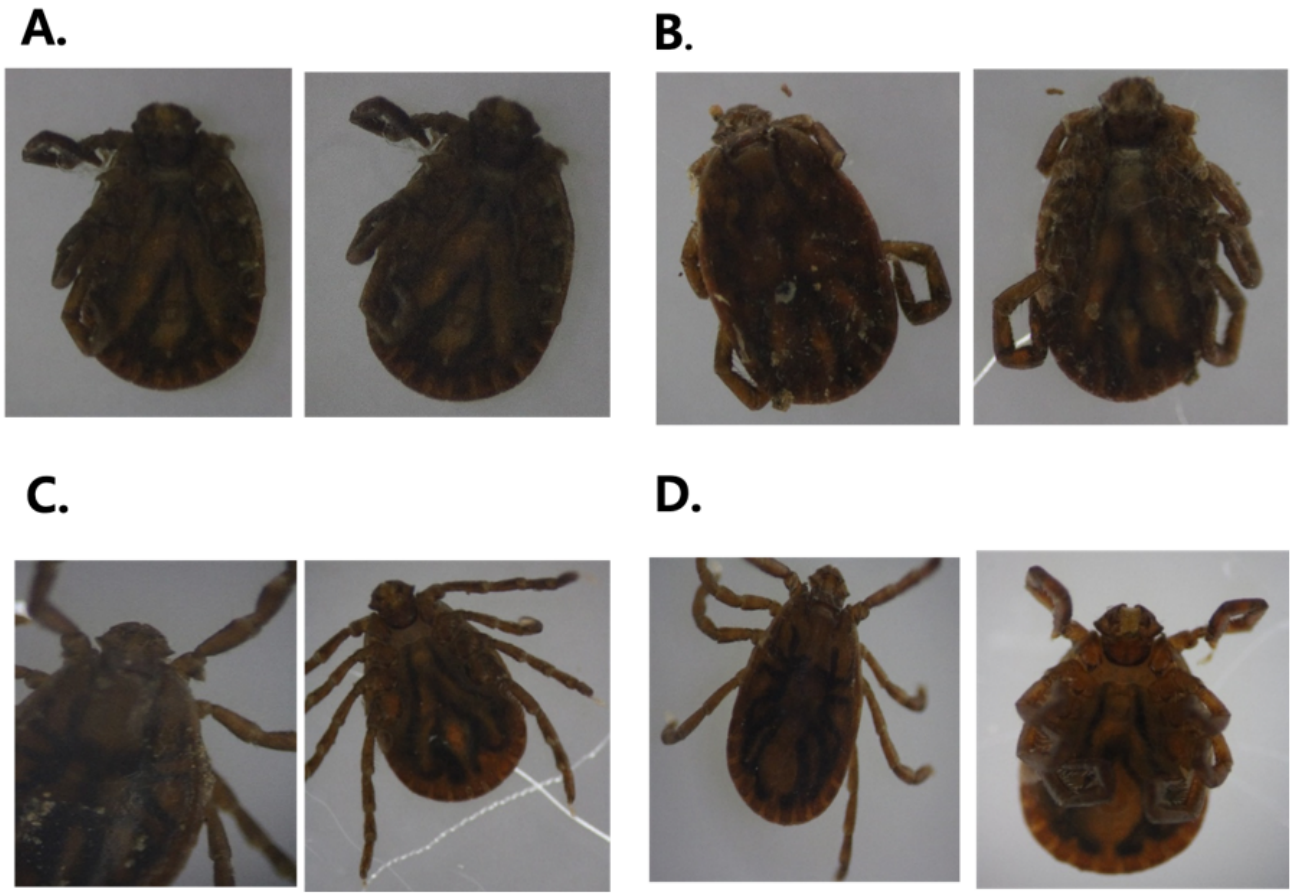

Figure 1

Micrographs of four ticks collected in this study. The images were acquired using a Stemi 508 stereomicroscope (Carl Zeiss Microscopy GmbH, Germany) at 25.6× magnification. The four ticks were found in the workplace of the patient after his hospitalization. A represents 2018-505-tick1. B represents 2018-505-tick2. C represents 2018-505-tick3. D represents 2018-505-tick4. According to morphological classification and molecular identification, the ticks were identified as adult female Haemaphysalis longicornis.

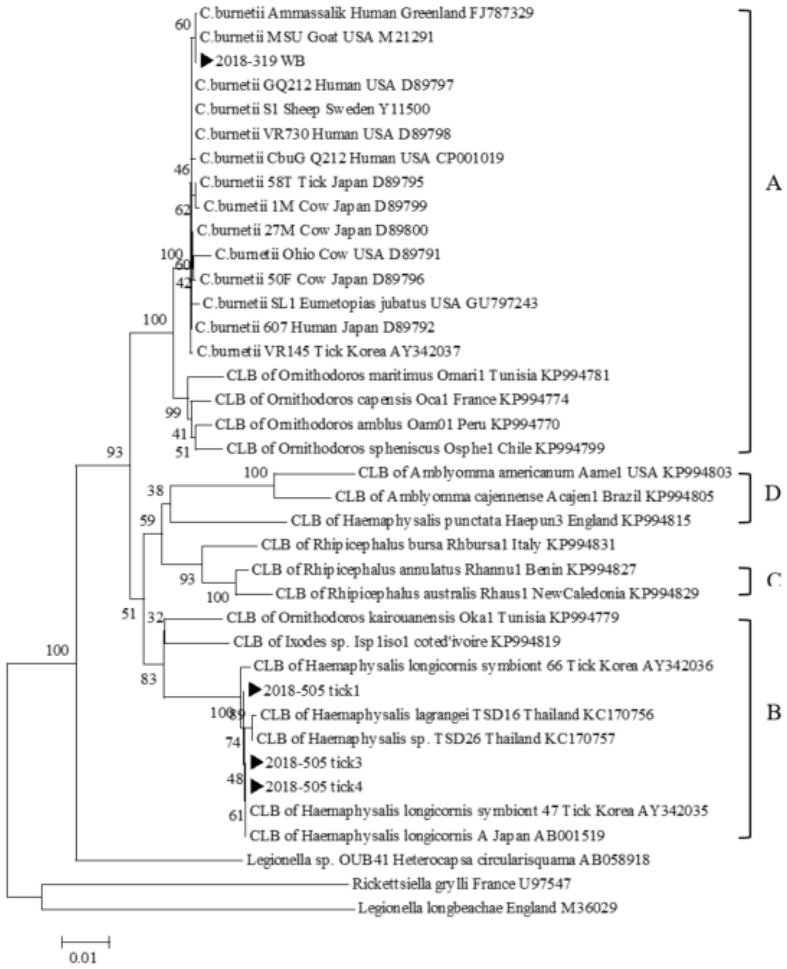

Figure 2

Phylogenetic tree based on partial 16S rRNA sequences (1210 bp) from GenBank and Coxiella-positive patient and three ticks collected in this study (▶). 2018-319 WB represents the bacteria infecting the patient, which was found to be Coxiella burnetii. Further below, the bacteria isolated from the ticks, 2018-505-tick 1, 3, and 4, are indicated. All ticks collected in this study were Haemaphysalis longicornis and they carried Coxiella-like bacteria (CLB), instead of C. burnetii. Coxiella clades (A–D) are indicated at right. Scale bars indicate 0.01 base substitutions per site. GenBank accession numbers are shown in the tree.