Molecular Demonstration of Hemotropic Mycoplasmas in Wild Japanese Monkeys (*Macaca fuscata*)

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ABSTRACT. The prevalence of hemotropic mycoplasmas in wild monkeys is largely unknown. Here, we report the presence of hemoplasmas in blood specimens collected from wild Japanese monkeys (*Macaca fuscata*) tentatively captured for ecological survey in Mie prefecture, Japan. We examined 9 monkeys using hemoplasma-specific real-time PCR and found all of them positive for a hemoplasma infection. The 16S rRNA gene and 16S to 23S rRNA intergenic spacer region of the hemoplasma detected in wild monkeys were amplified using end-point PCR. The nucleotide sequences of the PCR products were further determined and compared to those of other hemoplasmas. Our examinations revealed a wide prevalence of a hemoplasma strain in Japanese monkeys, which was similar to 'Candidatus Mycoplasma haemomacaque' reported in cynomolgus monkeys (*Macaca fascicularis*). Pathogenic traits of this hemoplasma strain remain unexplored.
KEY WORDS: hemoplasma, monkey, mycoplasma.

Hemotropic mycoplasma, also called hemoplasma, is a newly defined group of uncultivable pathogens, which has been classified solely using nucleotide sequences of the 16S rRNA or RNase P RNA genes. Hemotropic mycoplasma has been identified and classified in this manner, because of a lack of an appropriate means to examine its biological or serological property [14]. This group is composed of formerly *Eperythrozoon* and *Hemobartonella*(previously *Bartonella*) species and newly identified hemotropic mycoplasmas. Hemoplasma infection, which is accompanied by erythrocyte hemolysis, has been reported in a variety of mammalian species [14]. Hemoplasmas have been detected in some primates including squirrel monkeys (*Saimiri sciureus*), owl monkeys (*Aotus trivirgatus*), rhesus monkeys (*Macaca mulatta*), cynomolgus monkeys (*Macaca fascicularis*) and humans (*Homo sapiens*) as an etiological agent of infectious anemia [1, 3, 18, 22]. Provisional hemoplasma species in primates have been proposed as 'Candidatus Mycoplasma kahanei' in squirrel monkeys [17], '*Ca*. M. aoti' in owl monkeys [2], '*Ca*. M. haemomacaque' in cynomolgus monkeys [13] and '*Ca*. M. haemohominis' in humans [20]. However, the prevalence of hemoplasma infections in Japanese monkeys (*Macaca fuscata*) that live throughout Japan has remained largely unknown. In this report, we demonstrate a hemoplasma strain in Japanese monkeys that is similar to

'*Ca*. M. haemomacaque' reported in the U.S.A.

Ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood samples were collected by saphenous venipuncture from nine wild Japanese monkeys under anesthesia with Zoletil (Virbac, Peakhurst, NSW, Australia) in accordance with the Guidelines for Care and Use of Laboratory Animals of our institutions and stored at −80°C prior to examination. The blood samples were taken for ecological research purpose in Mie prefecture, Japan between October 2012 and February 2013, and the reminder was used for this study. All the monkeys, tentatively captured and released, were apparently healthy, and their age upon capture was unknown. No hematologic parameters were recorded in this study. Total DNA was extracted from 200 μl blood samples collected from Japanese monkeys using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls consisting of 200 μl phosphate-buffered saline solution were prepared with each batch. Extracted DNA samples were stored at −80°C prior to examination.

For preliminary screening of hemoplasma infection, specific PCR primers (forward primer: 5′-ATATTCCTAC-GGGAAGCAGC-3′ equivalent to nucleotide numbers 328 to 347 of *M. wenyonii* [AY946266] and reverse primer: 5′-ACCGCAGCTGCTGGCACATA-3′ equivalent to nucleotide numbers 503 to 522 of *M. wenyonii*) for the 16S rRNA gene of hemoplasmas were used in real-time PCR assay. Real-time PCR was performed in a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq*(TaKaRa Bio., Otsu, Japan) as described previously [11]. Of the nine blood samples tested using real-time PCR, all the monkeys were found to be positive for a hemoplasma infection. No signal was evident in negative controls.

After real-time PCR, the melting experiment was per-

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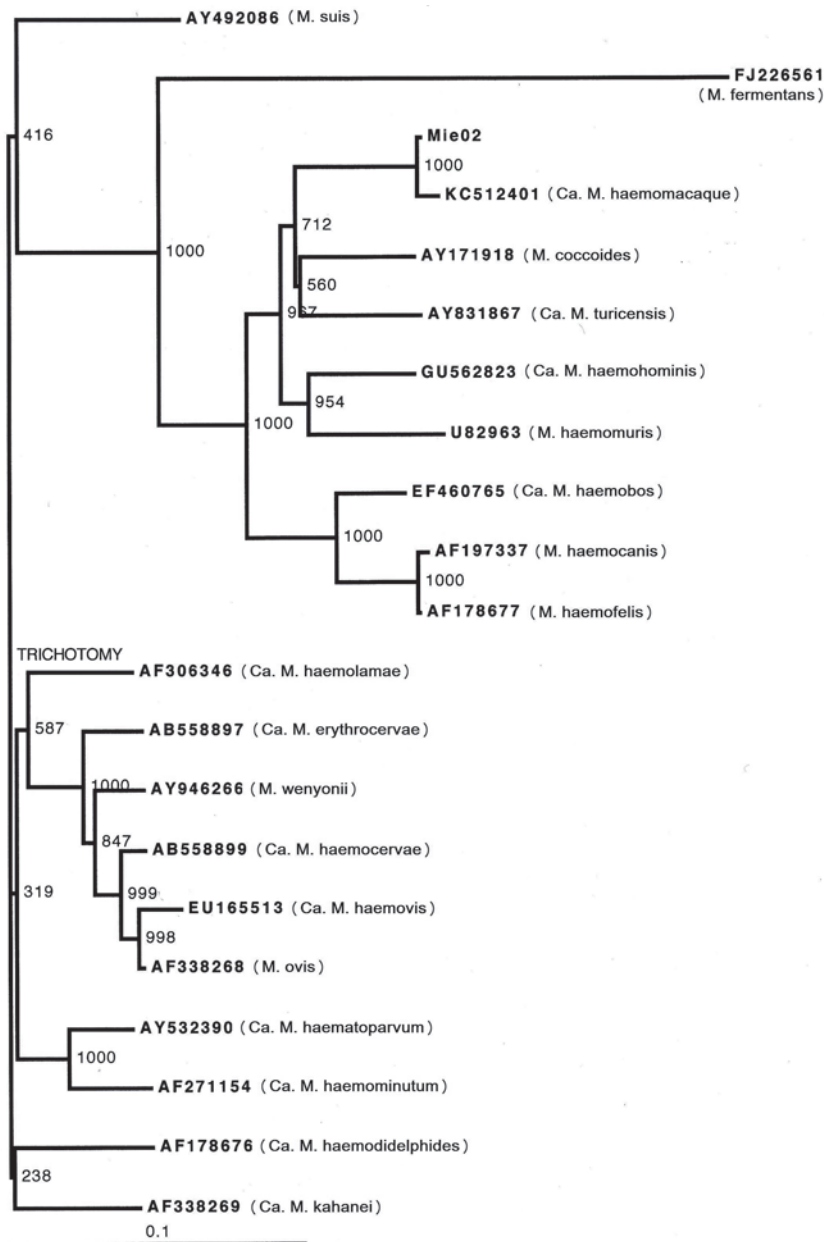   e-mail: harasawa-tky@umin.ac.jp

Fig. 1.   Comprehensive neighbor-joining phylogenetic tree generated by the nucleotide se-
quences of 16S rRNA genes showing the evolutionary relationship among 20 established
Mycoplasma species and a hemoplasma strain Mie02 detected in a Japanese monkey. *Myco-
plasma fermentans* PG18 (FJ226561) was included as an out-group. The bootstrap values are
indicated at the branch points. The tree is drawn to scale with branch lengths in the same units
as those of the evolutionary distances that were used to infer the phylogenetic tree (scale bar,
0.1 nucleotide substitutions per site).

formed as described previously [10]. The melting tempera-
ture (*Tm*) of the positive sample was depicted as a single
peak at about 84.5°C (data not shown), suggesting a specific
amplification. This *Tm* value, higher than those of bear he-
moplasmas [11], seemed peculiar to this hemoplasma strain
and useful for a differential marker of species identification

as described elsewhere [10]. Our previous experiments indi-
cated that the input amount of DNA, the copy number of the
target and the presence of co-infections with several targets
did not influence the *Tm* [10].

The nine positive samples in the real-time PCR experi-
ment were further subjected to end-point PCR to amplify

Fig. 2. Nucleotide sequence alignment of the ITS region from the seven hemoplasma sequences, (*M. haemofelis*, *M. haemocanis*, 'Candidatus M. haemobos' types 1 and 2, *M. haemomuris* subsp. *musculi*(Haemomuris-M) and *M. haemomuris* subsp. *ratti*(Haemomuris-R), bear strain [11] and the Mie02 monkey strain. Accession numbers for the nucleotide sequences of *M. haemomuris* subsp. *musculi, M. haemomuris* subsp. *ratti, M. haemofelis, M. haemocanis, 'Ca.* M. haemobos' types 1 and 2 and bear strain are AB080799, AB758434, AB638408, AF197337, AB638407, AB740010 and AB725596, respectively. Nucleotide sequence numbers are given from a consensus sequence. Homologous nucleotides are shown as inverted characters. Dashes indicate nucleotide gaps between adjacent nucleotides introduced for the alignment. BoxA and boxB are underlined as A and B, respectively.

the entire region of the 16S rRNA gene. End-point PCR was carried out with 50-μl reaction mixtures each containing 1 μl of DNA solution, 0.8 μl of Tks Gflex DNA polymerase

(1.25 units/μl), 25 μl of 2X Gflex PCR buffer (TaKaRa Bio.), 0.2 μl each of the forward primer (5′-AGAGTTT-GATCCTGGCTCAG-3′, equivalent to nucleotide numbers

11 to 30 of *M. wenyonii*(AY946266) or 5′- ATATTCCTAC-GGGAAGCAGC-3′, which is equivalent to nucleotide numbers 328 to 347 of *M. wenyonii*) and the reverse primer (5′- ACCGCAGCTGCTGGCACATA-3′, equivalent to nucleotide numbers 503 to 522 of *M. wenyonii* or 5′-TACCTT-GTTACGACTTAACT-3′, equivalent to nucleotide numbers 1446 to 1465 of *M. wenyonii*) (50 pmol/*μl* each) and water to a final volume of 50 *μl*. After initial denaturation at 94°C for 5 min, the reaction cycle was carried out 30 times with denaturation at 98°C for 10 sec, annealing at 60°C for 60 sec, extension at 68°C for 30 sec and final extention at 68°C for 5 min in a thermal cycler. The end-point PCR product from the 16S rRNA gene was fractionated on horizontal, submerged 1.0% SeaKem ME agarose gels (FMC Bioproducts, Rockland, ME, U.S.A.) in TAE buffer (40 mM Tris, pH8.0, 5 mM sodium acetate and 1 mM disodium EDTA) at 100 volts for 30 min. After electrophoresis, the gels were stained in ethidium bromide solution (0.4 *μg/ml*) for 15 min and visualized under a UV transilluminator. DNA was extracted using a 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.).

The nucleotide sequences of 16S rRNA gene in hemoplasmas from the nine Japanese monkeys were identical each other. The 16S rRNA gene sequence obtained from hemoplasmas in Japanese monkeys was compared with other hemoplasma sequences from the DNA database using Clustal W [21]. A phylogenetic tree was generated using the neighbor-joining method [19] from a distance matrix corrected for nucleotide substitutions using the Kimura 2-parameter model [12]. The data were re-sampled 1,000 times to generate bootstrap values (Fig. 1). The 16S rRNA gene nucleotide sequence of hemoplasma strain Mie02 detected in a Japanese monkey was most closely related to 'Ca. M. haemomacaque' in the tree. Sequence similarity between them was 97.9%, which was enough level for a same species [4]. 'Ca. M. haemomacaque' was first reported in the blood of cynomolgus monkeys in a research colony by assessing the 16S rRNA and RNase P RNA genes [13]. The 16S rRNA gene sequences are widely used in microbiology for identifying uncultivable microorganisms as new species; 16S rRNA gene sequences have also been the basis for the reclassification of hemotropic *Mycoplasma* species [15, 16]. We tried to amplify the RNase P RNA gene using PCR, but only to fail.

Subsequently, we amplified the 16S-23S rRNA intergenic transcribed spacer (ITS) region of the hemoplasmas detected in Japanese monkeys using end-point PCR with the forward primer (5′-GTTCCCAGGTCTTGTACACA-3′) and the reverse primer (5′-CAGTACTTGTTCACTGGTA-3′) as described previously [8]. Reaction was the same as used for the amplification of the 16S rRNA gene, except for the annealing temperature at 55°C instead of 60°C and the extention time for 15 sec instead of 30 sec. The ITS region nucleotide sequence of strain Mie02 was determined as described above and compared to those of other hemoplasmas (Fig. 2). The ITS region of the genus *Mycoplasma* is well conserved

within a species and has been used for a genetic marker for identification and classification of mycoplasmas [5]. No spacer tRNA gene was identified within the ITS region of this hemoplasma strain, which is a common feature that is consistent with the other species of the genus *Mycoplasma* [6, 7]. The hemoplasma strain detected in Japanese monkeys possessed the boxA and boxB motifs that are common to other mycoplasma species examined so far [9].

In the present study, we detected hemoplasmas in nine captive Japanese monkeys, which shared a same nucleotide sequence of the 16S rRNA gene and ITS region, suggesting that the same strain was circulating among Japanese monkeys in Mie prefecture, Japan. We then used 16S rRNA phylogenetic analysis to demonstrate the hemoplasma strain that was most closely related to 'Ca. M. haemomacaque' recently reported in the U.S.A. [13]. The incidence of hemoplasma infection in Japanese monkeys was 100% (9/9), despite a small number of examinations, whereas 84.6% (44/52) in cynomolgus monkeys in the U.S.A. [13]. Although we awaited our identification as 'Ca. M. haemomacaque' until examination of the RNase P RNA gene, our results suggested a wide prevalence of the hemoplasma strain similar to 'Ca. M. haemomacaque' in wild Japanese monkeys. It is particularly of interest that the genetically related hemoplasma strains were observed in geographically separate populations. The nucleotide sequence of the 16S rRNA gene combined with the ITS region of the monkey strain has been deposited to the DNA database under the accession number AB820288.

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