NOTE *Bacteriology*

Detection of Hemotropic Mycoplasmas in Free-Living Brown Sewer Rats (*Rattus norvegicus*)

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ABSTRACT. The prevalence of hemotropic mycoplasmas in wild rodents is largely unknown. Here, we report the presence of hemoplasmas in blood samples collected from brown sewer rats (*Rattus norvegicus*) trapped during rodent control around an animal hospital in Morioka, Japan. We examined nine rats using real-time PCR and end-point PCR, and found one rat (11.1%) that was positive for a hemoplasma infection. The 16S rRNA gene and 16S to 23S rRNA intergenic spacer region of the hemoplasma detected in a wild-caught rat were amplified using PCR. The nucleotide sequences of the PCR products were further determined and compared to those of other hemoplasmas. Our examinations revealed the presence of a hemoplasma that has not previously been described in rodents. The pathogenic traits of this hemoplasma remain unexplored.

KEY WORDS: hemoplasma, mycoplasma, wild-caught rat.

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Hemotropic mycoplasma, also called hemoplasma, is a newly defined group of uncultivable pathogens, which have been classified solely using nucleotide sequences of the 16S rRNA or RNase P RNA genes. Hemotropic mycoplasma has been classified in this manner, because of a lack of an appropriate means to examine its biological or serological properties [9]. 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 [9]. Hemoplasmas have been detected in rodents, including mice, rats and hamsters, as an etiological agent of infectious anemia or splenomegaly, and these hemoplasmas have collectively been identified as *Bartonella muris* based only upon microscopic observation [13]. However, the prevalence of hemoplasma infections in wild rodents has remained largely undocumented. In this report, we demonstrate a hemoplasma that has not been described previously; the hemoplasma was found in brown sewer rats (*Rattus norvegicus*) wild-caught near an animal hospital.

Nine wild-caught rats were trapped in July 2012 during sanitation measures at the Iwate University veterinary hospital (latitude 39.7N and longitude 141.1E) in Japan. Anti-coagulated blood samples were collected under ether anesthesia from seven of the rats, and blood was also taken from two of the rats that were found dead; blood samples were stored at −80°C prior to examination. No clinical sign was apparent in the rats. The protocol used in the present study was approved by the Animal Care and Use Committee of Iwate University, and all animal experiments were performed in accordance with the Guidelines for Care and Use of Laboratory Animals established by the Committee. Total DNA was extracted from 200 *µl* blood samples collected from rats 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 infections, universal PCR primers for the hemoplasmas' 16S rRNA gene were used, as described by Tasker *et al.* [16]. Real-time PCR was performed using a SmartCycler instrument (Cepheid, Sunnyvale, CA, U.S.A.) with SYBR Premix Ex *Taq* (Code #RR041A, TaKaRa Bio., Otsu, Japan). The reaction mixture contained 1 *µl* of each primer (10 pmol/*µl*), 12.5 *µl* of 2X premix reaction buffer and water to a volume of 23 *µl*. Finally, 2 *µl* (200 *p*g) of a DNA sample was added to this mixture as a template. 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, the melting experiment was performed from 60°C to 95°C at 0.2°C/sec with a smooth curve setting averaging one point. Melting peaks were visualized by plotting the first derivative against the melting temperature (*Tm*) as described previously [7]. The *Tm* was defined

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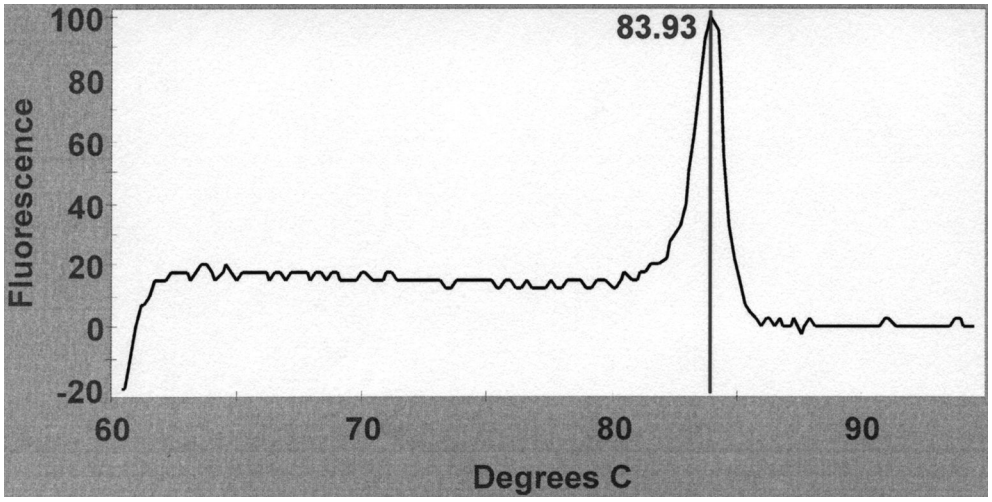

Fig. 1. Melting curve analysis for the real-time PCR product from the 16S rRNA gene of the hemoplasma strain detected in the peripheral blood of a wild-caught rat. Melting temperature was depicted as a single peak at 83.93°C.

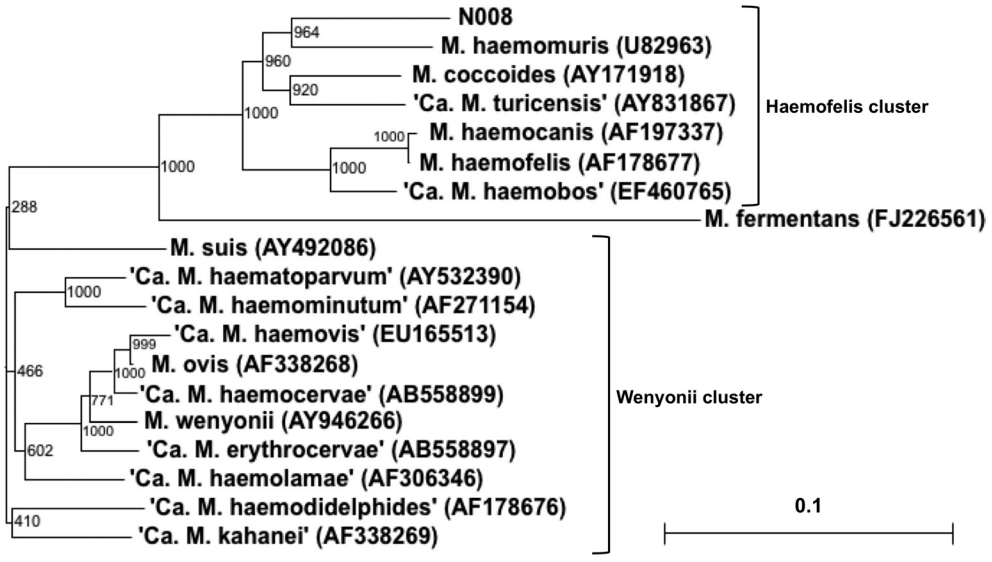

Fig. 2. Comprehensive neighbor-joining phylogenetic tree generated by the nucleotide sequences of 16S rRNA genes showing the evolutionary relationship among hemoplasmas (accession numbers are given in parentheses) and a newly identified rat hemoplasma strain, N008. *Mycoplasma fermentans* PG18 was included 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).

as a peak of the curve; if the highest point was a plateau, the mid-point was identified as the *Tm*. Of the nine blood samples tested using real-time PCR, one rat was found to be positive for a hemplasma infection. The *Tm* of the positive sample was 83.93°C (Fig. 1). Our previous experiments indicated 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* [12].

The positive sample from the real-time PCR experiment was further subjected to end-point PCR to amplify 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, 0.5 μl of TaKaRa LA *Taq*^{TM} (5 units/μl), 5 μl of 10X LA PCR^{TM} Buffer II (TaKaRa Bio.), 8 μl of 25

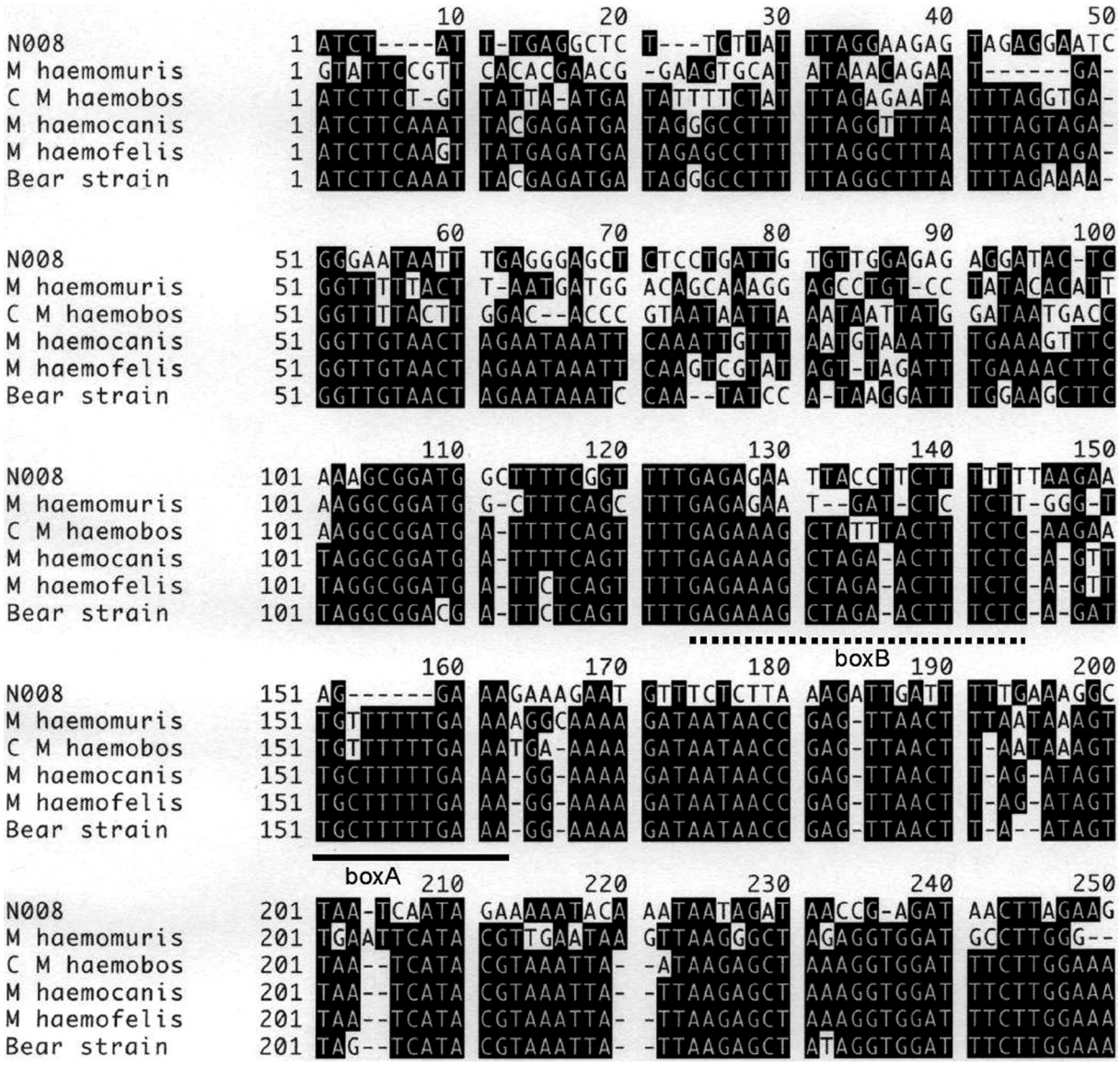

Fig. 3. Nucleotide sequence alignment of the ITS region from the six hemoplasma sequences, *M. haemofelis*, *M. haemocanis*, '*Candidatus* M. haemobos', *M. haemomuris*, bear strain and rat strain N008. Accession numbers for the nucleotide sequences of *M. haemomuris, M. haemofelis, M. haemocanis, 'Ca.* M. hamobos*'* and bear strain are AB080799, AB638408, AF197337, AB638407 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 is underlined, and boxB is shown by a dotted line. Notably, the boxA motif was missing in the rat hemoplasma strain, N008.

mM $MgCl_2$ (final 4.0 mM), 8 μl of dNTP mixture (2.5 mM each), 0.2 μl each of the forward primer (5′-AGAGTTTGATCCTGGCTCAG-3′, equivalent to nucleotide numbers 11 to 30 of *M. wenyonii*(AY946266) or 5′- ATATTCCTACGGGAAGCAGC-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′-TACCTTGTTACGACTTAACT-3′, equivalent to nucleotide numbers 1,446 to 1,465 of *M. wenyonii*) (50 pmol/μl each) and water to a final volume of 50 μl. After the mixture was overlaid with 20 μl of mineral oil, the reaction cycle was carried out 35 times with denaturation at 94°C for 30 sec, annealing at 58°C for 120 sec and extension at 72°C for 60 sec 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, 1 mM disodium ethylenediaminetetracetate) at 50 volts for 60 min. After electrophoresis, the gels were

stained in ethidium bromide solution (0.4 μg/m*l*) 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 sequence of this rat hemoplasma 16S rRNA gene was most similar to the sequence of *M. haemomuris* (formerly *Bartonella muris* or *Haemobartonella muris*) Shizuoka strain that was isolated from a small field mouse (*Apodemus argenteus*) in Japan [14]. Sequence homology between them was 87%, which was lower than the critical cut-off level of a same species [1].

The 16S rRNA gene sequence obtained from a rat hemoplasma was similar to other hemoplasma sequences from the DNA database using Clustal W [17]. A phylogenetic tree was generated using the neighbor-joining method [15] from a distance matrix corrected for nucleotide substitutions using the Kimura two-parameter model [8]. The data were re-sampled 1,000 times to generate bootstrap values (Fig. 2). The 16S rRNA gene nucleotide sequence of a hemoplasma strain detected in a sewer rat was distinct from those of other hemoplasmas. 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 [10, 11]. In our previous examinations, hemoplasmas were divided into two phylogenetic groups, Haemofelis and Wenyonii clusters [18]. The hemoplasma strain detected in a wild-caught rat belonged in the Haemofelis cluster in the present study.

Next, we amplified the 16S-23S rRNA intergenic transcribed spacer (ITS) region of this specimen using end-point PCR with the forward primer Hemo16-23S-F and the reverse primer, as described previously [2]. The ITS region nucleotide sequence was determined as described above and compared to those of other hemoplasmas (Fig. 3). 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 [3]. Nucleotide sequence homology at the ITS region between the rat strain N008 and *M. haemomuris* Shizuoka strain was 37%, and this suggests that they are genetically distinct. No spacer tRNA gene was identified within the ITS region of the rat hemoplasma strain, which is a common feature that is consistent with the other species of the genus *Mycoplasma* [4, 5]. The rat hemoplasma strain lacked the boxA motif that is common to other mycoplasma species examined so far [6], despite the presence of the boxB motif.

In the present study, we used 16S rRNA phylogenetic analysis to demonstrate, in a wild-caught rat, a hemoplasma strain that was most closely related to *M. haemomuris* Shizuoka strain [14]. Although *M. haemomuris,* previously called *Bartonella muris* or *Haemobartonella muris,* was a species name given to an anemic pathogen isolated from an albino rat [10, 11], the alleged 16S rRNA gene nucleotide sequence of *M. haemomuris* was determined using a morphologically identified hemoplasma strain isolated from a small field mouse [14]. Similarly, hemoplasmas detected in mice, rats or hamsters have been identified on the basis of morphology. This may pose a question whether all the hemoplasma strains detected in these rodents were identical or a same species. It is not certain that there is a genetic relationship among these hemotropic pathogens isolated from mice, rats and hamsters. Thus, our findings may address this problem by demonstrating differences in nucleotide sequences of the 16S rRNA gene and the ITS region of the hemoplasmas detected in mice and rats.

In conclusion, we used 16S rRNA phylogenetic analysis to demonstrate the presence of a hemoplasma, in a wild-caught brown rat, which was similar but not identical to *M. haemomuris*. Both murine hemoplasmas were distinct from each other in the primary and secondary structures of the ITS region. Our data indicate that there is genetic variation among murine hemoplasmas. The nucleotide sequence of the 16S rRNA gene combined with the ITS region has been deposited to the DNA database under the accession number AB752303.

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