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Western Immunoblot Analysis of *Haemobartonella muris* and Comparison of 16S rRNA Gene Sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis*

YASUKO RIKIHISA,[1]* MAKOTO KAWAHARA,[2] BOHAI WEN,[1] GARY KOCIBA,[1] PAUL FUERST,[3] FUMIHIKO KAWAMORI,[4] CHIHARU SUTO,[5] SHINICHIRO SHIBATA,[2] AND MASATAKA FUTOHASHI[2]

Department of Veterinary Biosciences, College of Veterinary Medicine,[1] and Department of Molecular Genetics,[3] The Ohio State University, Columbus, Ohio 43210, and Nagoya City Public Health Research Institute, Nagoya 467,[2] Shizuoka Prefectural Institute of Public Health and Environmental Science, Shizuoka 420,[4] and Department of Medical Zoology, Nagoya University School of Medicine, Nagoya 466,[5] Japan

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Infectious agents were isolated from the spleens of three wild mice (*Apodemus argenteus*) by intraperitoneal inoculation of the spleen homogenate into laboratory mice. The laboratory mice developed clinical signs and splenomegaly, and three isolates were maintained by passage in mice. Tetracyclines were effective in preventing infection of mice with these agents, but streptomycin and penicillin were ineffective. The agents did not grow in bacterial growth media or chicken embryos. In smears of blood from infected mice stained by the Giemsa or the indirect immunofluorescence method, numerous organisms were found on the surfaces of erythrocytes. Electron microscopy revealed cell wall-less pleomorphic cocci of 350 to 700 nm in diameter. On the basis of these results, the isolates were identified as *Haemobartonella muris*. There was no antigenic cross-reactivity with *Rickettsia* or *Ehrlichia* spp. or other related organisms. Western immunoblot analysis of three strains of *H. muris* with mouse antisera to *H. muris* revealed identical major antigens of 118, 65, 53, 45, and 40 kDa. By heteroduplex analysis of the three PCR-amplified segments of the 16S rRNA genes, the three strains of *H. muris* were found to be identical. The 16S rRNA genes of one of the *H. muris* strains, four strains of *H. felis*, and two strains of *Eperythrozoon suis* were sequenced and compared. The sequences of two strains of *H. felis* from cats in California were identical, as were the sequences of a strain from a cat in Ohio and a strain from a cat in Florida, but the similarity of sequences between the California and the Ohio-Florida strains was only 85%. The sequence of an *H. muris* strain was unique and was more closely related to that of the Ohio-Florida strain of *H. felis* (89%) than to that of the California strain of *H. felis* (84%). The sequence of *E. suis* from a pig in Illinois was identical to that from another pig from Taiwan. The similarity of the 16S rRNA gene sequence of *E. suis* with those of three *Haemobartonella* strains was 84 to 92%, with that of *E. suis* being most similar to that of the *H. felis* strain from California. In the phylogenetic analysis based on 16S rRNA gene sequences, the *Haemobartonella* spp. and *E. suis* formed a distinct clade more closely related to *Mycoplasma* spp. (79 to 83% similarity) than to *Anaplasma marginale* (72 to 75% similarity). Our results suggest that the *Haemobartonella* spp. and *E. suis* may be reclassified in the same genus in the family *Mycoplasmataceae*.

Haemobartonella spp. are very small (0.3 to 0.5 μm in diameter), gram-negative bacteria enveloped with a single membrane. They are pleomorphic microorganisms, including coccus or rod forms, that infect erythrocytes, sometimes causing severe anemia (23). Three different *Haemobartonella* spp. have been recognized on the basis of their mammalian hosts: *Haemobartonella muris* infects rats, mice, and hamsters (2, 14); *Haemobartonella felis* infects domestic cats (8, 10, 26); and *Haemobartonella canis* infects dogs (3, 24). There has been no report of a *Haemobartonella* sp. infecting pigs. *Eperythrozoon suis* is a organism that is similar to *Haemobartonella* spp. in morphology and that infects the erythrocytes of pigs, causing anemia and icterus (15). *Haemobartonella* spp. are located on or within erythrocytes but rarely in the plasma, while *Eperythrozoon* spp. occur on erythrocytes and free in the plasma at about equal frequency (23). Several reports have described *Haemobartonella*-like organisms infecting humans in the United States (1, 6, 12). Blood-sucking insects have been demonstrated to be or have been suggested to be vectors of *Haemobartonella* spp. and *Eperythrozoon* spp. (2, 10, 24, 25).

According to *Bergey's Manual of Systematic Bacteriology*, both *Haemobartonella* spp. and *Eperythrozoon* spp. are placed in the family *Anaplasmataceae* in the order *Rickettsiales*. This is mainly because they lack an outer membrane, are unculturable, and are parasitic to erythrocytes, like *Anaplasma* spp. (15, 23). However, unlike *Anaplasma* spp., which replicate in the membrane-bound inclusions of erythrocytes, both *Haemobartonella* spp. and *Eperythrozoon* spp. adhere to and primarily grow on the surface of the erythrocytes (5, 6, 11, 17, 28). The antigenic compositions and 16S rRNA gene sequences of this group of bacteria have not been reported. This group of organisms has never been cultured, and a type strain is not available for comparison.

In 1985, the agent infectious to erythrocytes was isolated from the spleens of three wild mice in Japan. In this report we describe the antigenic, ultrastructural, and pathologic characteristics of this agent. On the basis of these characteristics, the agent was identified as *H. muris*. To further characterize the phylogenetic relationship of this agent, the 16S rRNA genes of this agent and several other related organisms were sequenced and their similarities were compared.

* Corresponding author. Mailing address: Department of Veterinary Biosciences, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Rd., Columbus, OH 43210-1096. Phone: (614) 292-9677. Fax: (614) 292-6473.

MATERIALS AND METHODS

Isolation of the infectious agent from wild mice. In October 1985, 10 wild mice (*Apodemus argenteus*) were caught during an epidemiologic survey of rickettsial disease in Motonagakubo, Nagaizumi Town, Shizuoka Prefecture, in Japan. At necropsy, splenomegaly was noted in nine of the mice. The spleens were aseptically removed and homogenized in 10% (wt/vol) sucrose-phosphate-glutamate buffer (SPG; 0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutamate, 0.218 M sucrose [pH 7.0]). The spleen homogenate of each mouse was then inoculated intraperitoneally (i.p.) at 0.2 ml/mouse into two mice (ddY strain) at Shizuoka Laboratory Animal Center (Shizuoka, Japan). The inoculated mice developed clinical signs (lethargy, anorexia, and ruffled fur), and some of them died 7 to 15 days postinoculation (p.i.). At necropsy, splenomegaly was again observed. The agents could be maintained in mice by serial passage of the infected spleen cells in mice every 10 days. The spleen homogenates in SPG were frozen at −80°C until use. Of the nine isolates, three (strains TR8556, TR8563, and TR8564) were used in this study.

Infection of mice. BALB/c mice (age, 8 weeks) were purchased from Shizuoka Laboratory Animal Center. Five mice were used for each group. A 10% (wt/vol) homogenate in SPG was prepared with a Dounce homogenizer from the spleens of mice infected with strain TR8556, TR8563, or TR8564 on day 10 p.i. After centrifugation at 500 × *g* for 5 min, the supernatant was collected. A 0.2-ml dose of supernatant was inoculated i.p. into each of 80 mice. Every 5 days, up to 25 days p.i., groups of five mice each were killed, and their spleens were removed and weighed. Splenomegaly was used as an index of infectivity and was defined as a relative spleen weight (grams per 100 g of body weight) of greater than 2.5 (13). The infectious titer of the pooled spleen homogenate of mice was determined by inoculating 0.2 ml of serial 10-fold dilutions of the spleen homogenate (10^{-1} to 10^{-5}) i.p. into groups of five mice each. The infectious titer was defined as the dilution required to induce splenomegaly at 15 days p.i. in 50% of the inoculated BALB/c mice ($SMID_{50}$) with serially diluted homogenates and was calculated by the method of Reed and Muench (19).

Sensitivity to temperature and antibiotics. The 10% spleen homogenate from infected mice was incubated at 37 or 50°C for 5, 10, 15, 30, 45, and 60 min and was then inoculated i.p. into a group of five mice at 0.2 ml per mouse. The mice were killed on day 15 p.i., and the relative spleen weights were determined. Five antibiotics were tested for their effectiveness in protecting mice from infection with strain TR8563. Tetracycline hydrochloride and oxytetracycline were obtained from Takeda Chemical Industries Co., Ltd., Osaka, Japan. Minocycline hydrochloride was obtained from Pfizer Taito Co., Ltd., Tokyo, Japan. Streptomycin sulfate and benzylpenicillin potassium were obtained from Meiji Seika Co., Ltd., Tokyo, Japan. Eight milligrams of each antibiotic was administered i.p. into each of the five mice in each treatment group. After 30 min, each mouse was inoculated with 0.2 ml of the spleen homogenate ($10^{2.5}$ $SMID_{50}$s). The mice were killed on day 15 p.i., and relative spleen weights were calculated.

Growth in bacteriologic media. The spleen homogenate was spread by the use of bent glass rods onto plates of Luria-Bertani (LB) broth medium (Difco, Detroit, Mich.), thioglycolate medium (Difco), brain heart infusion medium (Difco), tryptic soy broth (Difco), and sheep erythrocyte agar medium (Nippon Biosupply Center, Tokyo, Japan). The plates were incubated in ambient air at 37°C and were examined for bacterial growth every day for 2 weeks.

Yolk sac inoculation. The yolk sacs of five eggs containing 7-day-old embryos were each inoculated with 0.2 ml of 10% homogenates in SPG. On day 7 p.i., the yolk sac was removed and a 10% (wt/vol) homogenate was prepared in SPG. This homogenate was used to inoculate five more embryos (0.2 ml per egg), after which a second yolk sac homogenate was prepared as described before. This homogenate was then inoculated i.p. into five mice at 0.2 ml per mouse.

Growth in rats. Three 8-week-old Wistar rats were inoculated i.p. with the spleen homogenate containing strain TR8563. Blood smear samples obtained from the tail veins of the rats on days 10 and 15 p.i. were stained by the Giemsa method or by the indirect fluorescent antibody (IFA) assay described here. Spleen homogenates were examined by PCR with the EC9 and EC10 primers (see below).

Weil-Felix reaction. Murine antiserum against strain TR8563 was inactivated by incubation at 60°C for 20 min and was then serially diluted 1:10 to 1:640 in phosphate-buffered saline (PBS). Weil-Felix reagent (Denka Seiken Co., Ltd., Tokyo, Japan) was applied to an aliquot of each antiserum dilution. After incubation at 4°C overnight, the dilution tubes were examined for the presence of agglutination.

IFA testing. Strains TR8556, TR8563, and TR8564 and *Ehrlichia sennetsu*, *Ehrlichia muris* AS145, *Orientia tsutsugamushi* Karp, *Coxiella burnetii* Nine Mile, and *Rickettsia japonica* YH (spotted fever group) were used as antigens in the IFA test. *O. tsutsugamushi*, *C. burnetii*, and *R. japonica* were kindly provided by the Toyama Institute of Health, Toyama, Japan; Gifu University, Gifu, Japan; and the National Institute of Health, Tokyo, Japan, respectively. The remainder of the organisms were isolated and maintained in our laboratories (13). Blood smear preparations of blood taken from the tail veins of mice infected with strains TR8556, TR8563, and TR8564 on day 10 p.i. were used as the antigen. A 10-μl suspension of Vero cells (1.0×10^7 cells per ml) infected with *C. burnetii* or *R. japonica* was cultured in each well of sterile multiwell slides (Flow Laboratories, McLean, Va.) at 37°C for 2 h in a humidified atmosphere of 5% CO_2 and 95% air. After being washed twice with PBS, the cells were fixed with acetone for 10 min. These slides were then dried and used as antigens for the IFA test. Antigens of *E. sennetsu*, *E. muris*, and *O. tsutsugamushi* were prepared in mice or L929 cells as described previously (13). Antisera to three strains were made in mice. Each of five mice was inoculated i.p. or intradermally four times at 7-day intervals with 0.2 ml of the homogenate of the spleen of mice infected with one of the previously described agents. Blood samples were obtained 2 weeks after the last inoculation. The sera were sterilized by membrane (Millipore, Bedford, Mass.) filtration and were stored at −80°C until use. The IFA test was carried out as described in a previous report (13).

Western immunoblot analysis of isolated organisms. Sodium dodecyl sulfate (SDS)-polyacrylamide slab gel electrophoresis of *H. muris* TR8556, TR8563, and TR8564 dissociated from the mouse erythrocyte surfaces by incubation in PBS at 37°C for 1 h was performed as described previously (13). The microorganism was dissolved in 0.125 M Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.05% pyronine Y at a concentration of 2 mg of protein per ml by being heated at 100°C for 3 min. Solubilized microorganisms (2 mg/ml) were applied onto a 10% polyacrylamide gel at 20 μl per lane. A mixture of proteins with known molecular weights (Bio-Rad Laboratories, Richmond, Calif.) was electrophoresed on one lane of each gel. Electrophoresis was performed at a constant amperage (30 mA per gel) for 4 h in 0.025 M Tris-HCl buffer (pH 8.3) containing 0.192 M glycine and 0.1% SDS. After electrophoresis, the separated proteins were transferred to a nitrocellulose membrane filter (Schleicher & Schuell, Keene, N.H.) by electrophoresis in a semidry electroblotter (Integrated Separation Systems, Hyde Park, Mass.) at a constant amperage (136 mA per gel) for 45 min. After being immersed in 5% (wt/vol) nonfat dried milk in PBS at 37°C for 30 min, the membrane filters were incubated with mouse anti-TR8556, anti-TR8563, or anti-TR8564 sera at a 1:50 dilution in PBS-nonfat dried milk at 37°C for 2 h. After three successive 10-min washes in PBS–0.002% Tween 20 and a 20-min wash in PBS, the membrane filters were incubated in alkaline phosphatase-conjugated affinity-purified goat anti-mouse immunoglobulin G (heavy and light chains; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) at a 1:1,000 dilution in PBS-nonfat dried milk at 37°C for 2 h. The membrane filters were washed as described before and were immersed in substrate solution containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Light and electron microscopic observation. For light microscopy, blood smear preparations of blood taken from the tail veins of infected mice on day 10 p.i. were stained by the Giemsa method and erythrocytes were examined for the presence of microorganisms. For electron microscopy, the erythrocyte samples were taken from the hearts of infected mice on day 10 p.i. and were washed in PBS by centrifugation at 200 × *g* for 5 min. The samples were prepared as described elsewhere (22).

Blood samples from cats infected with *H. felis*. One blood sample (sample 2642) collected in 1989 was from a cat infected with *H. felis* that had originally been isolated in Florida and that had been passaged in cats in Ohio (Florida strain). Another sample (sample 0796) was obtained from a cat diagnosed as having severe haemobartonellosis in 1996 in Ohio (Ohio isolate). Additional blood samples from two cats infected with a small (21) or large strain of *H. felis* that had originally been derived from different cats and that had been maintained separately by passage in cats were kindly provided by N. C. Pedersen (University of California, Davis). A single blood sample from an *E. suis*-infected pig was kindly provided by J. F. Zachary, University of Illinois, Champain-Urbana, and a second swine blood sample was kindly provided by Chung-Nan Wen (Pig Research Institute Taiwan, Miaoli, Taiwan). The erythrocytes of all blood samples were found to be parasitized with the organisms when examined by Giemsa staining or immunofluorescence labeling with the homologous serum. The blood samples were frozen at −80°C after the addition of 20% dimethyl sulfoxide.

Extraction of DNA from the blood samples and amplification of the 16S rRNA gene of *Haemobartonella* spp. and *E. suis* by PCR. The DNA was extracted from the blood samples with the QIAamp tissue kit (QIAGEN, Inc., Chatsworth, Calif.) by the protocol recommended by the manufacturer. This DNA was used as the template in the PCR. The 16S rRNA genes of *Haemobartonella* spp. could not be amplified by the use of A-17 and 3-17, universal 5′ and 3′ end primers, respectively, for the 16S rRNA gene sequences of prokaryotes. Therefore, one DNA fragment of approximately 600 bp of the 3′ end of the 16S rRNA gene was at first amplified with a pair of universal primers: EC9 (5′-AAGGATCCTACC TTGTTACGACTT-3′) and EC10 (5′-AATCTAGATTAGATACCCTAGTAG TCC-3′). The 5′ end of the gene was amplified as a fragment of approximately 900 bp by using primers A-17mod (5′-AGAGTTTGATCCTGG-3′) and Haem (5′-GGTAAGGTTTTTCGTG-3′). PCR was performed as described previously (29). The reactions were carried out at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 40 cycles. The amplified DNA fragments were purified from a 1% low-melting-temperature agarose gel (GIBCO BRL, Gaithersburg, Md.) by using a PCR DNA purification kit (Promega, Madison, Wis.). This DNA was used as the template for DNA sequencing.

Nucleotide sequencing of the 16S rRNA gene product obtained by PCR. Base sequences of both DNA strands were determined by dideoxynucleotide sequencing with a double-stranded DNA sequencing system (GIBCO BRL) by the protocol recommended by the manufacturer. The following forward (F) and reverse (R) primers were used for sequencing: EC9, EC10, A-17mod, Haem, F312 (5′-CTACGGGAAGCAGCAG-3′), R385 (5′-TTCATCGTTCACATGG-3′), F715 (5′-GTGGGGAGCAAATGG-3′), R768 (5′-ATACCCATCGTTTAC

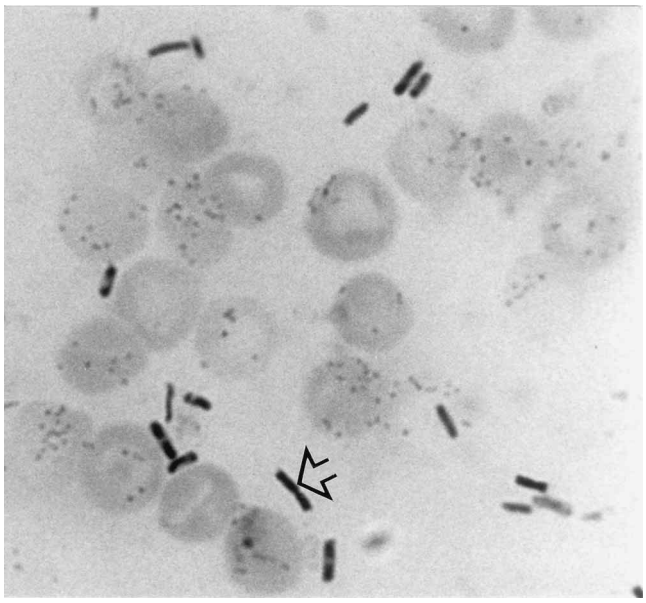

FIG. 1. Microorganisms of strain TR8563 on the surface of mouse erythrocytes stained by the Giemsa method. They are much smaller than *Escherichia coli* (arrow) which was added to the mouse blood for size comparison. Magnification, ×1,800.

G-3′), F1112 (5′-ACGTCAAGTCATCATG-3′), and R1159 (5′-ATTGTAGCA CGTTTG-3′). The number in the primers identifies the location of the first base of the primer in the base sequence of the 16S rRNA gene of *H. muris* determined in this study. A 5% Long Ranger gel (AT Biochem, Malvern, Pa.) was used for sequencing in order to determine more than 400 bases with each primer.

Comparison of DNA sequences. A homology search was carried out with the databases of GenBank by using the basic logical alignment search tool software in the BLAST network service (National Center for Biotechnology Information, Bethesda, Md.). Sequences were aligned manually with the program ESEE (4). The corrected levels of nucleotide divergence of the 16S rRNA genes were calculated by using the program DNADIST in PHYLIP, version 3.1, with the Kimura two-parameter correction (7). A phylogenetic tree of the taxa studied was constructed by the neighbor-joining method (NEIGHBOR in PHYLIP) (7) on the basis of the matrix of corrected nucleotide divergence. The analysis excluded sites at which the 16S rRNA gene sequences could not be aligned unambiguously.

DNA heteroduplex analysis. Three segments (~400 bp) of the 16S rRNA gene were amplified by PCR with three pairs of primers (A-17mod and R385, F312 and R768, and F1112 and EC9). Heteroduplexes and homoduplexes were produced by mixing the two PCR products and incubating the mixture at 96°C for 5 min, 55°C for 10 min, 45°C for 10 min, 37°C for 10 min, and 26°C for 15 min. Triple dye loading buffer (National Diagnostics, Atlanta, Ga.) was added to each reaction product, and the mixture and heteroduplex control DNA (National Diagnostics) were loaded onto SequaGel MD (National Diagnostics) and electrophoresed at 20 V/cm.

DNA sequence accession numbers. The GenBank accession numbers of the 16S rRNA gene sequences used in this study are as follows: *Anaplasma marginale*, M60313; *Bacillus azotoformans*, X60609; *Clostridium thermoautotrophicum*, L09168; *Cyanobacterium* sp., M62776; *Lactobacillus vitulinus*, M23727; *Mycoplasma imitans*, L24103; *Mycoplasma pirum*, M23940; *Mycoplasma muris*, M23937; *Spiroplasma* sp., M24475; *Ureaplasma canigenitalium*, D78648; *Ureaplasma urealyticum*, U06096; *Erysipelothrix rhusiopathiae*, M23728; *Acholeplasma laidlawii*, M23932; *Bartonella henselae*, M73229; *H. felis* Florida-Ohio, U88563; *H. felis* California, U88564; *H. muris*, U82963; and *E. suis*, U88565.

RESULTS

Isolation of infectious agent. Starting at about day 7 p.i., mice inoculated with homogenates of spleens from the infected wild mice developed ruffled fur, inactivity, and anorexia. Some of them died at approximately 7 to 15 days p.i. At necropsy, hypertrophy of the spleen, liver, and superficial lymph nodes and mild ascites were present. The relative spleen weight (grams per 100 g of body weight) for infected mice ($n = 5$) was initially 0.4 ± 0 g/100 g and began to increase on day 5 p.i., reached a maximum of about 5.3 ± 0.8, 5.1 ± 0.6, and 4.9 ± 0.9 g/100 g for strains TR8556, TR8563, and TR8564, respectively, on day 15 p.i., and then decreased to 2.2 ± 0.2, 1.7 ± 0.3, and 2.1 ± 0.2 g/100 g for strains TR8556, TR8563, and TR8564, respectively, until day 25 p.i., at which time the experiment was terminated. Thus, the maximum spleen size was about 10 times the normal size. No significant differences in pathology among the three strains were noted.

The infectious titer of the 10% spleen homogenate of infected BALB/c mice was $10^{4.5}$ on day 10 p.i. The mortality rates of the mice inoculated with $10^{3.5}$, $10^{2.5}$, $10^{1.5}$, and $10^{0.5}$ $SMID_{50}$s of strain TR8563 were approximately 80% (four of five mice), 60% (three of five mice), 20% (one of five mice), and 10% (zero of five mice), respectively. The infectivity of the spleen homogenate was thermosensitive, as in the case for *Ehrlichia muris* (13). The relative spleen weights (grams per 100 g of body weight) for mice ($n = 5$) determined at day 15 p.i. were 1.8 ± 1.2 g/100 g when the TR8563 strain-infected spleen inoculum was pretreated at 37°C for 45 min, 0.4 ± 0 g/100 g when the spleen inoculum was pretreated at 50°C for 5 min, and 4.2 ± 0.5 g/100 g for control infected mice. Strains TR8556, TR8563, and TR8564 did not grow in chick embryos (38°C) or any of several bacterial growth media. The results also indicate the absence of contaminating microorganisms in the isolate. The relative spleen weights (grams per 100 g of body weight) for mice ($n = 5$) determined at day 15 p.i. were 0.6 ± 0.6, 0.6 ± 0.1, and 1.5 ± 0.9 g/100 g for mice inoculated with 8 mg of tetracycline hydrochloride, minocycline hydrochloride, or oxytetracycline hydrochloride, respectively. However, the relative spleen weights (grams per 100 g of body weight) for mice ($n = 5$) determined at day 15 p.i. were 4.4 ± 0.8, 4.4 ± 1.1, and 5.1 ± 0.6 g/100 g for mice inoculated with 8 mg of streptomycin sulfate, potassium benzylpenicillin, or no drug (control infected mice), respectively.

No organisms were detected in blood smears of rats inoculated i.p. with TR8563 on days 10 and 15 p.i. by the Giemsa

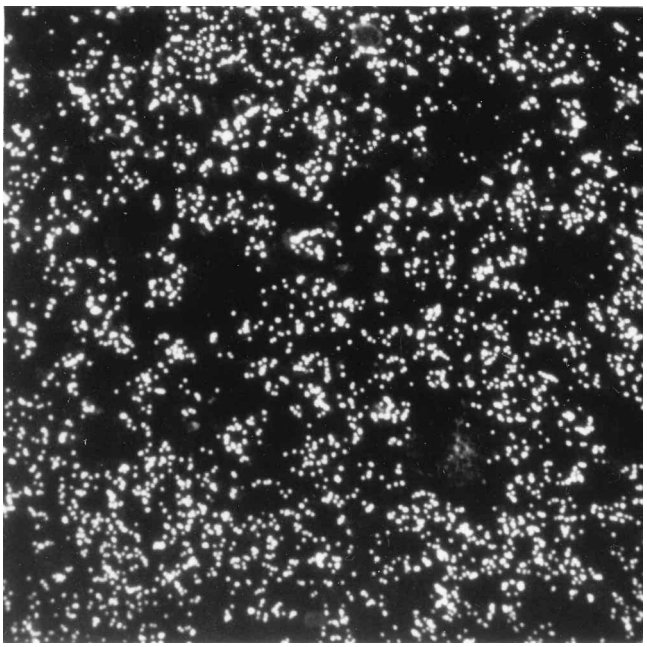

FIG. 2. IFA labeling of erythrocyte smears infected with strain TR8563. Note the heavy parasitemia. Magnification, ×700.

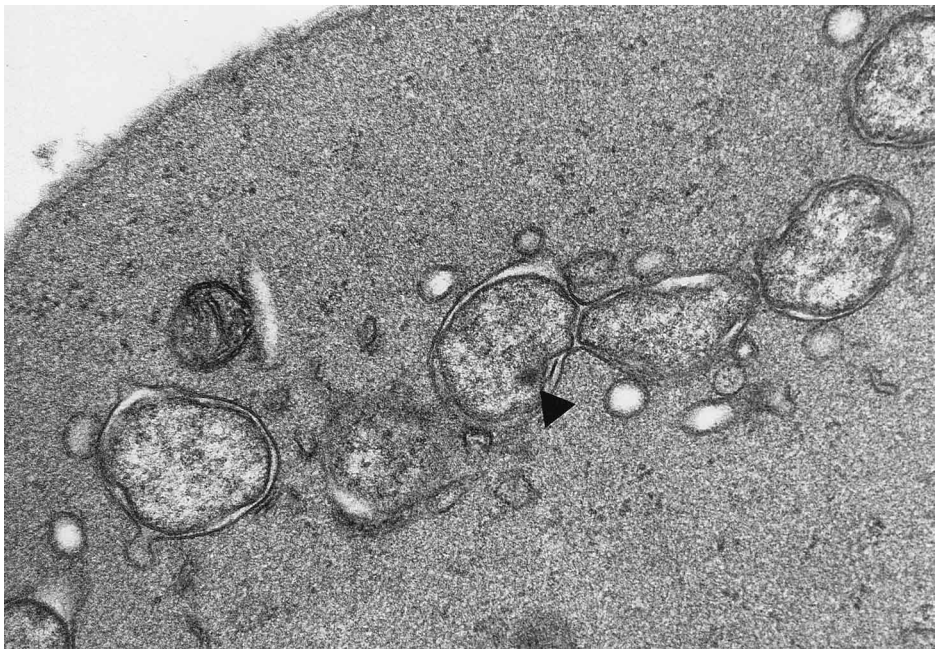

FIG. 3. Transmission electron micrograph of *H. muris* surrounded by the host membrane in the peripheral cytoplasm of a reticulocyte in the blood of a mouse at 10 days postinfection. Note a dense particulate inclusion-like body (black arrowhead). Magnification, ×38,000.

method or the IFA assay method. However, a 700-bp PCR product of the 16S rRNA gene obtained with a pair of primers (primers EC9 and EC10) was detected in the spleens of rats inoculated with TR8563 on days 10 and 15 p.i.

Light and electron micrographs of the organism. When the blood smears of the infected mice were stained with Giemsa on day 10 p.i., numerous small cocci stained bluish purple (Fig. 1). Most of them were attached to the erythrocytes, not to leukocytes, and they were rarely seen free in the plasma. These cocci were seen singly or in chains of two or more. By the IFA test method, numerous organisms were seen on the erythrocyte in blood smears (Fig. 2). Electron microscopic studies were performed with blood samples obtained on day 10 p.i. Most organisms originally attached to the surfaces of the erythrocytes appeared to have been dissociated after the fixation and lost during sample preparation. However, several organisms were observed in deep surface indentations of erythrocytes and reticulocytes and in the membrane-bound inclusions which may be connected to the extracellular space out of the plain of the ultrathin section. The organisms were spherical or ellipsoidal and 350 to 700 nm in diameter. They had a single limiting membrane, no cell wall, ribosomes, fine DNA strands, and occasionally, a dense particulate inclusion similar to those reported previously (27) (Fig. 3).

IFA and Western immunoblotting. The titers of anti-TR8556 (homologous titer, 1:640), anti-TR8563 (homologous titer, 1:640), or anti-TR8564 (homologous titer, 1:1,280) serum against another two strains determined by the IFA test were identical. None of these antisera reacted with any of the rickettsial agents tested. Anti-*E. sennetsu* (homologous titer, 1:1,280), anti-*E. muris* (homologous titer, 1:640), anti-*C. burnetii* (homologous titer, 1:320), anti-*O. tsutsugamushi* (homologous titer, 1:640), and anti-*R. japonica* (homologous titer, 1:640) sera did not react with three isolates or with the other rickettsial agents tested. On the basis of these pathologic, morphologic, biologic, and serologic findings, we conclude that the organism isolated is *H. muris.*

We found that *H. muris* can be dissociated from the erythrocyte surface by incubating it in PBS at 37°C for 1 h. Since *H. muris* causes heavy bacteremia at day 10 p.i., almost pure bacteria could be recovered in the pellet by centrifugation of the dissociated organisms. Western immunoblot analysis of organisms freed from erythrocytes revealed five major antigens (118, 65, 53, 45, and 40 kDa) (Fig. 4). Antisera against three isolates gave identical antigenic profiles when all three isolates were used as antigens.

Comparison of 16S rRNA genes of three strains of *H. muris*. DNA-heteroduplex analysis can detect single base substitutions within a DNA fragment (30). DNA heteroduplex analysis of three PCR-amplified segments of the 16S rRNA genes of three strains of *H. muris* showed that they were identical (Fig. 5).

Amplification of the 16S rRNA gene. By using the universal primers (primers EC9 and EC10) which amplify the 3′ half of the 16S rRNA gene, an expected fragment of 700 bp was

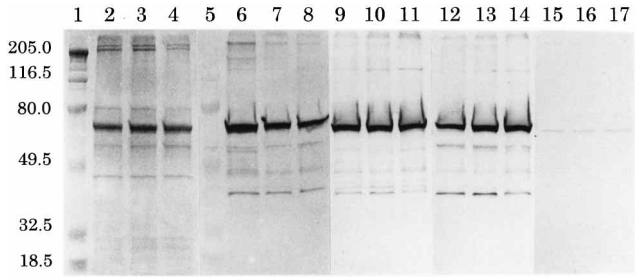

FIG. 4. Western immunoblot analysis of *H. muris* TR8556 (lanes 2, 6, 9, 12, and 15), TR8563 (lanes 3, 7, 10, 13, and 16), and TR8564 (lanes 4, 8, 11, 14, and 17) with mouse anti-TR8556 (lanes 6 to 8), anti-TR8563 (lanes 9 to 11), anti-TR8564 (lanes 12 to 14) sera, and nonimmune mouse sera (lanes 15 to 17). Coomassie-blue stained protein profiles of the three strains are also shown (lanes 2 to 4). Molecular size standards are in lane 2 (Coomassie blue) lane 5 (amido black stain). Molecular sizes are indicated on the left (in kilodaltons).

FIG. 5. PCR-heteroduplex analysis of three segments of the 16S rRNA genes of *H. muris* TR8556 (A), TR8563 (B), and TR8564 (C) amplified with three pairs of primers (segment 1, primers A-17mod and R385; segment 2, primers F312 and R768; segment 3, primers F1112 and EC9). The heteroduplexes of the three strains are as follows: lane 1, A and C; lane 2, A and B; lane 3, B and B; lane 4, B and C.

amplified from one strain of *H. muris*, four strains of *H. felis*, and two strains of *E. suis*. On the basis of consideration of the DNA sequences of these amplified fragments, one internal primer (Haem) was designed. By using primer Haem and a universal 5′ primer (A-17mod), a single fragment of ~900 bases, representing the 5′ portion of the gene, was amplified from all seven strains.

Sequence analyses. The two amplified fragments from all seven strains were sequenced. The sequences of the two amplified fragments overlapped by approximately 200 bases, yielding single continuous partial fragments from each strain representing more than 90% of the 16S rRNA gene (*H. muris*, 1,401 bases; *H. felis* Ohio-Florida, 1,401 bases; *H. felis* California, 1,430 bases). The sequences of two *H. felis* California strains were found to be identical, as were the sequences of the *H. felis* Ohio and Florida strains, while the similarity of the sequences between *H. felis* California and *H. felis* Ohio-Florida was 85%. The sequence of *H. muris* was most similar to that of *H. felis* Ohio-Florida (overall sequence similarity level, 89%; Table 1). In contrast, it was less similar to the sequence of *H. felis* California (similarity level, 85%).

The sequences of the 16S rRNA gene from *E. suis* strains infecting pigs in Illinois and Taiwan were determined for 1,487 and 1,200 bases, respectively (approximately 200 bases could not be obtained from the 3′ end of the Taiwan strain; the reason could not be determined). The sequences of the 1,200 bases that overlapped were found to be identical to each other. Comparison of the *E. suis* sequence with those of the three strains of *Haemobartonella* spp. showed that it was most similar to that of *H. felis* California (92% sequence similarity) and less related to those of the other two strains (approximately 84% sequence similarity; Table 1).

A BLAST search results indicated that the 16S rRNA gene sequences of *Haemobartonella* spp. and *E. suis* were most closely related to those of *Mycoplasma* spp., followed by those of *Ureaplasma* and *Spiroplasma* spp. (Table 1). The similarity of the 16S rRNA gene sequences of *Haemobartonella* spp. and

TABLE 1. Matrix of corrected nucleotide substitution distances and nucleotide sequence similarity

Organism no.	Organism	Distance or percent[a]													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*H. muris*		0.1210	0.1888	0.1772	0.2249	0.2099	0.1983	0.2389	0.2481	0.2780	0.3279	0.3077	0.3379	0.3286	0.3373
2	*H. felis* Ohio-Florida	89.03		0.1793	0.1712	0.2170	0.2087	0.2062	0.2280	0.2451	0.2625	0.3004	0.2888	0.3246	0.2924	0.3127
3	*E. suis*	83.55	84.24		0.0849	0.2429	0.2479	0.2492	0.2790	0.2897	0.2811	0.3603	0.3255	0.3483	0.3397	0.3564
4	*H. felis* Calif.	84.48	84.90	92.03		0.2335	0.2339	0.2372	0.2684	0.2816	0.2784	0.3317	0.3144	0.3344	0.3233	0.3566
5	*M. imitans*	80.93	81.48	79.59	80.28		0.0411	0.1075	0.1186	0.1257	0.1900	0.2617	0.2208	0.2536	0.2770	0.2757
6	*M. pirum*	81.96	82.06	79.80	80.21	96.02		0.1004	0.1149	0.1167	0.1904	0.2596	0.2228	0.2401	0.2567	0.2662
7	*M. muris*	82.78	82.26	79.14	80.00	90.08	90.68		0.1085	0.1130	0.2003	0.2606	0.2288	0.2468	0.2556	0.2874
8	*U. urealyticum*	79.80	80.59	77.11	77.81	89.09	89.41	89.98		0.0368	0.1982	0.2746	0.2420	0.2782	0.2936	0.2997
9	*U. canigenitalium*	79.90	79.33	76.35	76.88	88.48	89.25	89.56	96.41		0.2026	0.2740	0.2410	0.2774	0.2948	0.3068
10	*Spiroplasma* sp.	77.07	78.11	76.85	77.07	83.36	83.32	82.55	82.68	82.34		0.1923	0.1654	0.2114	0.2130	0.2437
11	*L. vitulinus*	73.88	75.52	71.83	73.49	78.11	78.25	78.15	77.26	77.27	83.20		0.1605	0.2067	0.2247	0.2769
12	*B. azotoformans*	75.20	76.33	73.91	74.67	81.11	80.89	10.42	79.44	79.53	85.27	85.62		0.1244	0.1879	0.1940
13	*C. thermoautotrophicum*	73.15	73.93	72.41	73.37	78.64	79.54	79.09	76.89	76.94	81.75	82.00	88.58		0.1741	0.1998
14	*Cyanobacterium* sp.	73.73	76.00	73.00	74.07	77.08	78.39	78.43	75.87	75.77	81.65	80.68	83.51	84.55		0.2307
15	*A. marginale*	73.05	74.54	71.76	71.92	77.05	77.69	76.23	75.44	74.98	79.38	77.11	83.02	82.57	80.31	

[a] Data above the diagonal blank space are corrected average nucleotide substitutions (distance) per site by using the Kimura two-parameter model; data below the diagonal blank space are average percent nucleotide sequence similarity per site. Distance and similarity were calculated for 1,308 bases of aligned sequence remaining after removal of regions of ambiguous alignment.

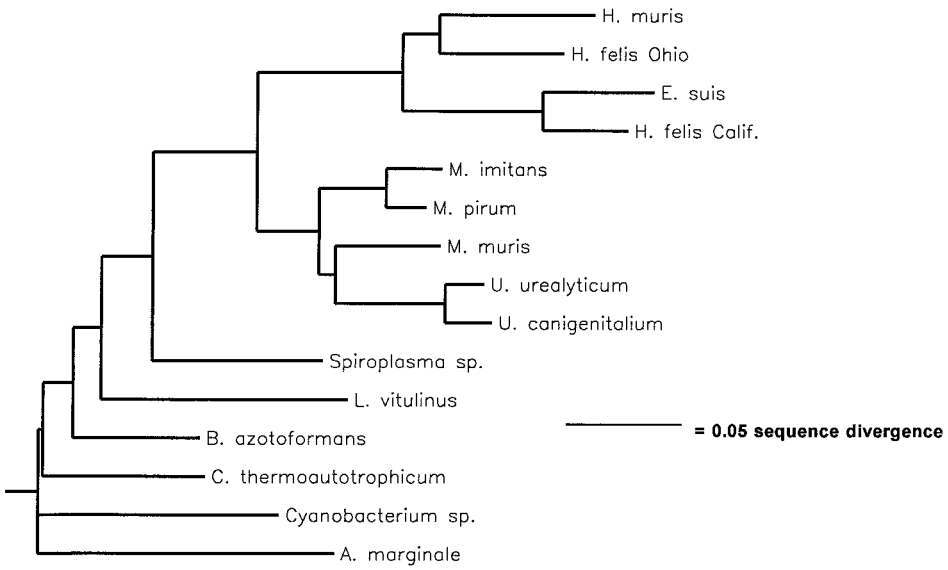

FIG. 6. Phylogenetic relationships of *Haemobartonella* spp. and *E. suis* to *Mycoplasma* spp. and other related bacteria. H. felis Ohio, *H. felis* Ohio-Florida. The bar indicates 5% sequence divergence.

E. suis to those of *Mycoplasma* spp. was approximately 79 to 83%. The sequence similarity to *A. marginale* was significantly lower (72 to 75%). The 16S rRNA gene sequences of the *Haemobartonella* spp. and *E. suis* were less related to those of *Acholeplasma* spp. (the matches between *A. laidlawii* and *H. muris*, *H. felis* Ohio-Florida, *H. felis* California, and *E. suis* were 67.7, 66.7, 64.0, and 69.8%, respectively), *Erysipelothrix* spp. (the matches between *E. rhusiopathiae* and *H. muris*, *H. felis* Ohio-Florida, *H. felis* California, and *E. suis* were 62.6, 66.6, 70.0, and 69.0%, respectively), or *Bartonella* spp. (the matches between *B. henselae* and *H. muris*, *H. felis* Ohio-Florida, *H. felis* California, and *E. suis* were 62.5, 64.8, 55.9, and 55.4%, respectively). The sequences of the 16S rRNA genes of 12 organisms showing the highest degree of homology with the sequences of the three different *Haemobartonella* spp. and the sequence of *E. suis* and representing diverse bacterial groups were chosen to be included in a phylogenetic analysis. Because Bergey's manual includes *Haemobartonella* and *Eperythrozoon* in the family *Anaplasmataceae*, the sequence of the 16S rRNA gene of *A. marginale* was used as a reference. In the phylogenetic analysis, *H. muris*, *H. felis* Ohio-Florida, *H. felis* California, and *E. suis* were joined together in a monophyletic clade that was most closely related to the *Mycoplasma* spp. (Fig. 6). As expected, among the forms of *Haemobartonella*, *H. felis* California clusters with *E. suis* and is separated from *H. felis* Ohio-Florida and *H. muris*, yielding a unique branch (Fig. 6). *E. suis*, the three *Haemobartonella* spp., the *Mycoplasma* spp., the *Ureaplasma* spp., and the *Spiroplasma* spp. clustered together, yielding a large group which was separated from another group that included the gram-positive organisms *Bacillus*, *Lactobacillus*, and *Clostridium* spp. The levels of sequence similarity and evolutionary distances used for phylogenetic analysis are presented in Table 1.

DISCUSSION

The results of this study demonstrate that, on the basis of morphological and antigenic comparisons, the microorganisms that were isolated from wild mice in Japan and that caused splenomegaly and infection of erythrocytes in wild and laboratory mice are *H. muris*. During epidemiological surveys with various rickettsiae or other zoonotic agents, researchers have encountered splenomegaly and/or hepatomegaly in laboratory mice inoculated with the spleen and/or liver homogenates of field-caught rodents. *Babesia* sp., *Eperythrozoon* sp., and *Haemobartonella* sp. found in the blood of wild mice *Apodemus speciosus* or *A. argenteus* have been suspected of being the causative agent of splenomegaly (16, 20). Recently, Fujita et al. (9) reported the prevalence and properties of *Haemobartonella*-like microorganisms isolated from field-caught rodents in Fukushima Prefecture (northeastern Japan). The features of their isolates were very similar to those of the present organisms, isolated in central Japan, suggesting a widespread prevalence of the agent in Japan. They also reported that *A. argenteus* is a species commonly infected with the agent. Besides these hemotropic microorganisms, *E. muris*, a newly recognized monocytic rickettsia in wild mice, is also a causative agent of splenomegaly in wild and laboratory mice (13, 29).

On the basis of biologic and morphologic characteristics, *Haemobartonella* spp. and *Eperythrozoon* spp. have been classified in the family *Anaplasmataceae* (15, 23). However, our results indicate that, on the basis of sequences of the 16S rRNA gene, *Haemobartonella* spp. and *E. suis* do not belong to the family *Anaplasmataceae* or even within the order *Rickettsiales*, but rather are most closely related to forms of *Mycoplasma*. The pathogenic mycoplasmas usually parasitize the mucosal surfaces of the respiratory and urogenital tracts, with rare penetration into the submucosa or blood (18). Although unlike *Haemobartonella* spp. and *Eperythrozoon* spp. many *Mycoplasma* spp. can be cultured in cell-free media, mycoplasmas have limited biosynthetic abilities and require complex media for growth, including amino acids, nucleic acid precursors, lipids, vitamins, and inorganic ions and glucose as an energy source, and certain strains grow poorly or not at all in artificial media (18). In addition, haemobartonellas, eperythrozoons, and mycoplasmas are all sensitive to tetracyclines (15, 18, 23), which specifically inhibit protein synthesis in procaryotes, but because they lack a cell wall, these organisms are not sensitive to penicillin, whose primary target is the biosynthesis of peptidoglycan, a main component of bacterial cell walls.

Although *Haemobartonella* spp. are often speculated to be related to *Bartonella* spp., which are hemotropic parasites and culturable in modified blood agar, our study showed that they are not related at all. Morphologically, *Bartonella* spp. and *Haemobartonella* spp. are quite distinct. *Bartonella* spp. have two membranes (outer and inner membranes), are much larger rods (0.25 to 0.5 by 1.0 to 3.0 μm), and possess unipolar flagella. *Bartonella* spp. are associated with both endothelial cells and erythrocytes, but *Haemobartonella* spp. are associated exclusively with erythrocytes (23a).

The sequence of *E. suis* was most closely related to that of *H. felis* California in the *Haemobartonella* group, suggesting that *E. suis* should be reclassified into the same genus with the three forms of *Haemobartonella*. Although both strains caused anemia in cats, *H. felis* Ohio-Florida and *H. felis* California represented two different species of *Haemobartonella*, since a large divergence (>15%) in their 16S rRNA gene sequences was found, and this divergence separated them. Whether there are any differences in morphology and biologic and pathogenic features among the different *Haemobartonella* strains and *E. suis* remain to be studied. Since the *H. muris* strains studied here produce massive parasitemia and clinical disease in laboratory mice within 10 days p.i., mice provide a convenient laboratory animal model for studying the pathogenesis of *Haemobartonella* strains and the immune response to haemobartonellosis. The heavy parasitemia of *H. muris* also provides sufficient numbers of organisms for antigenic, cellular, and molecular analyses of the organism which are needed in order to further define its taxonomic position.

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