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Specific Molecular Detection of *Anaplasma* sp. Closely Related to *Anaplasma phagocytophilum* in Ixodid Ticks and Cattle in a Pastureland in Hokkaido, Japan

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Abstract

Recent molecular analyses of the *Anaplasma* sp. closely related to *Anaplasma phagocytophilum* (previously believed to be *A. phagocytophilum*) in Japan have clarified its distinct phylogenetic position. PCR methods relying on 16S rRNA- and P44/MSP2-based primers designed to detect this species have low sensitivity and specificity. In this study, a highly sensitive and specific nested PCR method using newly designed primers based on heat-shock operon gene (*groEL*) was developed to detect this species. The method was later used in an epidemiological study testing DNA samples from 85 Ixodid ticks (collected by flagging) and 50 cattle from the same pastureland in Nakaosobetsu, Hokkaido, Japan. Results revealed prevalence rates of 2.4% (2 of 85) in ticks and 2% (1 of 50) in cattle. The present study also reported the first molecular detection of the *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan in *H. douglasii*, and established a new reliable PCR method that detects this *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan.

Key Words: *Anaplasma* sp.—*Anaplasma phagocytophilum*—Heat-shock operon—Ticks—Cattle.

Introduction

The phylogenetic position of *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan has been established as distinct from *A. phagocytophilum* (Ybañez et al. 2012). Many research studies in Japan have previously considered this species as *A. phagocytophilum* and have used 16S rRNA-based PCR (Ohashi et al. 2005; Kawahara et al. 2006; Jilintai et al. 2009; Yoshimoto et al. 2010; Masuzawa et al. 2011) and P44/MSP2-based PCR (Ohashi et al. 2005; Wuritu et al. 2009; Murase et al. 2011) for its detection.

16S rRNA based-primers used in previous studies have low specificity that can detect any of the 2 organisms (Jilintai et al. 2009; Yoshimoto et al. 2010). Designing specific primers based on the 16S rRNA gene can be difficult due to a very close homology with *A. phagocytophilum* (Ybañez et al. 2012). On the other hand, P44/MSP2-based primers may also have low sensitivity because those 16S rRNA-based PCR-positive samples have negative results in the PCR assay using the former gene (Masuzawa et al. 2011). P44/MSP2-based PCR may also yield varied nucleotide product lengths due to a hypervariable region found within the gene (Wuritu et al. 2009); hence, relying on visual confirmation after gel electrophoresis of product amplicons maybe questionable without further sequencing. Blood smear examination may also be unreliable. So far, animals found to be PCR positive with this *Anaplasma* sp. were negative in the blood smears (Jilintai et al. 2009; Murase et al. 2011). Thus, molecular detection can be considered the most reliable detection method for this species.

In this study, a heminested PCR method using newly designed primers based on *groEL* was developed to detect *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan. The method was tested in previously tested 16S rRNA-positive samples and in several other *Anaplasma* and *Ehrlichia* species to evaluate its sensitivity and specificity. The method was later used in DNA samples from ticks and cattle.

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Materials and Methods

DNA Samples

Initially, DNA from 20 *Anaplasma* sp. closely related to *A. phagocytophilum*-positive samples that were determined by using 16S rRNA-based analysis was used to evaluate the sensitivity of the new PCR method. This included 10 Sika deer blood samples (*Cervus nippon yesoensis*) from Shizunai, Hokkaido, Japan (Jilintai et al. 2009) and 10 *Ixodes persulcatus* field samples from Sikaoi, Hokkaido, Japan. DNA from the following species were also used to evaluate the specificity of the new PCR method: *Ehrlichia canis* (supplied by Dr. Simonne Harrus, Israel), *Ehrlichia muris* (Tamamoto et al. 2007), *Ehrlichia* sp. from *I. ovatus* (supplied by Dr. H. Fujita, Japan), *A. bovis* (Jilintai et al. 2009), *A. marginale* (Ooshiro et al. 2009), *A. centrale* (Inokuma et al. 2001), *A. platys* (Inokuma et al. 2002), *A. phagocytophilum* from human (formerly human granulocytic ehrlichia, supplied by Dr. P. Brouqui, France), horse (formerly *Ehrlichia equi*, supplied by Dr. P. Brouqui, France), and cattle (supplied by Dr. G. Jouncour, France).

Additionally, ticks and cattle blood were collected from pastureland in Nakaosobetsu, Shibecha, Hokkaido, Japan, in May, 2011. A total of 85 Ixodid ticks, including 35 *Haemaphysalis douglasii*, 37 *I. persulcatus*, and 13 *I. ovatus* collected by flagging and 50 EDTA blood samples from cattle, were used. DNA was extracted using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA), eluted with 200 μL of TE buffer, and stored at −30° until further use.

Primer design, DNA amplification, and purification methods

Primers were designed based on the *groEL* nucleotide sequences of *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan (JN055359, JN055358). The sequences of the designed primers used in this study are indicated in Table 1. In the first phase of the heminested PCR, a final volume of 10 μL was used. It is composed of 4.5 μL of distilled water, 1 μL of 2 mM dNTP,1 μL of 10× PCR buffer, 0.4 μL $MgCl_2$ (50 mM), 0.5 μL of 10 μM of each primer, 0.1 μL *Taq* DNA polymerase (5 U/μL), and 1 μL of DNA template. The step-down cycling conditions were the following: Initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 72°C (with a 2° incremental decrease until reaching final annealing temperature at 65°C) for 30 sec and 72°C for 1.5 min, and final extension at 72°C for 5 min. In the second phase of the heminested PCR, a final volume of 25 μL was set. It was composed of 15.35 μL of distilled water, 2.5 μL of 2 mM deoxyribonucleotide triphosphates (dNTP), 2.5 μL 10×PCR buffer, 1 μL of $MgCl_2$, 1.25 μL of 10 μM of each primer, 0.1 μL of 5 units Taq polymerase, and 1 μL of the first PCR amplicon. The same cycling conditions as the first PCR were used. The negative and positive controls used were distilled water and *Anaplasma* sp. closely related to *A. phagocytophilum* from Sika deer in Japan (Ybañez et al. 2012), respectively. Amplification products were visualized using 1.5% agarose gel after migration, and were purified using either QIAquick PCR purification kit (QIAGEN, USA) or QIAquick Gel Extraction Kit (QIAGEN, USA).

Sensitivity and specificity of the PCR method

To evaluate its sensitivity, the *groEL*-based PCR method was tested in 20 *Anaplasma* sp. closely related to *A. phagocytophilum*-positive samples (using 16S rRNA) from Sika deer (*Cervus nippon yesoensis*) (Jilintai et al. 2009) and Ixodid tick samples. Random sequencing of 2 amplicons was performed to confirm positivity. DNA from an infected deer (Ybañez et al. 2012) was serially diluted using double-distilled water until 1×10^{-8}, and was used as template in the 16S rRNA-based standard PCR using the internal primers and in the *groEL*-based standard PCR using internal primer and first outer reverse primer. 16S rRNA-based nested PCR and *groEL*-based heminested PCR were also performed using the same template. To evaluate specificity, these methods were tested using DNA from 3 *A. phagocytophilum* strains. The *groEL*-based method was further tested in 7 other *Ehrlichia* and *Anaplasma* species.

Sequencing and analyses

A direct sequencing method was performed using the same PCR internal primers. In cases where the sequence result was short, the amplicons were cloned into a vector using TOPO TA cloning (Invitrogen, USA) and sequenced using the primers provided with the product. Nucleotide sequence results were initially checked using BLAST for comparison to other known sequences. Percent identities were computed using EMBOSS pairwise alignment (using the local method) hosted by the European Bioinformatics Institute (www.ebi.ac.uk/Tools/emboss/align/index.html). Gaps were not considered in the final computation of % identities. The multiple alignment analysis was performed using MUSCLE program (Edgar, 2004) using the default parameters (also hosted by the European Bioinformatics Institute Website). Phylogenetic analyses were performed using a neighbor-joining method- maximum composite likelihood substitution

Table 1. Oligonucleotide Sequences of Primers Used

Primer Name	*Oligonucleotide (5′(3′)*	*Reference*
groEL		
APJgR-324-F1	TGGGTCTGACATTGTAAGTATTCGG	This study
APJGr-364F2	AGCAAAAGAGGCGGTACTATCATCTCTG	This study
AnagroE712R	CCGCGATCAAACTGCATACC	Ybanez et al.,in press
16S rRNA		
EC12A	TGATCCTGGCTCAGAACGAACG	Kawahara et al. 2006
EC9	TACCTTGTTACGACTT	Kawahara et al. 2006
SSAP2f	GCTGAATGTGGGGATAATTTAT	Kawahara et al. 2006
SSAP2r	ATGGCTGCTTCCTTTCGGTTA	Kawahara et al. 2006

model and a maximum likelihood method using the MEGA software version 5.05 (Tamura et al, 2011). The tree stabilities were estimated by bootstrap analysis for 1000 replications. Separate analyses using translated amino acid sequence characters were performed for comparison.

Nucleotide accession numbers

The nucleotide accession numbers of sequences used for comparison are indicated beside the organism's name (Fig. 1). *groEL* sequences of *Anaplasma* spp. obtained in this study were registered at GenBank with accession numbers JQ317186 (from *H. douglasii*) and JQ317185 (from Sika deer).

Results

All samples that were found to be positive using the 16S rRNA-based PCR (Kawahara et al. 2006) were also found to be positive with the newly developed heminested PCR method (based on *groEL*). Direct sequencing from 2 randomly selected amplicons from the positive samples resulted in the targeted 228-bp nucleotide. All sequences revealed

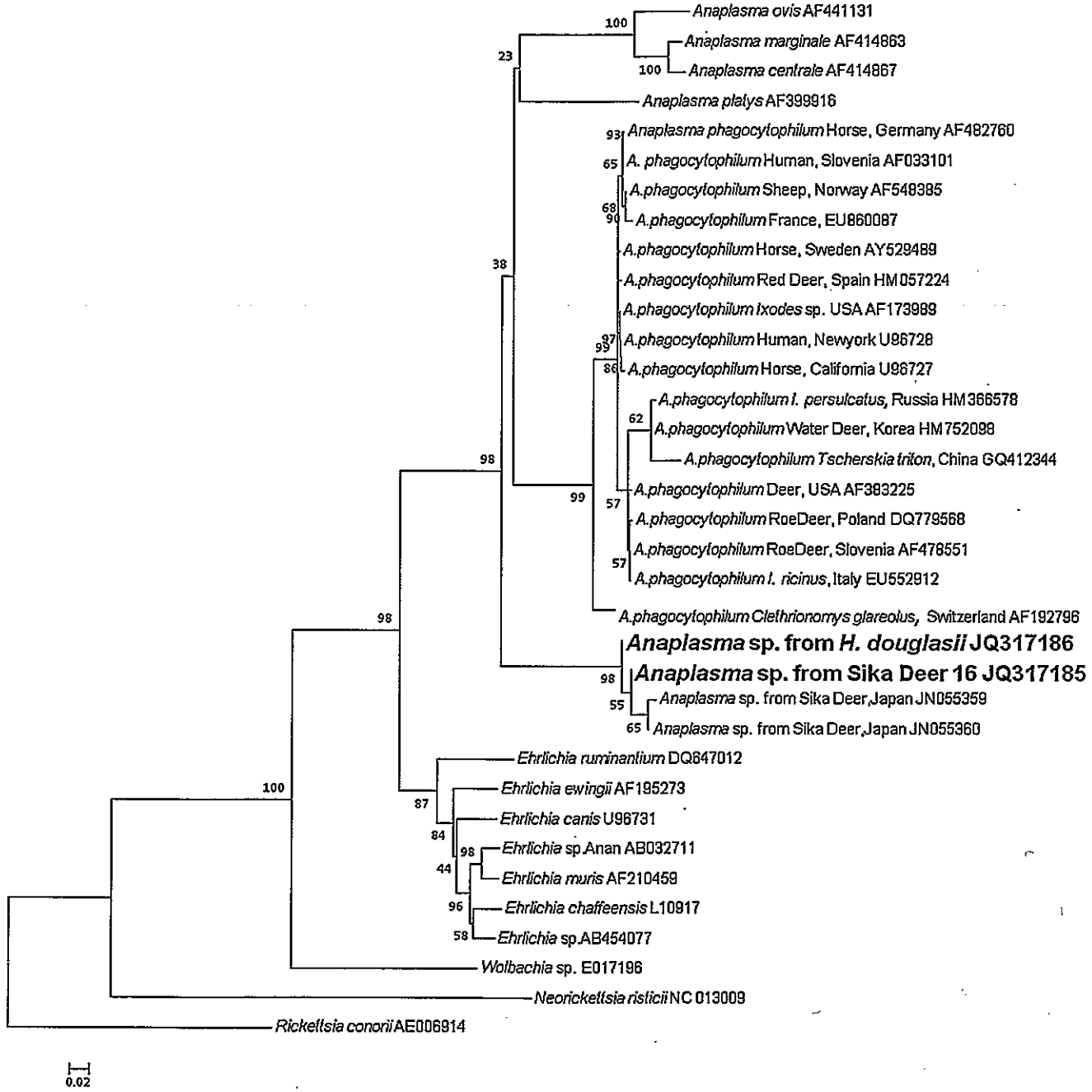

FIG. 1. Phylogenetic relationship of *Anaplasma* sp. from *Haemaphysalis douglasii* from Nakaosobetsu, Hokkaido, Japan, and Sika deer from Shizunai, Japan, with other Anaplasmataceae organisms based on *groEL*. The tree was analyzed using nucleotide sequences by neighbor-joining method(pairwise deletion) and supported by 1000 bootstrap replications. *Rickettsia conorii* was used as an outgroup.

99.6% (1-bp difference) homology with JN055360 (*Anaplasma* sp. closely related to *A. phagocytophilum* from Sika deer in Japan). For the *groEL*-based PCR, serial dilution assays showed a sensitivity limit of 1×10^{-3} for the standard PCR, and at least up to 1×10^{-8} for the heminested protocol (all of which demonstrated strong bands). On the other hand, 16S rRNA-based PCR only had sensitivity limits of 1×10^{-2} and 1×10^{-4} for the standard and nested PCR assay, respectively (Fig. 2).

The *groEL*-based PCR method was only positive in the *Anaplasma* sp. closely related to *A. phagocytophilum*, whereas the 16S rRNA-based PCR was positive in all these strains (Fig. 3). Moreover, the *groEL*-based PCR method was also negative in all other species of *Ehrlichia* and *Anaplasma* species used in this study (data not shown).

Testing the DNA samples obtained from Nakaosobetsu, Hokkaido, Japan, revealed prevalence rates of 2.4% (2 of 85) and 2% (1 of 50) in Ixodid ticks and cattle, respectively. The positive tick samples were 1 adult male and 1 nymph identified as *H. douglasii*. Sequencing of the strongest positive amplicon (from *H. douglasii* adult male) after cloning showed 228-bp nucleotide results that were 99.1% (2-bp difference) similar with JN055360. Phylogenetic analyses also demonstrated that this sequence clustered with the *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan (JN055359 and JN055360) and not with other *Anaplasma* species. The clade was also supported by a high bootstrap value (Fig. 1). Similar results were also obtained when deduced amino acid sequences and/or maximum likelihood method was employed (data not shown).

Discussion

The newly developed PCR method based on *groEL* appears to be highly sensitive and specific in detecting *Anaplasma* species closely related to *A. phagocytophilum* in Japan as compared to the 16S rRNA-based PCR. The new method detected 100% of all 16S rRNA-positive samples and had a higher detection limit in both the standard and heminested PCR protocol than the 16S rRNA-based PCR. Results indicate that compared with the 16S rRNA-based PCR, the *groEL*-based PCR in the present study is 10 times more sensitive using the standard PCR protocol and at least 1000 times more sensitive using the heminested PCR protocol. The increased sensitivity of the *groEL*-based PCR over the 16S rRNA method needs further investigation and may also be useful in designing *groEL*-based molecular detection methods in other species. Moreover, It was also found to be more specific than

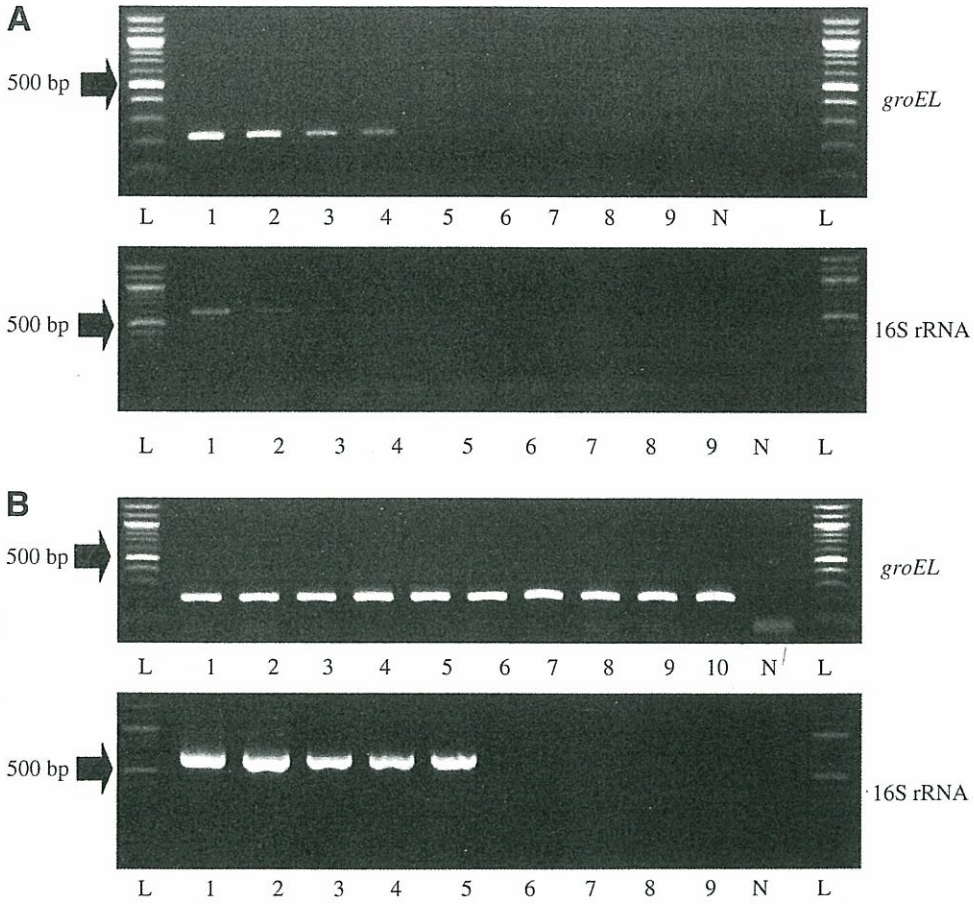

FIG. 2. Sensitivities of the *groEL*- and 16S rRNA-based PCR assay by 10-fold serial dilution of DNA in standard PCR using internal primers (**A**) and heminested and nested PCR (**B**). DNA was obtained from blood of an infected Sika deer (Ybañez et al, in press). Lanes 1–10, represent assays of the same template dilutions of 1, 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, $1-10^{-9}$ for each panel, respectively. L and N represent the DNA ladder and negative control (distilled water), respectively.

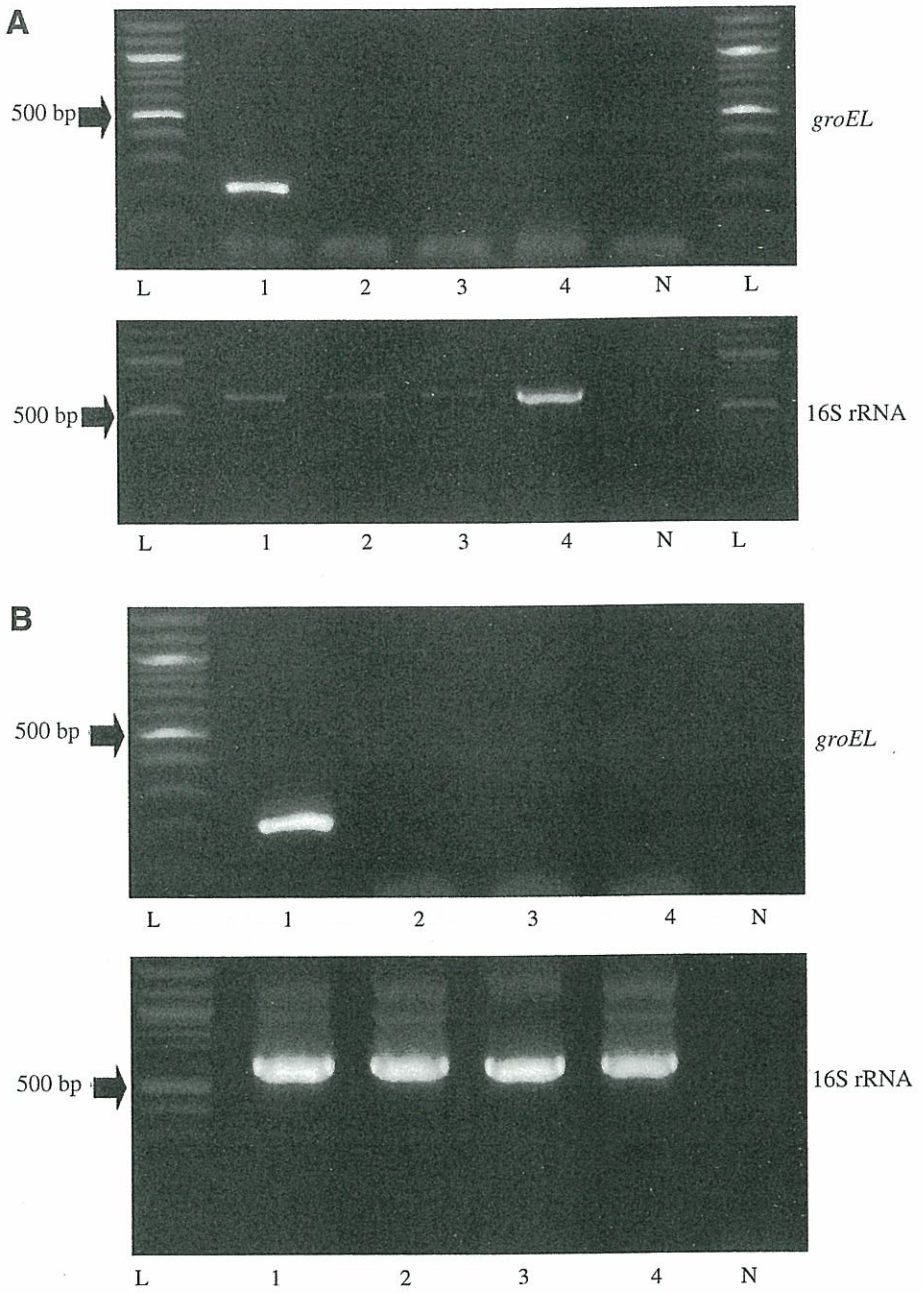

FIG. 3. Specificities of the *groEL*- and 16S rRNA-based PCR assay in standard PCR using internal primers (**A**) and hemi-nested and nested PCR (**B**). Lanes 1–4, *Anaplasma* sp. closely related to *A. phagocytophilum* from infected deer, *A. phagocytophilum* (HGE strain), *A. phagocytophilum* from horse, and *A. phagocytophilum* from cattle, respectively. L and N represent the DNA ladder and negative control (distilled water), respectively.

the 16S rRNA-based PCR because it excluded other *Ehrlichia* and *Anaplasma* species. The use of *groEL* in designing a PCR method appears to be more advantageous because it is highly conserved at the intraspecies level than the P44/MSP2 (Wuritu et al. 2009), and more divergent at the interspecies level than the 16S rRNA gene (Sumner et al. 1997). Several studies on *Anaplasma* have been conducted using this gene (Sumner et al. 1997; Chae et al. 2000; Inokuma et al. 2002a; Inokuma et al. 2002b). Ybañez et al. (2012) also used this gene for the amplification of the *Anaplasma* species closely related to *A. phagocytophilum* in Japan, but its specificity and sensitivity were not evaluated. Moreover, the previous method amplifies the complete *groEL* fragment, whereas the *groEL*-based PCR in the present study amplifies only a partial fragment.

The prevalence rate in cattle (2.0% or 1 of 50) from Nakaosobetsu, Hokkaido, Japan, was within previously reported range of 1.0–3.4% (Jilintai et al. 2009; Murase et al. 2011), while that in Ixodid ticks (2.2% or 2 of 89) was lower than the previous report (7.1% by Murase et al. 2011). Molecular detection of *Anaplasma* sp. closely related to *A. phagocytophilum* in *H. douglasii* is the first report in such species so far. This pathogen has been detected before in *I. persulcatus*, *I. ovatus* (Ohashi et al. 2005) and *H. megaspinosa* (Yoshimoto

et al. 2010). This result provides additional evidence of distinction of this *Anaplasma* sp. in Japan from *A. phagocytophilum* due to a varied range of possible tick vectors.

The *Anaplasma* sp.–positive cattle in this study was asymptomatic, similar to the findings of Jilintai et al. (2009). This is also similar to *A. phagocytophilum* infection in horses, wherein the persistent infection of the pathogen is not associated with any detectable clinical or pathological abnormalities (Franzen et al. 2009). However, the absence of symptoms and negative blood smears in relation to the pathogenesis of the *Anaplasma* sp. closely related to *A. phagocytophilum* needs further study. In conclusion, this study established a new reliable PCR method to detect the *Anaplasma* sp. closely related to *A. phagocytophilum* in Japan.

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Author Disclosure Statement

No competing financial interests exist.

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