

## A Clinical Case of Severe Anemia in a Sheep Coinfected with *Mycoplasma ovis* and ‘*Candidatus Mycoplasma haemovis*’ in Hokkaido, Japan

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(Received 17 June 2011/Accepted 11 August 2011/Published online in J-STAGE 25 August 2011)

**ABSTRACT.** A 2-year-old East Friesian sheep imported from Australia exhibited severe anemia after contagious pustular dermatitis in Hokkaido, Japan. Hemoplasma infection was confirmed in blood smears. Both *Mycoplasma ovis* and ‘*Candidatus Mycoplasma haemovis*’ were detected by PCR and sequence analyses. In the epidemiological analysis, dual pathogens were detected in 6 of 12 (50.0%) sheep imported from Australia with the infected ewe at the same time, 1 of 5 (20.0%) sheep introduced from a domestic farm in Hokkaido, and in 1 of 16 (6.3%) sheep from an epidemiologically unrelated ranch. It is the first clinical case of sheep to confirm coinfection of these pathogens in Japan.

**KEY WORDS:** ‘*Candidatus Mycoplasma haemovis*’, case report, Japan, *Mycoplasma ovis*, sheep.

doi: 10.1292/jvms.11-0296; *J. Vet. Med. Sci.* 74(1): 99–102, 2012

*Haemobartonella* and *Eperythrozoon* cause infectious anemia in several mammalian species [8, 17]. These pathogens have recently been reclassified as genus *Mycoplasma* on the basis of 16S rRNA gene analysis and morphological similarities [8]. *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) causes ovine hemoplasmosis, which is characterized by poor weight gain, severe anemia, and even mortality in lambs and, occasionally, young adult sheep [1]. On the other hand, chronic sustained infection can occur asymptotically in older sheep [4]. *M. ovis* also causes a more severe disease in goats [7]. Recently ‘*Candidatus Mycoplasma haemovis*’, a new ovine hemoplasma, has been detected in Hungary [5]. This is the first case report to describe *M. ovis* and ‘*Candidatus M. haemovis*’ coinfection in a sheep presenting with severe anemia in Japan.

A 2-year-old East Friesian ewe imported from Australia in July 2009 showed contagious pustular dermatitis a month after import. Because anorexia continued after recovery, a blood test was carried out. Severe anemia with hematocrit of 14% was recorded. Erythrocytes with coccoid parasites on their surface were detected in a blood smear (Fig. 1), which strongly suggested infectious hemoplasma. An EDTA-anticoagulated blood sample was collected from the infected ewe. For the epidemiological study, blood was randomly collected from 12 sheep imported from Australia with the infected ewe at the same time, and were managed at

a same sheep building until an investigation and, 5 sheep introduced from a domestic farm in Hokkaido. Additionally blood samples were also collected from 16 sheep kept in an epidemiologically unrelated ranch. It was geographically apart from the first ranch, and no movement of sheep has been recorded between the two ranches.

Total DNA was extracted from 200  $\mu$ l of blood using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) and stored at  $-30^{\circ}\text{C}$  until use. For screening, PCR amplification was performed in a 25  $\mu$ l reaction mixture containing 5  $\mu$ l of each DNA template and an F2/R2 hemoplasma-specific primer set [6]. Longer 16S rRNA

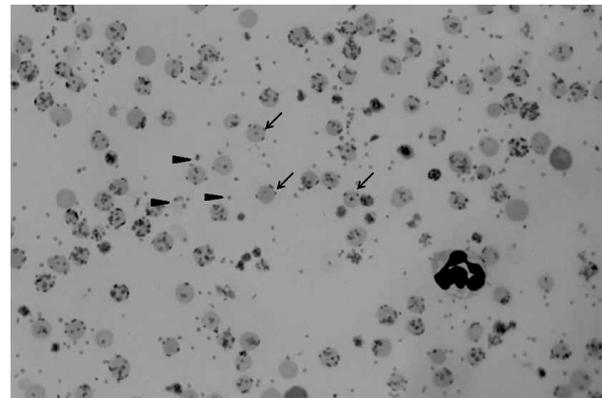


Fig. 1. Giemsa-stained blood smear from the anemic sheep with *Mycoplasma ovis* and ‘*Candidatus M. haemovis*’. Hemoplasmas were detected in plasma (arrow head) and on the surface of erythrocytes (arrow).

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sequences, including the divergent region near the 5-end, were amplified using primers fD1 and Rp2 [16]. Blood samples were also screened for the presence of Anaplasmataceae family members by conventional PCR using primers EHR16SD and EHR16SR as described previously [10]. PCR products were purified using a QIAquick PCR purification kit (QIAGEN). Purified PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen) and transformed into *Escherichia coli* Mach T1 cells. Plasmid sequence analysis was performed as previously described [15]. Sequences were identified using the BLAST search program (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>). Finally, a phylogenetic classification was carried out using CLUSTAL W (DDBJ, <http://clustalw.ddbj.nig.ac.jp/top-j.html>) and TreeView programs (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

Hemoplasma-specific PCR analysis of infected peripheral blood revealed two bands of approximately 200 bp (Fig. 2). Anaplasma-specific PCR was negative. TA cloning was performed and eight long clonal 16S rRNA genes (1314–1374 bp) were obtained. The 16S rRNA gene sequences obtained from clones 1, 2, 3, 6 and 7 were closely related to '*Candidatus M. haemovis*' (EU166531), with 99.19–99.41% identity. But these identities with the *M. ovis* were 96.89–97.10%. Sequences of clones 4, 5 and 9 shared a higher degree of identity (99.42–99.71%) with *M. ovis* (AF166510). And these identity with the '*Candidatus M. haemovis*' were 96.86–97.84%. The phylogenetic analysis of the 8 clones also revealed that '*Candidatus M. haemovis*' and *M. ovis* belong to the same clade (Fig. 3). The accession numbers of hemoplasma clones detected from the present study are as follows; '*Candidatus M. haemovis*': JF931131, JF931132, JF931133, JF931136 and JF931137, and *M. ovis*: JF931134, JF931135 and JF931138.

In the epidemiological analysis, dual bands were detected in 6 of 12 (50.0%) sheep imported from Australia with the infected ewe at the same time, 1 of 5 (20.0%) sheep introduced from a domestic farm in Hokkaido, and in 1 of 16 (6.3%) sheep from an epidemiologically unrelated ranch. All positive samples showed dual bands. The pathogen was not identified in the blood smear of the positive specimen, and no abnormalities were detected in hematocrit or plasma protein level. TA cloning and gene analysis were carried out on positive samples from two randomly selected sheep imported from Australia and one sheep from an unrelated ranch. Bands of 132 bp and 149 bp were detected in the two samples from sheep imported from Australia. In these samples, 8 clones detected from the 132 bp fragment were the most closely related to '*Candidatus M. haemovis*' (EU166531), and the 6 clones from the 149 bp fragment shared 100% identity with *M. ovis* (AF166510). Two bands were also detected in the positive specimen from the unrelated ranch. Six clones detected from one fragment were all completely identical to '*Candidatus M. haemovis*' (EU166531) and 5 clones from the other fragment were most closely related to *M. ovis* (AF166510).

Ovine hemoplasmosis is mainly observed in lambs, and is

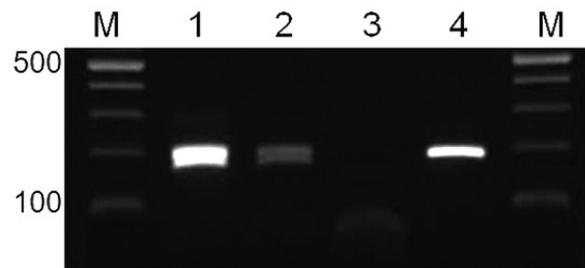


Fig. 2. Representative results of PCR analysis using the hemoplasma-specific F2/R2 primer set. Two bands of approximately 200 bp were detected. Lane 1: infected ewe with anemia; Lane 2: 3-month-old lamb, which lived with the infected ewe; Lane 3: negative control (DW); Lane 4: positive control (*M. haemofelis*); Lane M: molecular size marker.

rare in adults [11]. *M. ovis* is prevalent all over the world, but '*Candidatus M. haemovis*', a new ovine hemoplasma, has only been reported in Hungary [5]. In the present study, both *M. ovis* and '*Candidatus M. haemovis*' were detected in the peripheral blood of a sheep with severe anemia using PCR analysis and TA cloning. This is the first clinical case report to confirm coinfection of sheep with *M. ovis* and '*Candidatus M. haemovis*' in Japan.

Hemoplasmas were observed in both plasma and on the surface of erythrocytes in the Giemsa-stained blood smear from the anemic sheep (Fig. 2). In the blood smear of a sheep infected with 2 different hemoplasma, the pathogens were recorded only on erythrocytes in the previous report [5]. There are no data available to morphologically distinguish the two hemoplasma species; *M. ovis* and '*C. M. haemovis*' at this moment.

Various hemoplasmas have been detected in other animals [12, 13, 18], and pathogenic differences among them have been reported [2, 3, 14]. The phylogenetic analysis revealed that both '*Candidatus M. haemovis*' and *M. ovis* belong to the same clade, which contains lower pathogenic species that infect other animals. However, severe anemia was recorded in the present case, which may have been due to the dual hemoplasma infection. Alternatively, the sheep may have been immunosuppressed because of the contagious pustular dermatitis that preceded anemia. It was also possible that the case might have immunosuppressive diseases before infection of the contagious pustular dermatitis; however, the onset factor of the anemia was undetermined.

Epidemiological analysis revealed a high rate of coinfection by *M. ovis* and '*Candidatus M. haemovis*' in sheep imported from Australia with the infected ewe. It is possible that the sheep had already been infected with these pathogens in Australia, and severe anemia had developed after occurring contagious pustular dermatitis. Dual infection was also detected in a sheep introduced from a domestic farm in Hokkaido, and a sheep from another ranch, suggesting that both *M. ovis* and '*Candidatus M. haemovis*' may already exist in domestic sheep in Japan. Because most of sheep kept in Japan are imported from foreign countries

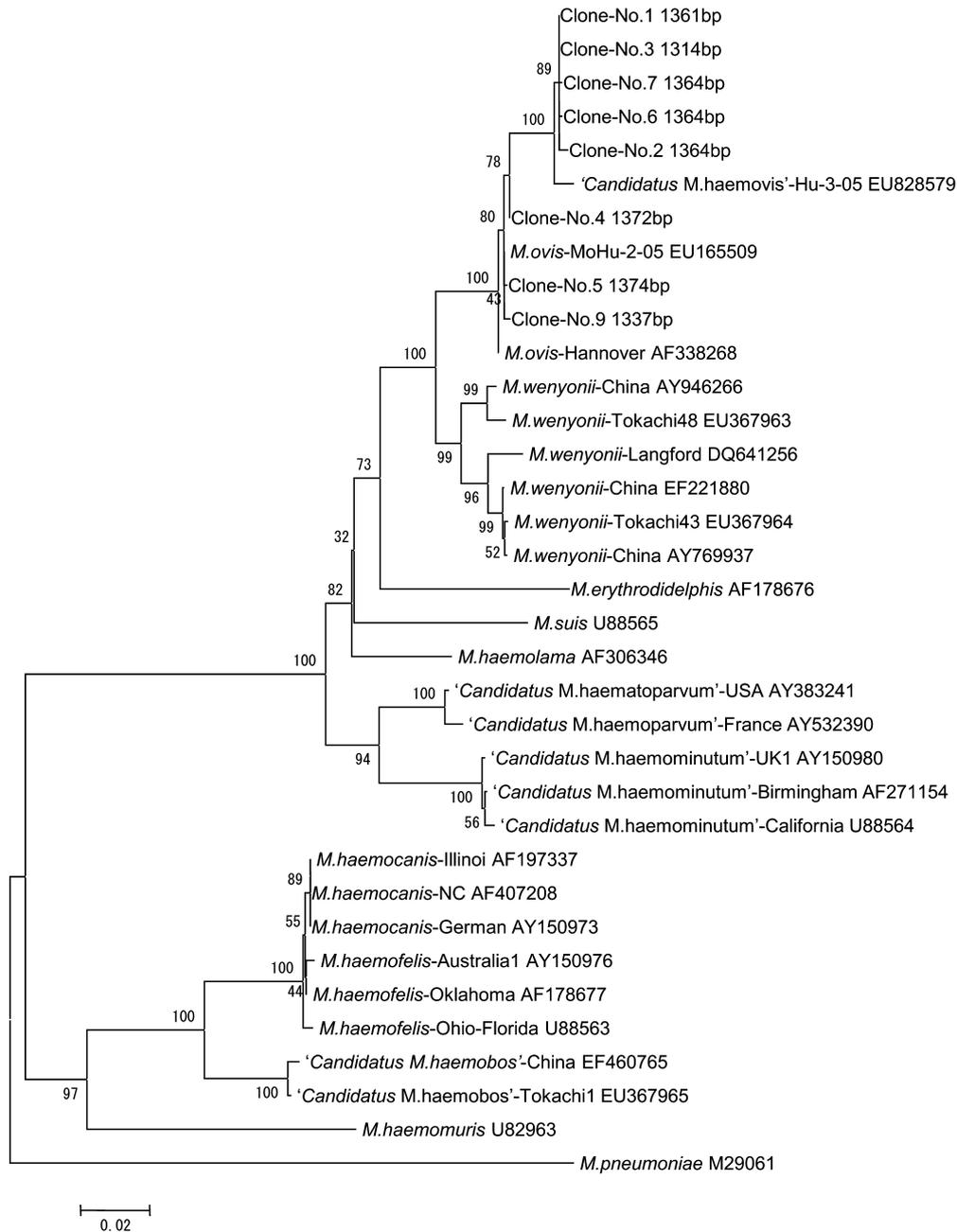


Fig. 3. Phylogenetic relationships among various hemoplasmas based on partial 16S rRNA gene sequences. The neighbor-joining method was used to construct the phylogenetic tree with the Clustal W program. The scale bar represents 2% divergence. Numbers at the nodes are the proportions of 100 bootstrap re-samplings supporting the topology shown. The GenBank accession numbers for the 16S rRNA gene sequences used to analyze percent identities and construct a phylogenetic tree are as follows: *M. wenyonii* strains, China, AY769937, EF221880, and AY946266; Langford, Canada, DQ641256; Tokachi, Japan, EU367964 and EU367963; '*Candidatus M. haemobos*' strains, China, EF460765; Tokachi, Japan, EU367965; *M. ovis* strains, Hannover, Germany, AF338268, Hungary, EU165509; '*Candidatus M. haemovis*' strains, Hungary, EU828579; *M. suis*, U88565; *M. pneumoniae*, M29061; *M. haemolama*, AF306346; *M. erythroidelphis*, AF178676; '*Candidatus M. haemominutum*' strains, UK, AY150980; Birmingham, USA, AF271154; California, USA, U88564; *M. haemofelis* strains, Australia, AY150976; Oklahoma, USA, AF178677; Ohio, Florida, USA, U88563; '*Candidatus M. haemoparvum*' strains, France, AY532390; USA, AY383241; *M. haemocanis* strains, North Carolina, USA, AF407208; Illinois, USA, AF197337; Germany, AY150973; and *M. haemomuris*, U82963.

such as Australia and New Zealand, these pathogens might also be introduced to Japan from imported sheep. As reported previously, *M. ovis* was detected in Japanese serows; *Capricornis crispus* [9], however, the relationship between this wild ruminant and domestic sheep is unknown. A large-scale epidemiological investigation is required to determine the status of hemoplasma infection in domestic sheep in Japan.

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