Specific molecular detection and characterization of *Anaplasma marginale*

in Mongolian cattle

Adrian Patalinghug YBAÑEZ\(^1,2\)), Thillaiampalam SIVAKUMAR\(^3\), Badgar BATTSETSEG\(^4\), Banzragch BATTUR\(^4\), Khukhuu ALTANGEREL\(^3\), Kotaro MATSUMOTO\(^1\), Naoaki YOKOYAMA\(^2,3\), and Hisashi INOKUMA\(^1,2\) *

\(^1\)Department of Veterinary Clinical Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

\(^2\)United Graduate School of Veterinary Sciences, Gifu University, Gifu 501-1193, Japan

\(^3\)National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, Japan

\(^4\)Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Zaisan 210153, Ulaanbaatar, Mongolia

*CORRESPONDENCE TO: INOKUMA, H.

Department of Veterinary Clinical Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080-8555, Japan. e-mail: inokuma@obihiro.ac.jp

RUNNING HEAD: ANAPLASMA MARGINALE IN MONGOLIAN CATTLE
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
Anaplasma marginale is a gram-negative, obligate intracellular pathogen belonging to the family Anaplasmataceae, order Rickettsiales [20]. It causes bovine anaplasmosis characterized by a mild to severe hemolytic disease, resulting in a considerable economic loss to the cattle industry [19]. *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 [37].

*A. marginale* infection in cattle is diagnosed by peripheral blood smear examination (PBSE) [34], serological tests [8], molecular methods [2], and sub-inoculation of blood into splenectomized calf [2]. PBSE has low sensitivity [34], while serological tests are prone to cross-reactions [36]. 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 [22, 28, 47], heat-shock protein (*groEL*) [22], major surface protein-1a (*Msp1a*) [21, 25], *Msp4* [13, 25], and *Msp5* [8, 25, 34]. 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 [17, 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 [8, 17, 36]. 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* [8].

Mongolia is bordered by Russia and China which have already reported the detection of *A. marginale* infection in their respective territories [18, 23, 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 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 [28], 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 [29, 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* [38], *Ehrlichia* sp. from *Ixodes ovatus* (supplied by Dr. H. Fujita, Japan), *Anaplasma bovis* [16], *A. centrale* [14], *A. platys* [15], *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 \textit{E. equi}, supplied by Dr. P. Brouqui, France) and cattle (supplied by Dr. G. Jouncour, France), \textit{Theileria orientalis}, \textit{Babesia bovis}, and \textit{B. ovata} [35]. Subsequently, a total of 300 field bovine blood DNA samples prepared from Mongolian cattle in May 2010 [3, 4, 35] were also used in the present study.

\textbf{Development of nPCR methods for \textit{A. marginale} detection:} Initially, nPCR methods based on \textit{Msp5} and \textit{groEL} genes (\textit{Msp5} nPCR and \textit{groEL} nPCR, respectively) were evaluated for the detection of \textit{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 \textit{A. marginale} \textit{Msp5} (NC012026, NC004842, JF270381, NZABOP01000021, NZABOQ01000016, NZABOR01000017, DQ379973, AY714547, M93392, AY245428, AY527217, AY054384, EF546443, DQ317448, DQ317449, DQ100450) and \textit{groEL} (CP001079, CP000030) gene sequences already registered in GenBank. In the first phase of the \textit{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 x 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 seconds, 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 x 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 \textit{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).

\textit{Specificity and sensitivity of the Msp5 and groEL nPCR methods:} The \textit{Msp5} and \textit{groEL} nPCR methods were preliminarily tested using 14 control DNA samples that were positive for \textit{A. marginale} as described above. Further evaluations on their specificities were performed using the DNA samples prepared from several other \textit{Ehrlichia}, \textit{Anaplasma}, \textit{Babesia}, and \textit{Theileria} species, which were also mentioned above. Moreover, their sensitivities were also evaluated. From an \textit{A. marginale}-positive DNA sample, longer amplicons were produced by standard PCR using \textit{Msp5} and \textit{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 \textit{Escherichia coli} using the TA Cloning kit (Invitrogen). The transformed \textit{E. coli} was grown overnight in a shaker at 37°C in Luria Bertani broth (Invitrogen) supplemented with 1 µl/ml of 50 mg/ml ampicillin (Wako Pure Chemical Laboratories, Ltd., Osaka, Japan). Plasmids were extracted and purified from the pelleted \textit{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/µl (approximately 2 x 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. [29], 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. [23]. 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 % identities. The multiple alignment analysis was performed using the MUSCLE program [10] 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 [39]. 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 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 $2 \times 10^2$ and $2 \times 10^3$ 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, 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 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, USA, 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 (Figs. 3A, 3B, and 3C, respectively) demonstrated that A. marginale isolates prevalent in Mongolia clustered with the sequences of A. marginale isolated from other countries (China, USA, 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 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 [8, 24, 27]. 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) [12] 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 [30, 40]. These 2 species have already been detected in the 2 aforementioned neighboring countries of Mongolia [31, 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 [12]. 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. [22], 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 [9, 22, 26]. 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 USA isolates. While in the 16S rRNA phylogenetic tree, the Mongolian isolates appeared to be closer to USA 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 [19].

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 [32]. Moreover, several temperate-regions strains of *A. marginale* have already been identified [11]. 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 [17]. 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 [8, 25, 33, 36]. 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.

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**REFERENCES**


34. Singh, H., Jyoti, Haque, M., Singh, N. K. and Rath, S. S. 2012. Molecular detection of


42. Walder, G., Lkhamsuren, E., Shagdar, A., Bataa, J., Batmunkh, T., Orth, D., Heinz, F. X.,
evidence for tick-borne encephalitis, borreliosis, and human granulocytic anaplasmosis in
Acad. Sci. 990: 45–53.
45. Ybañez, A. P., Matsumoto, K., Kishimoto, T. and Inokuma, H., Molecular analyses of a
potentially novel Anaplasma species closely related to Anaplasma phagocytophilum
46. Zaugg, J. L. 1986. Experimental anaplasmosis in American bison: persistence of
infections of Anaplasma marginale and non-susceptibility to A. ovis. J. Wildl. Dis. 22:
169-172.
analysis of the genus Anaplasma in Southwestern China based on 16S rRNA sequence.
Figure Legends

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 x 10^{-1}, 1 x 10^{-2}, 1 x 10^{-3}, 1 x 10^{-4}, 1 x 10^{-5}, 1 x 10^{-6}, 1 x 10^{-7}, and 1 x 10^{-8} for each panel, respectively. Lane 9 in (B) represents the negative control (double distilled water). L represents 100-bp DNA ladder marker.

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

<table>
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<th>Sequence (5' to 3')</th>
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<td>CATGAATACAGCTGCR*AGTGACACAGCCA</td>
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<td>variable</td>
<td>Lew et al, 2003</td>
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*Degenerate primer: R= A or G
<table>
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<th>Province</th>
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<th>Positive samples (%)</th>
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<td>Dadal</td>
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<td>Undurkhan</td>
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<td><strong>Total</strong></td>
<td><strong>300</strong></td>
<td><strong>26 (8.7)</strong></td>
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Fig. 1. The specificity of the newly developed MspS nPCR assay. The following are 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 x 10^-1, 1 x 10^-2, 1 x 10^-3, 1 x 10^-4, 1 x 10^-5, 1 x 10^-6, 1 x 10^-7, and 1 x 10^-8 for each panel, respectively. Lane 9 in (B) represents the negative control (double distilled water). L represents 100-bp DNA ladder marker.
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.