FULL PAPER *Bacteriology*

Specific Molecular Detection and Characterization of *Anaplasma marginale* in Mongolian Cattle

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(Received 14 August 2012/Accepted 30 October 2012/Published online in J-STAGE 13 November 2012)

ABSTRACT. *Anaplasma marginale* is an etiologic agent of bovine anaplasmosis. This study aimed to molecularly detect and characterize *A. marginale* that is prevalent in Mongolian cattle populations. A highly specific and sensitive nested PCR (nPCR) method based on the *Msp5* gene was developed to detect *A. marginale* (*Msp5* nPCR). The method detected *A. marginale* from the positive DNA samples obtained from different countries, while no amplicons were observed from DNA samples of several other bovine blood pathogens tested. The detection limit of *Msp5* nPCR was determined to be 2 copies/*μl*. The method was tested against field blood DNA samples prepared from 300 Mongolian cattle in 2010. Results indicated a prevalence rate of 8.7% (26 of 300). On the other hand, partial DNA fragments of an *Anaplasma* sp. closely related to *A. ovis* (with 95.0% identity) were detected using a different nPCR method based on *groEL* gene. The phylogenetic analyses based on the *Msp5, groEL* and 16S rRNA genes demonstrated that *A. marginale* isolates in Mongolia were not divergent from the isolates distributed in other countries. The present study successfully established a new nPCR assay that can detect *A. marginale*, and reported the first molecular detection and characterization of *A. marginale* and an *Anaplasma* sp. closely related to *A. ovis* in Mongolian cattle populations.

KEY WORDS: *Anaplasma marginale*, cattle, Mongolia, *Msp5*, PCR.

doi: 10.1292/jvms.12-0361; *J. Vet. Med. Sci.* 75(4): 399–406, 2013

Anaplasma marginale is a gram-negative, obligate intracellular pathogen belonging to the family Anaplasmataceae, order Rickettsiales [19]. It causes bovine anaplasmosis characterized by a mild to severe hemolytic disease, resulting in a considerable economic loss to the cattle industry [18]. *A. marginale* has a worldwide distribution [1], and together with *Babesia bovis* and *B. bigemina*, it has been considered to be one of the most prevalent and costly tick borne diseases of cattle globally [36].

A. marginale infection in cattle is diagnosed by peripheral blood smear examination (PBSE) [33], serological tests [39], molecular methods [2] and sub-inoculation of blood into splenectomized calf [2]. PBSE has low sensitivity [33], while serological tests are prone to cross-reactions [35]. On the other hand, the method using sub-inoculation of blood is relatively time-consuming and expensive [2]. Therefore, molecular methods like PCR have been the preferred methods in several cross-sectional epidemiological studies.

Common molecular detection methods of *A. marginale* have been based several genes, including 16S rRNA [21, 27, 47], heat-shock protein (*groEL*) [21], major surface protein-1a (*Msp1a*) [20, 24], *Msp4* [12, 24] and *Msp5* [24, 33, 39]. However, evaluation of the previous methods has apparently been limited, because either only few controls for the specificity testing have been used, or the sensitivity detection limits have not been determined. Some genes are also conserved in several *Anaplasma* spp. [45], and thus PCR assay designed on these genes may have lower specificity. Therefore, developing a highly specific and sensitive molecular method would be advantageous in the conduct of epidemiological studies dealing with *A. marginale* infection.

The *Msp5*, a highly conserved 19-kDa protein, is encoded by a single-copy 633-bp gene on the genome of *A. marginale*. Detection of anti-*Msp5* antibody has been recognized to be potentially useful in identifying persistently *A. marginale*-infected cattle [16, 41]. The recombinant *Msp5* of *A. marginale* has been used as a diagnostic antigen for competitive enzyme-linked immunosorbent assay (cELISA) for the detection of bovine anaplasmosis [16, 35, 39]. A nested PCR method based on *Msp5* has also been previously used to detect low levels of rickettsemia in cattle experimentally infected with *A. marginale* [39].

Mongolia is bordered by Russia and China which have already reported the detection of *A. marginale* infection in their respective territories [17, 22, 44]. Thus, there is an apparent risk that the pathogen may have already crossed from the bordering countries. Due to the potential economic loss that can be caused by the pathogen, conducting an epidemiological study dealing with it in Mongolia is beneficial

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Table 1. Nucleotide sequences of primers used in the present study

Primer	Sequence (5' to 3')	bp size	Reference
Msp5			
AM-49F1	GTGTTCCTGGGGTACTCCTATGTGAACAAG	547	This study
AM-595R1	AAGCATGTGACCGCTGACAAACTTAAACAG		This study
AM-211F2	AAGCACATGTTGGTAATATTCGGCTTCTCA	195	This study
AM-376R2	AATTCTCGCATCAAAAGACTTGTGGTACTC		This study
16S rRNA			
fD1	AGAGTTTGATCCTGGCTCAG	~1,500	[28]
Rp2	ACGGCTACCTTGTTACGACTT		[28]
AM-87F	TACGCAGCTTGCTGCGTGTATG	877	This study
AM-963R	GCCCTTCTGTTAAGAAGGATCTAG		This study
groESL			
AMgroES-111F1	AGAGCTCGAAGGAAAGAAGTTCATAGT	1,668	This study
AMgroEL1557R1	CATGAATACAGCTGCR*AGTGACACAGCCA		This study
AMgroES-67F2	TAATCGCTAAGGAGGCGTAGTC	580	This study
AMgroEL513-R2	GTCTTTGCCAACTTCCCTTACGCACTGTG		This study
MSP1a			
MSPa3134R1	TCACGGTCAAAACCTTTGCTTACC	variable	[21]
MSPa733F1	TGTGCTTATGGCAGACATTTCC	variable	[21]
MSPa2957R2	AAACCTTGTAGCCCCAACTTATCC		[21]

*Degenerate primer: R= A or G.

for the surveillance of the disease. Therefore, the present study was endeavored to develop a new PCR method that is highly sensitive and specific in detecting *A. marginale*, and subsequently use this method to determine the molecular prevalence of the pathogen in Mongolian cattle. In addition, the study also aimed to perform molecular characterization and phylogenetic analyses on the detected pathogen based on *Msp5*, 16S rRNA, *Msp1-a* and *groEL* genes.

MATERIALS AND METHODS

DNA samples: A total of 14 DNA samples extracted from 6 bovine blood and 7 *Rhipicephalus (Boophilus) microplus* ticks that were collected in the Philippines (Personal communication) and from 1 bovine blood that was collected in Japan [27], were initially used as the positive control samples to evaluate the newly developed nPCR methods. All of these DNA samples were found to be positive for *A. marginale* using a screening PCR assay based on the 16S rRNA gene [28, 43]. DNA samples extracted from the following species were also used as the negative controls to evaluate the specificities of the developed nPCR methods: *Ehrlichia canis* (supplied by Dr. S. Harrus, Israel), *E. muris* [37] , *Ehrlichia* sp. from *Ixodes ovatus* (supplied by Dr. H. Fujita, Japan), *Anaplasma bovis* [15], *A. centrale* [13], *A. platys* [14], *Anaplasma* sp. closely related to *A. phagocytophilum* of Japan [45], *A. phagocytophilum* from human (formerly human granulocytic ehrlichia, supplied by Dr. P. Brouqui, France), horse (formerly *E. equi,* supplied by Dr. P. Brouqui, France) and cattle (supplied by Dr. G. Jouncour, France), *Theileria orientalis*, *Babesia bovis* and *B. ovata* [34]. Subsequently, a total of 300 field bovine blood DNA samples prepared from Mongolian cattle in May 2010 [3, 4, 34] were also used in the present study.

Development of nPCR methods for A. marginale detection: Initially, nPCR methods based on *Msp5* and *groEL* genes (*Msp5* nPCR and *groEL* nPCR, respectively) were evaluated for the detection of *A. marginale* infection. The oligonucleotide sequences of newly designed primers used in the present study are indicated in Table 1. PCR primers were designed based on several *A. marginale Msp5* (NC012026, NC004842, JF270381, NZABOP01000021, NZABOQ01000016, NZABOR01000017, DQ379973, AY714547, M93392, AY245428, AY527217, AY054384, EF546443, DQ317448, DQ317449 and DQ100450) and *groEL* (CP001079 and CP000030) gene sequences already registered in GenBank. In the first phase of the *Msp5* nPCR, a final volume of 10 μl was set. It was composed of 4.9 μl double distilled water (DDW), 1 μl of 2 mM dNTP, 1 μl of 10 × PCR buffer, 1 μl of 10 pmol of each outer primer (AM-49F1 and AM-595R1), 0.1 μl of 5 units/μl AmpliTaq Gold polymerase (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ, U.S.A.) 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, 74°C (with 2°C incremental decrease until reaching final annealing temperature at 68°C) for 30 sec and 72°C for 1.5 min, and then final extension at 72°C for 5 min. In the second phase, a final volume of 25 μl was set. It was composed of 16.35 μl of DDW, 2.5 μl of 2 mM dNTP, 2.5 μl of 10 × PCR buffer, 1.25 μl of 10 pmol of each inner primer (AM-49F1 and AM-595R1), 0.15 μl of 5 units/μl AmpliTaq Gold polymerase (Applied Biosystems) and 1 μl of the 1st PCR product. The same cycling conditions as the first PCR were used for the second PCR. On the other hand, similar PCR protocol was also set for the partial *groEL* amplification (Outer primers: AMgroES-111F1/ AMgroEL1557R1, inner primers: AMgroES-67F2/ AMgroELGr513R2), except for

the starting and final annealing temperatures (The first PCR: 71–61°C, second PCR: 68–58°C). The amplification products were visualized in 1.5% agarose gel after migration, and then purified using either QIAquick® PCR Purification Kit (Qiagen, Hilden, Germany) or QIAquick® Gel Extraction Kit (Qiagen).

Specificity and sensitivity of the Msp5 and groEL nPCR methods: The *Msp5* and *groEL* nPCR methods were preliminarily tested using 14 control DNA samples that were positive for *A. marginale* as described above. Further evaluations on their specificities were performed using the DNA samples prepared from several other *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* species, which were also mentioned above. Moreover, their sensitivities were also evaluated. From an *A. marginale*-positive DNA sample, longer amplicons were produced by standard PCR using *Msp5* and *groEL* outer primers, ligated into a pCR 2.1 plasmid (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, U.S.A.), and subsequently cloned into One Shot Top10 chemically competent *Escherichia coli* using the TA Cloning kit (Invitrogen). The transformed *E. coli* was grown overnight in a shaker at 37°C in Luria Bertani broth (Invitrogen) supplemented with 1 μl/m*l* of 50 mg/m*l* ampicillin (Wako Pure Chemical Laboratories, Ltd., Osaka, Japan). Plasmids were extracted and purified from the pelleted *E. coli* using QIAprep® Spin Miniprep Kit (Qiagen), and subsequently eluted with 50 μl of purified DDW. Concentrations of the plasmids were adjusted to a starting concentration of 1 $ng/\mu l$ (approximately 2×10^8 copies/μl), and later 10-fold serially diluted using DDW until 10^{-8} for use as DNA templates for the standard PCR (using only the inner primers) and nPCR methods. To evaluate its field applicability, 15 field bovine blood samples that were prepared from the Undurkhan district of Khentii province, Mongolia were tested. DNA sequencing was performed on randomly selected amplicons. Consequently, the nPCR method with better specificity and sensitivity was chosen.

Detection of A. marginale infection in Mongolian cattle: The remaining field blood DNA samples prepared from 285 Mongolian cattle (a total of 300 including the 15 samples tested earlier during the preliminary evaluation of the nPCR methods) were analyzed using the *Msp5* nPCR. The samples were sourced from the following districts: Bogd in Uvurkhangai province, Ulaangom in Uvs province, and Tsenkher-Mandal, Jargalt-Khan, Dadal and Binder in Khentii province in Mongolia [3]. The nucleotide sequences of 10 randomly selected *Msp5* nPCR amplicons were also determined.

Longer Msp5, 16S rRNA and Msp1-a gene amplifications: To obtain a longer DNA fragment of the *Msp5* gene, the inner forward primer (AM-211F2) and outer reverse primer (AM-595R1) were used in the second PCR. The amplification products (414 bp) were visualized in 1.5% agarose gel after migration, and then purified using either QIAquick® PCR Purification Kit (*Qiagen*) or QIAquick® Gel Extraction Kit (Qiagen). Further characterization was attempted by determining the nucleotide sequences of 16S rRNA and *Msp1a* genes detected from the *Msp5*-positive DNA samples. For 16S rRNA gene amplification, the first PCR with outer primers fD1 and Rp2 was performed according to the protocol of Parola *et al.* [28], while the second PCR with inner primers (AM-87F and AM-963F) was done using the same protocol as described above for the *Msp5* gene amplification. The nPCR amplification for *Msp1a* gene was performed according to the protocol of Lew *et al.* [20]. The nucleotide sequences of PCR primers used in the present study are indicated in Table 1.

DNA sequencing and phylogenetic analyses: Direct DNA sequencing method was basically performed using the same PCR primers in the present study. If the sequence result was of low quality, the amplicon was cloned into a plasmid vector using a TOPO TA cloning kit (Invitrogen), and then sequenced using the primers provided with the kit. Nucleotide sequences were initially checked using a BLAST search hosted by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for the comparison with other known nucleotide sequences. Percent identities were computed using an EMBOSS pairwise alignment (using the local method) hosted by the European Bioinformatics Institute (http://www. ebi.ac.uk/Tools/emboss/align/index.html). Gaps were not considered in the final computation of percent identities. The multiple alignment analysis was performed using the MUSCLE program [9] with the default parameters (also hosted by the European Bioinformatics Institute Website). Phylogenetic analysis was performed by neighbor-joining method (maximum composite likelihood substitution model) and maximum likelihood method using the MEGA software, version 5.05 [38]. The tree stability was estimated by a bootstrap analysis for 1,000 replications. Separate analyses using the translated amino acid sequence characters were also performed for their comparison.

Genbank accession numbers for nucleotide sequences: The accession numbers of nucleotide sequences used for comparison are indicated beside the organism's name (Fig. 3). All representative nucleotide sequences obtained in the present study were registered at GenBank and DNA Data Bank of Japan with the following accession numbers: *A. marginale Msp5* genes (Jargalt-Khan: AB703241, JQ735905, Bogd: AB703240, JQ735906), *A. marginale* 16S rRNA genes (Bogd, Ulaangom, Tsenker-Mandal, Jargalt-Khan, Undurkhan: JQ735904), *A. marginale groEL* (Undurkhan: JQ735902), *Anaplasma* sp. closely related to *A. ovis groEL* (Undurkhan: JQ735903).

RESULTS

Msp5-PCR primers (Table 1) were designed, based on the multiple alignments of all available *A. marginale Msp5* gene sequences registered in Genbank. Based on the resulting alignment, there were 25 locations of different base positions in the whole span of the *Msp5* gene, in which American, Carribean, African and Asian isolates differ from each other (data not shown). The nucleotide sequences of the inner primers (AM-211F2 and AM-376R2) were obtained from the conserved region, targeting a 195-bp DNA fragment. The resulting DNA fragment will only have 1–2 bp difference on all the recorded sequences (data not shown). On the other hand, *groEL*-PCR primers (Table 1) were designed based on

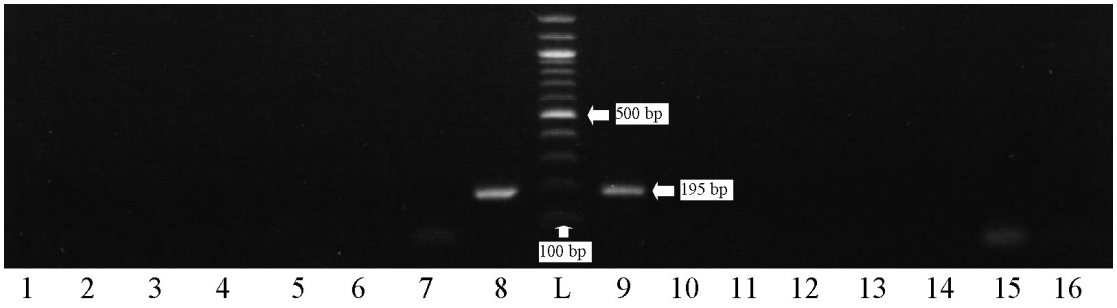

Fig. 1. The specificity of the newly developed *Msp5* nPCR assay. The following is represented in the respective lanes: 1) *Anaplasma phagocytophilum*-equine strain, 2) *A. phagocytophilum*-human strain, 3) *A. phagocytophilum*-bovine strain, 4) *Anaplasma* sp. closely related to *A. phagocytophilum* of Japan, 5) *A. bovis*, 6) *A. centrale*, 7) *A. platys*, 8) *A. marginale* from Philippine cattle, 9) *A. marginale* from Japanese cattle, 10) *Ehrlichia canis*, 11) *E. muris*, 12) *Ehrlichia* sp. from *Ixodes ovatus*, 13) *Theileria orientalis*, 14) *Babesia bovis*, 15) *B. ovata* and 16) negative control (double distilled water). L represents 100-bp DNA ladder marker.

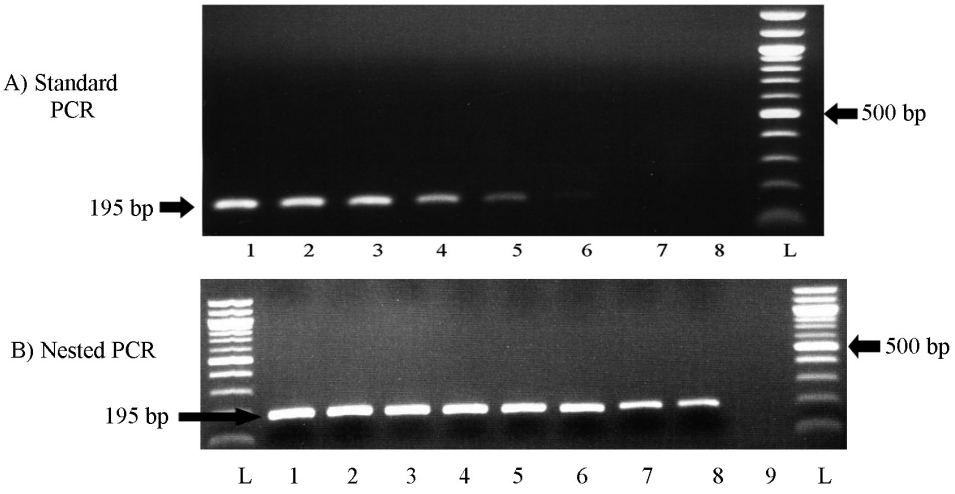

Fig. 2. The sensitivity detection limits of the newly developed *Msp5* nPCR assay (B) by using 10-fold serially diluted plasmid DNA that consists of a 547-bp partial *Msp5* gene fragment. Standard *Msp5* PCR method (A) was also performed using the inner primers. Lanes 1-8 represent assays of the same template dilutions of 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, and 1×10^{-8} for each panel, respectively. Lane 9 in (B) represents the negative control (double distilled water). L represents 100-bp DNA ladder marker.

2 registered *A. marginale* sequences, which covered a portion of the intergenic spacer between the *groES* and *groEL* genes for the forward primers. The *groEL* nPCR targeted a conserved 513-bp, partial *groEL* fragment.

In the preliminary evaluation, all of the 14 *A. marginale* 16S rRNA PCR-positive control DNA samples were found to be positive in the *Msp5* and *groEL* nPCR methods. Moreover, both nPCR methods showed high specificity, since they detected only the *A. marginale*-positive DNA control while excluding all other negative control DNA samples set in the present study (Fig. 1a for *Msp5* nPCR, and figure not shown for the *groEL* nPCR). Further evaluation of the nPCR methods by 10-fold serial dilution sensitivity tests yielded the same detection limits of 2 copies/PCR, respectively (Fig. 2a for *Msp5* nPCR, and figure not shown for the *groEL* nPCR). On the other hand, standard protocol using the inner primers yielded detection limits of 2×10^2 and 2×10^3 copies/PCR for the *Msp5*(Fig. 2) and *groEL* PCR assays, respectively.

Initial testing of the *Msp5* and *groEL* nPCR methods using the 15 field bovine blood DNA samples from the Undurkhan district showed various results. While both nPCR methods detected 1 *A. marginale*-positive sample, the *groEL* nPCR also detected partial *groEL* fragments of an *Anaplasma* sp. closely related to *A. ovis* (513 bp, JQ735903) from 10 other field bovine blood DNA samples from the same district, with 95.0% and 90.0% identities to *A. ovis* (FJ460441 and AF441131) and *A. marginale* (AF414861 and AF41485), respectively. The partial *groEL* nucleotide sequence of the detected *A. marginale* isolate was 99.8% identical to the closest *A. marginale* gene isolates from other countries (Florida,

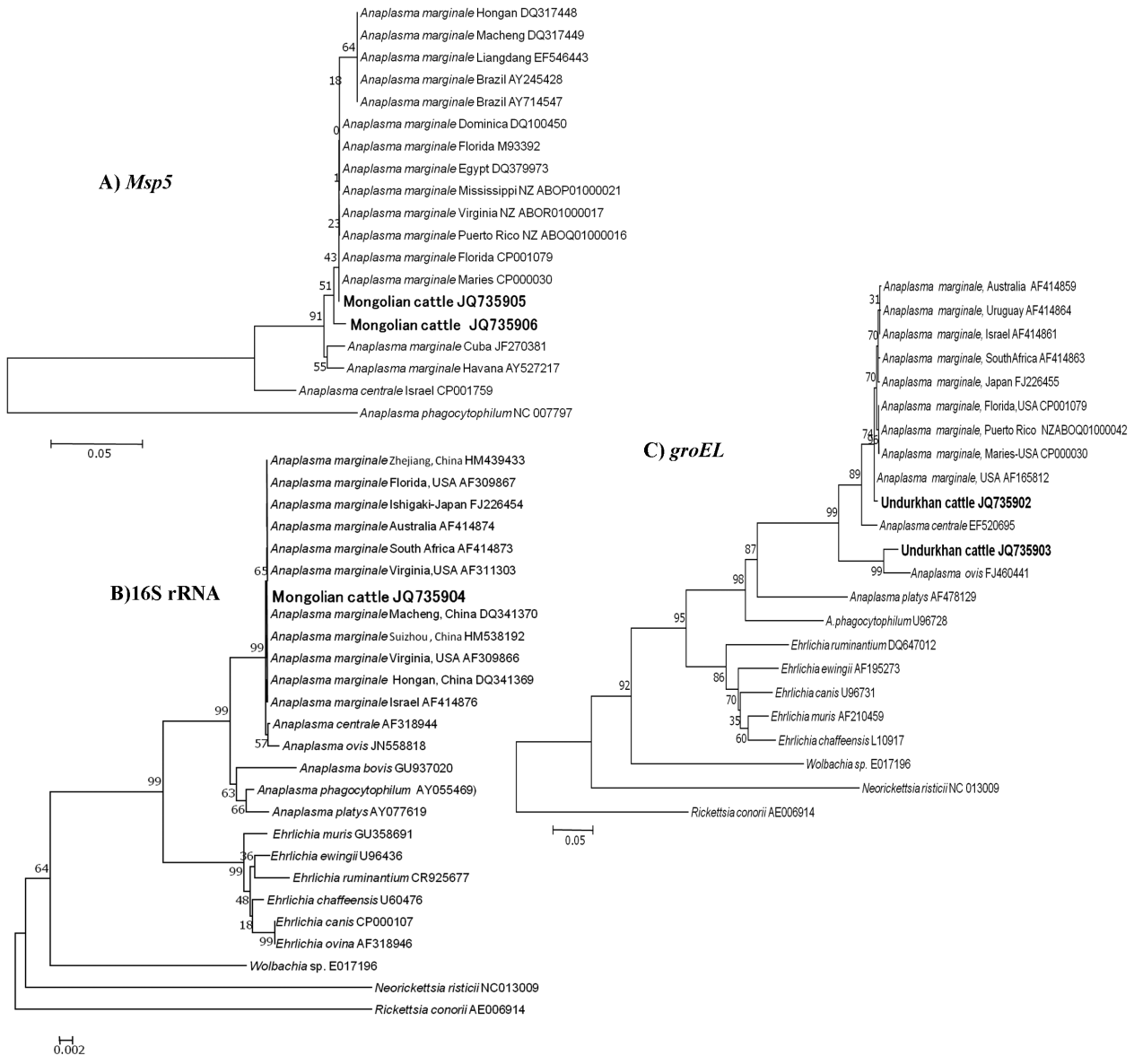

Fig. 3. Phylogenetic relationships of *Anaplasma marginale* with other Anaplasmataceae organisms based on a) *Msp5*, b) 16S rRNA and c) *groEL* genes. The trees were analyzed by neighbor-joining method, supported by 1,000 bootstrap replications. Outgroups used were *A. phagocytophilum* for the *Msp5* gene tree, and *Rickettsia conorii* for the 16S rRNA and *groEL* gene trees, respectively.

US-CP001079, Israel-AF414862 and Australia-AF414860). On the other hand, DNA sequencing of the positive amplicon from the Undurkhan district using the *Msp5* nPCR resulted to the targeted nucleotide sequence (195 bp; AB703241). Therefore, only the *Msp5* nPCR assay was determined to be more reliable.

Testing the field blood DNA samples prepared from Mongolian cattle populations using the *Msp5* nPCR indicated a prevalence rate of 8.7% (26 of 300), as shown in Table 2. The Jargalt-Khan district in the Khentii province had the highest percentage of *A. marginale* infection in cattle (22.6%), while none could be detected from the cattle populations grazed in

Table 2. PCR detection results in Mongolian cattle populations

Province	District	Total no. of samples	Positive samples (%)
Uvurkhangai	Bogd	38	3 (7.9)
Uvs	Ulaangom	50	6 (12.0)
Khentii	Tsenker-Mandal	44	2 (4.5)
	Jargalt-Khan	62	14 (22.6)
	Dadal	45	0 (0.0)
	Binder	46	0 (0.0)
	Undurkhan	15	1 (6.7)
	Total	300	26 (8.7)

the Dadal and Binder districts of the same province (Table 2). The identity among the shorter *Msp5* sequences (195 bp) was determined to be 99.5–100%. From 2 of these samples, longer representative *Msp5* sequences of 414 bp each (Jargalt-Khan: JQ735905, Bogd: JQ735906) were obtained and used for the phylogenetic analyses (Fig. 3A).

PCR amplification and subsequent DNA sequencing of the 16S rRNA gene fragment (877 bp) were performed from 5 *Msp5* nPCR-positive DNA samples (1 from each district). Results of DNA sequences (JQ735904) showed 100% identity to other registered sequences of *A. marginale* isolates, including China, U.S.A., Japan, Australia and South Africa (Fig. 3B). On the other hand, PCR amplification targeting the *Msp1a* showed negative results, despite being positive with *Msp5*, *groEL* and 16S rRNA genes.

Phylogenetic analyses based on the obtained nucleotide sequences of *Msp5,* 16S rRNA and *groEL* genes (Fig. 3A, B, C, respectively) demonstrated that *A. marginale* isolates prevalent in Mongolia clustered with the sequences of *A. marginale* isolated from other countries (China, U.S.A., Japan, Australia, Uruguay, Egypt, Brazil, Dominica, Puerto Rico, Israel and South Africa). The clades were also supported by high bootstrap values. Similar results were also obtained when the deduced amino acid sequences and/or maximum likelihood method were employed in *Msp5* and *groEL* trees (data not shown).

DISCUSSION

In the present study, a newly developed *Msp5* nPCR assay was proven to be highly sensitive and specific for detecting *A. marginale*. Only the *Msp5* nPCR was further utilized because of its high specificity than the *groEL* nPCR (the latter assay also detected an *Anaplasma* sp. closely related to *A. ovis*). The *Msp5* nPCR detected all of the 14 *A. marginale*-positive control samples, and had a high detection limit level (2 copies/PCR), which was 100 times more sensitive than that of the standard PCR assay (200 copies/PCR). These results corroborated with previous observations that the nPCR assay is usually 10–1,000 times more sensitive than the standard PCR assay [23, 26, 39]. Thus, the *Msp5* nPCR developed in the present study was proven to be useful for detecting low levels of *A. marginale* infection. Moreover, the *Msp5* nPCR was found to be highly specific for *A. marginale*. DNA samples from closely related Anaplasmataceae species and other bovine intra-erythrocytic parasites were also tested to ensure that the established nPCR assay could exclude other common organisms, which may confound the PCR results.

The prevalence rate of *A. marginale* in the tested Mongolian cattle populations was determined to be 8.7% (26 of 300). The detection of the pathogen indicates that it may have crossed from the bordering countries due to cattle movement to and from Mongolia, or that the Mongolian cattle might have already harbored their own *A. marginale*. This result provides an evidence of possible endemicity of the pathogen in the country. Previously, only 2 *Anaplasma* species have been reported in Mongolia: *A. ovis* in Mongolian reindeer (*Rangifer tarandus*) [11] and *A. phagocytophilum* in humans [42]. *A. phagocytophilum* is also an important agent of bovine tick-borne disease, which may induce a clinical or subclinical infection in cattle [29, 40]. These 2 species have already been detected in the 2 aforementioned neighboring countries of Mongolia [30, 47].

Detection of the partial *groEL* fragments derived from an *Anaplasma* sp. closely related to *A. ovis* in Mongolia suggests that the possible reservoir, Mongolian reindeer, may already have a close contact or exposure to Mongolian cattle populations. Experimental infection of *A. ovis* did not produce any clinical signs in American bison [46], and there is no report of any clinical bovine anaplasmosis caused by *A. ovis*. However, the *A. ovis* detected in the Mongolian reindeer population appeared to be associated with sudden death and clinical signs like fever, lethargy and pale mucous membranes [11]. Therefore, further studies are essential to characterize the pathogenesis of this *Anaplasma* sp. closely related to *A. ovis* in cattle.

For the *Msp1a* nPCR established by Lew *et al.* [19], not one of the *Msp5* nPCR-positive bovine DNA samples from Mongolia were amplified using the method. The *Msp1a* gene has been used as a stable genetic marker for analyzing the genetic diversity of *A. marginale* isolates, because it is known to exhibit variable tandem repeats [8, 20, 25]. Further studies may be required to establish a new *Msp1a* nPCR assay that can determine the genetic diversity of *A. marginale* isolates, particularly in Mongolia.

Consensus results of the phylogenetic analyses based on *Msp5,* 16S rRNA and *groEL* genes revealed non-divergent positions of *A. marginale* isolates detected in the Mongolian cattle populations. In the *Msp5* and *groEL* phylogenetic trees, the Mongolian *A. marginale* isolates appeared to be closer to U.S.A. isolates. While in the 16S rRNA phylogenetic tree, the Mongolian isolates appeared to be closer to U.S.A. and China. However, the latter observation is not conclusive because of the low bootstrap values supporting the subclades, and of the partial 16S rRNA nucleotide sequence having 100% identity with several other countries. The 16S rRNA gene may not provide a better resolution of phylogenetic trees, as compared to other genes [5, 7, 45]. It will be interesting to include the *Msp5* and *groEL* gene sequences of isolates from Russia and China (which were not available during the time of present study) in the phylogenetic analyses to determine if they will assume a position near those of the Mongolian isolates, since the 2 countries are geographically proximate to Mongolia.

Dermacentor nuttali is the common tick infesting the cattle in Mongolia [3]. The current potential tick vectors of *A. marginale* do not include *D. nuttali* [2]. Therefore, the potential role of *D. nuttali* in the transmission of *A. marginale* and/or searching for appropriate vectors is also highly interesting in Mongolia. Although *Rhipicephalus (Boophilus) microplus*, a tropical cattle tick and a known vector of *A. marginale* [6] is absent, mechanical transmission by arthropods is possible, and is considered to be the major route of dissemination of *A. marginale* in areas where the tropical tick is absent [18].

The detection of *A. marginale* in Mongolia, which has a

temperate climate, corroborated with previous findings that this pathogen can also be found in temperate regions [31]. Moreover, several temperate-regions strains of *A. marginale* have already been identified [10]. In another study, a tropical pathogen, *Babesia bovis*, was also detected in Mongolian cattle populations [4].

The use of *Msp5* appears to be advantageous in designing a PCR method for *A. marginale* detection, since this gene is highly conserved [16]. Several studies on the detection of *A. marginale* have been conducted using the *Msp5*, but most of the primer sequences often have close homologies with *A. centrale, A. ovis* or both, because they have been designed based on a single or limited set of the nucleotide sequences [24, 32, 35, 39]. The *Msp5* primers of the present study were designed based on highly conserved nucleotide sequences, which are found in most, if not all, of the available *A. marginale Msp5* gene sequences registered in GenBank.

In conclusion, the present study successfully established a new *Msp5* nPCR assay that can detect *A. marginale* infection in cattle with high specificity and sensitivity. The first molecular detection and characterization of *A. marginale* is also reported in Mongolian cattle populations. A larger sampling frame covering more provinces and districts may be needed to establish the actual prevalence all throughout the country, since some districts turned out to be negative in the present study. Nevertheless, the prevention and treatment of *A. marginale* infection should now be included in the cattle health management in Mongolia.

ACKNOWLEDGMENTS. We are grateful for the kind cooperation provided by the staff members of the farms and Laboratory of Molecular Genetics, Institute of Veterinary Medicine in Mongolia. In addition, we thank Ms. Hiroko Yamamoto and Ms. Rochelle Haidee D. Ybañez for their excellent technical assistance. This study was supported by a program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN), a grant from the Global COE Program from the Japanese Ministry of Education, Science, Sports, Culture, and Technology, and Grants-in-Aid for Scientific Research from the Japan Society for Promotion of Science, Japan.

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