

Molecular Identification of ‘*Candidatus Mycoplasma haemovis*’ in Sheep with Hemolytic Anemia

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ABSTRACT. We examined the presence of hemoplasmas, hemotropic mycoplasmas, among 11 sheep (*Ovis aries*) with regenerative and hemolytic anemia and found six of them were positive by real-time PCR. The positive samples were then subjected to conventional PCR for direct sequencing of the 16S rRNA gene. Nucleotide sequences of all the positive samples were identified as the 16S rRNA gene of ‘*Candidatus Mycoplasma haemovis*’ by phylogenetic analysis, demonstrating the infections with this particular hemoplasma species in Japan.

KEY WORDS: hemoplasma, mycoplasma, ovine, rRNA, sheep.

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Hemoplasmas are tiny epierythrocytic prokaryotes that lack a cell wall like other mycoplasmas and are susceptible to tetracyclines, but have never been cultured *in vitro*. Infections may lead to hemolytic anemia in animals, but veterinary investigation had been hampered by the lack of appropriate diagnostic procedures. Although most studies relied on cytological identification of the organisms on blood smears, this method has a low diagnostic sensitivity and cannot differentiate the species [4]. Besides, this diagnostic method may misidentify the hemoplasmas as Howell-Jolly bodies, since the both appear frequently after splenectomy, associate with anemia, and contain DNA. Currently only two hemoplasma species, *Mycoplasma ovis* (previously known as *Eperythrozoon ovis*) [5] and ‘*Candidatus Mycoplasma haemovis*’ [3] are recognized in sheep (*Ovis aries*). Although *M. ovis* infection among sheep is prevalent throughout the world, ‘*Candidatus M. haemovis*’ has not been detected in Japan so far. In the present study we investigated an outbreak of infection with ‘*Candidatus M. haemovis*’ in anemic sheep on an experimental farm in Iwate University, Morioka (latitude 39.7N and longitude 141.1E), Japan.

The disease manifested between October and December of 2010, and mainly affected yearlings, which were purchased from a commercial farm in the Iwate prefecture. Hematological examination was carried out on 11 animals with mild regenerative anemia and blood smears were prepared for Giemsa staining. Small, coccoid, epicytular parasites were detected on erythrocytes by microscopic examination of the Giemsa-stained blood smears (Fig. 1). Hematocrit, hemoglobin, and red blood cells values of these animals were 9.6 to 25.7%, 2.5 to 7.1 g/dl, and 350 to 800 ×

10⁴/dl, respectively, being below the normal ranges.

Total DNA was extracted from 200 μ l EDTA-anticoagulated blood samples collected from sheep using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Negative controls consisting of 200 μ l phosphate-buffered saline were prepared with each batch. Extracted DNA samples were stored at –20°C prior to use. To detect the hemoplasmas in real-time PCR, specific primers for the 16S rRNA gene were originally designed. Forward primer Hemo-F, 5’-TCCATA TTCCTACGGGAAGCAA-3’, and reverse primer Hemo-R, 5’-TACCCCTTGATTAAGTCTAA-3’. Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex Taq (Code #RR041A, TaKaRa Bio, Shiga, Japan). The reaction mixture contained 1 μ l of each primer (10 pmol/ μ l), 12.5 μ l of

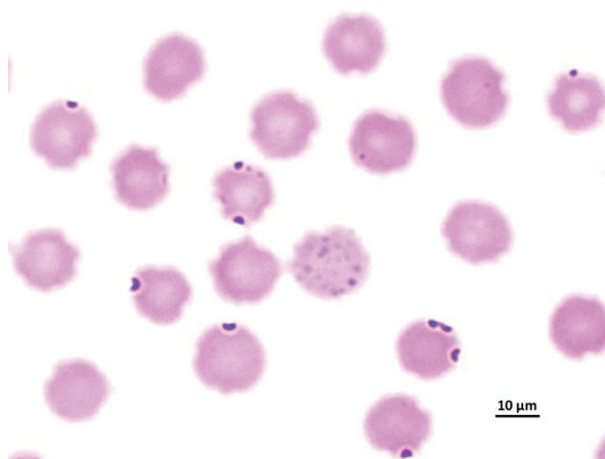


Fig. 1. Giemsa-stained blood smear from an affected sheep with ‘*Candidatus M. haemovis*’. Hemotropic mycoplasma cells were seen on the surface of erythrocytes.

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2× premix reaction buffer and water to volume of 23 μ l. Finally, 2 μ l of DNA samples as templates were added to this mixture. Amplification was achieved with 40 cycles of denaturation at 95°C for 5 sec, renaturation at 57°C for 20 sec, and elongation at 72°C for 15 sec, after the initial denaturation at 94°C for 30 sec. Fluorescence readings in a channel for SYBR Green I were taken throughout the experiments.

After real-time PCR, melting experiment was performed from 60 to 95°C at 0.2°C/sec with smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (T_m) as described previously [2]. The T_m was defined as a peak of the curve, and if the highest point was a plateau, then the mid-point was identified as the T_m . The input amount of DNA, the copy number of the target as well as presence of co-infections with several targets did not influence the T_m . Since nucleotide sequences and sizes bracketed by the primers are specific to species, melting curve analysis of the amplified products may serve as a differential marker for hemoplasma speciation. Six ovine blood samples, Hitsuji1, Hitsuji5, Hitsuji6, Hitsuji7, Hitsuji8 and Hitsuji9, were pos-

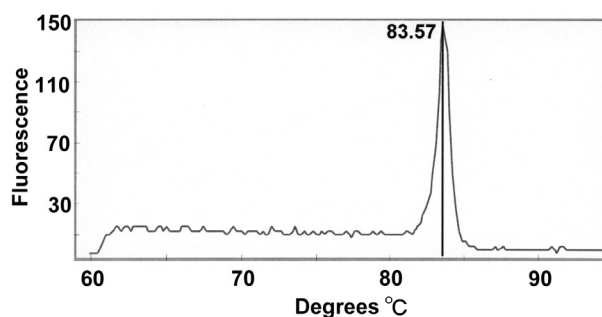


Fig. 2. Thermal melting curve of the PCR products depicted by using SYBR Green I. A characteristic melting temperature at 83.57°C was evident in '*Candidatus M. haemovis*' infection.

itive in the real-time PCR. The T_m (mean \pm SE.) values of the PCR products from these six ovine hemoplasma were estimated to 83.60 \pm 0.09°C (Fig. 2).

The positive samples were further examined by conventional PCR targeting the 16S rRNA gene for nucleotide sequencing. The conventional PCR was carried out with 50-

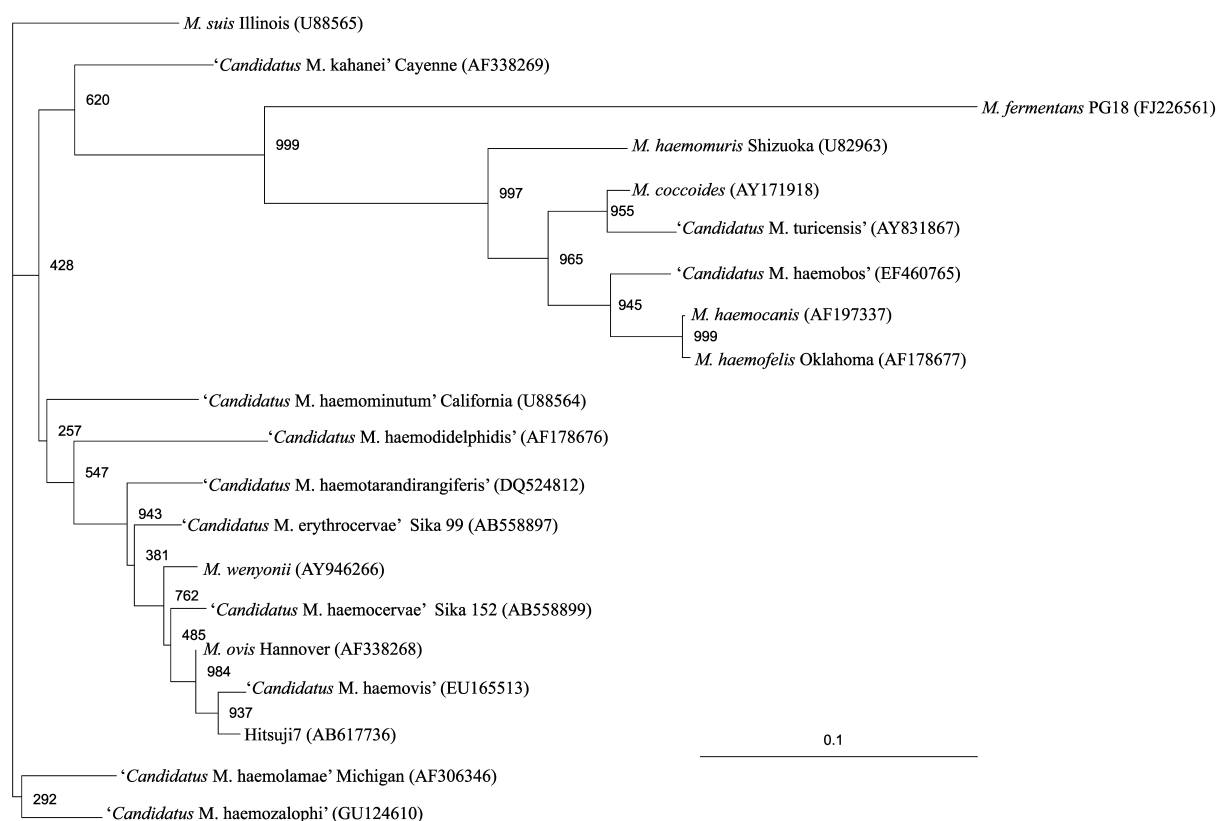


Fig. 3. A phylogenetic tree based on the 16S rRNA gene sequence comparison among mycoplasmas including 18 hemoplasma species (accession numbers are given in a parenthesis). Genetic distances were computed with CLUSTAL W [9]. Ovine strain Hitsuji7 representing Hitsuji1, Hitsuji5, Hitsuji6, Hitsuji8 and Hitsuji9 was included in the '*Candidatus M. haemovis*' clade. A nucleotide sequence of the 16S rRNA gene of *M. fermentans* PG18 strain with accession number FJ226561 was included as an out-group. Numbers in the relevant branches refer to the values of boot-strap probability of 1,000 replications. Scale bar indicates the estimated evolutionary distance.

μ l reaction mixtures containing 1 μ l of DNA solution, 0.5 μ l of TaKaRa LA *Taq*TM (5 units/ μ l), 5 μ l of 10X LA PCRTM Buffer II, 8 μ l of 25 mM MgCl₂ (final 4.0 mM), 8 μ l of dNTP mixture (2.5 mM each), 0.2 μ l (50 pmol/ μ l) of forward primers Ana-F1 (5'-GAGTTTGATCCTGGCT-CAGG-3') or Hemo-F10 (5'-ATATTCCTACGGGAAGCAGC-3'), 0.2 μ l (50 pmol/ μ l) of reverse primers Hemo-R11 or Hemo-R2 (5'-TACCTTGTTACGACTTAAC-3') and water to a final volume of 50 μ l. After the mixture was overlaid with 20 μ l of mineral oil, the reaction cycle was carried out 35 times with denaturation at 94 for 30 sec, annealing at 60 for 120 sec and extension at 72 for 60 sec in a thermal cycler.

The PCR products were fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME, U.S.A.) in TAE (40 mM Tris, pH8.0, 5 mM sodium acetate, 1 mM disodium ethylenediaminetetracetate) buffer at 50 volts for 60 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 μ g/ml) for 15 min and visualized under UV transilluminator. DNA was extracted by using NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany) and was subjected to direct sequencing in a 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, U.S.A.). In our experiments, results between the real-time PCR and conventional PCR were always consistent. The nucleotide sequences of the partial 16S rRNA gene have been deposited in the DDBJ, EMBL, GSDB and NCBI nucleotide sequence databases under the accession numbers AB617733 to AB617738. A single nucleotide substitution was apparent among these six sequences, suggesting a same lineage.

Nucleotide sequences of the 16S rRNA gene from the ovine strain along with the 19 established mycoplasma species were aligned using CLUSTAL W (version 1.83; DDBJ, Mishima, Japan) with further adjustment made manually by eyes as necessary [9]. A phylogenetic tree constructed by the algorithms implemented in the PHYLIP program (DDBJ, Japan) using the neighbor-joining method [7] indicated that the hemoplasma strain detected in sheep was classified as '*Candidatus Mycoplasma haemovis*' (Fig. 3). Besides, the nucleotide sequence '*Candidatus M. haemovis*' detected in the present study were most closely to *Mycoplasma* sp. TX1294-A (accession number GU230141) and TX1294-E (accession number GU383116) strains both detected from humans [8], suggesting an anthroponotic pathogen.

'*Candidatus M. haemovis*' was first demonstrated from a sheep flock with fatal hemolytic anemia in Hungary [3].

Prevalence of this hemoplasma species is currently unknown, because there has been no report on this particular species in other countries though *M. ovis* is prevalent throughout the world [1, 6]. We demonstrated this hemoplasma species in anemic sheep in Japan. Variations in the hematological values might be attributed to infection stages rather than differences in the hemoplasma strains, which were almost identical in the 16S rRNA sequences. It is most likely that the animals had been infected in the commercial farm, since the affected sheep in the present study was the only flock in the experimental farm of the Iwate University.

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