Comparison of *Ehrlichia muris* Strains Isolated from Wild Mice and Ticks and Serologic Survey of Humans and Animals with *E. muris* as Antigen

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In metropolitan Tokyo, the *Ehrlichia muris* seropositivity rate of 24 wild mice was 63% in Hinohara Village, but in the surrounding areas, it was 0 to 5%. This finding suggests that the reservoir of *E. muris* is focal. Among the 15 seropositive mice, ehrlichiae were isolated from 9 *Apodemus speciosus* mice and 1 *A. argenteus* mouse, respectively. Five ehrlichial isolates were obtained from 10 ticks (*Haemaphysalis flava*) collected in Asuke Town, Aichi Prefecture, where the *E. muris* type strain had been isolated. These new isolates were compared with the *E. muris* type strain. The mouse virulence and ultrastructure of the new isolates were similar to those of the type strain, and all of them were cross-reactive with each other, as well as with the type strain, by indirect immunofluorescent-antibody test. The levels of similarity of the base sequences of the 16S rRNA gene of one of the *A. speciosus* isolates and one of the tick isolates to that of the *E. muris* type strain were 99.79 and 99.93%, respectively. We suggest that all of these isolates are *E. muris*; that *E. muris* is not limited to *Eothenomys kageus* but infects other species of mice; and that *E. muris* is present at locations other than Aichi Prefecture. It appears that *H. flava* is a potential vector of *E. muris*. Twenty (1%) of 1803 humans from metropolitan Tokyo were found to be seropositive for *E. muris* antibodies. A serological survey revealed that exposure to *E. muris* or organisms antigenically cross-reactive to *E. muris* occurred among dogs, wild mice, monkeys, bears, deer, and wild boars in Gifu Prefecture, nearby prefectures, and Nagoya City, central Japan. However, human beings and *Rattus norvegicus* rats in this area were seronegative. These results indicate broader geographic distribution of and human and animal species exposure to *E. muris* or related *Ehrlichia* spp. in Japan.

Ehrlichioses are known as important emerging tick-borne diseases in humans, as well as in domestic animals (18–20), and are caused by infection with *Ehrlichia* spp. *Ehrlichia* spp. are obligate intracellular bacteria that belong to the Family *Rickettsiaceae*. *Ehrlichia* spp. can be divided into three distinct genetic groups on the basis of their 16S rRNA gene sequences (19, 20). Group 1 includes *Ehrlichia canis* and *E. ewingii* isolated from dogs outside Japan, *E. chaffeensis*, and a Venezuelan human ehrlichia, likely a strain of *E. canis*, recently isolated from a human (1, 16). In 1983, we isolated an infectious agent inducing splenomegaly in laboratory mice from a wild mouse, *Eothenomys kageus*, caught in Asuke Town, Aichi Prefecture, Japan. This agent was identified as a member of the genus *Ehrlichia* on the basis of morphological and antigenic comparisons (9). Analysis of the sequence of its 16S rRNA gene revealed that the agent is a new *Ehrlichia* sp. designated *E. muris* (21). *E. muris* also belongs to group 1. Recently dogs seropositive for *E. canis* were identified in Japan (24), suggesting the existence of *E. canis* in Japan, but this has yet to be proven. It is unknown whether *E. chaffeensis* exists in Japan. Group 2 includes *E. equi* isolated from horses, *E. phagocytophila* from sheep and goats, and the human granulocytic ehr-

lichiosis agent (3). These three organisms are very closely related and probably belong to the same species. The presence in Japan of *Ehrlichia* spp. from this group has not been examined yet. Group 3 includes *E. risticii*, *E. sennetsu*, and the SF agent from *Stellantchasmus falcatus*. *E. sennetsu* was isolated from a patient's blood in 1953 in Japan (15). Sennetsu fever, caused by *E. sennetsu*, is distributed in southwestern Japan and in Malaysia. The SF agent was isolated from the metacercaria of *S. falcatus* in Japan (8) and is probably a strain of *E. risticii* (22).

So far, only one strain of *E. muris* has been described (9); hence, the strain divergence is unknown. The extent of its geographic distribution, the rodent reservoir, the vector, and the exposure of humans and animals other than wild mice to *E. muris* are also unknown. In this study, we compared *E. muris* isolates from wild mice caught in metropolitan Tokyo and from *Haemaphysalis flava* ticks collected in Aichi, where the *E. muris* type strain was isolated. We also performed a seroepidemiologic study to assess the extent of exposure of humans and animals to group 1 *Ehrlichia* spp.

MATERIALS AND METHODS

Isolation of the infectious agent from wild mice and ticks. In November 1992, 24 wild mice (*Apodemus speciosus* and *A. argenteus*) were caught by using Sherman live traps (H. B. Sherman Traps, Inc., Tallahassee, Fla.) with peanuts as bait in Hinohara Village, Nishitama County, metropolitan Tokyo (Fig. 1). The spleens were aseptically removed and homogenized in 10% (wt/vol) sucrose-phosphate-glutamate buffer (0.0038 M KH_2PO_4, 0.0072 M K_2HPO_4, 0.0049 M L-glutamate, 0.218 M sucrose, pH 7.2). Each homogenate was inoculated intra-

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FIG. 1. The area in metropolitan Tokyo where *E. muris* isolates were collected and seroepidemiological survey of humans and wild mice was performed. ▲, survey point where wild mice were caught.

TABLE 1. Species of wild mice caught in metropolitan Tokyo and numbers of seropositive mice

Area	Total no. of wild mice (no. seropositive)			
	A. speciosus	*A. argenteus*	*E. kageus*	Total
Akikawa City	17			17
Hinohara Village	18 (14)	6 (1)		24 (15)
Hinode Town	30	70	22	122
Hachioji City	73 (4)	5		78 (4)
Oume City	18	13		31

peritoneally into two ddY strain mice at 0.2 ml per mouse. Ten isolates were obtained. All isolates were passaged twice in ddY strain mice.

Ten *H. flava* nymphs collected by flagging over vegetation in Asuke Town from June to September 1994 were tested. One *H. flava* was attached to each of 10 BALB/c mice kept individually in cages placed over a water-filled pan to prevent the ticks from escaping. The five isolates obtained from enlarged spleens were passaged once in mice by inoculating the spleen homogenate as described above. An additional 10 mice with no attached ticks served as controls.

Clinical signs, relative spleen weights, and titers of antibody against the *E. muris* type strain. The 10% spleen homogenates of each mouse infected with *E. muris*, 10 isolates from wild mice caught in Tokyo, and 5 isolates from ticks in Aichi Prefecture were inoculated intraperitoneally at 0.2 ml per mouse into five mice (BALB/c strain). The clinical signs (ruffled fur, inactivity, anorexia, and death) of mice inoculated were observed for up to 20 days postinoculation (p.i.) and compared with those of mice infected with the *E. muris* type strain. After blood collection, the relative spleen weights (in grams per 100 g of body weight) of all mice inoculated were estimated, and titers of antibody against *E. muris* were measured by indirect immunofluorescent-antibody assay (IFA) (9).

Serological comparison between *E. muris* isolates and *E. sennetsu*. The *E. muris* type strain, three isolates (I-268, I-289, and I-306) from *A. speciosus*, one isolate (I-269) from *A. argenteus*, one isolate (NA-1) from a tick, and *E. sennetsu* (Miyayama strain) were used as antigens in the IFA. The antigen and antisera were prepared, and the IFA was performed as previously described (9).

Light and electron microscopic observations. Smear preparations of peritoneal cells of infected mice killed on day 10 p.i. were stained by the Diff-Quik method (International Reagents Corp., Kobe, Japan). For electron microscopy, peritoneal cells of mice infected with strains I-268 and NA-1 were collected at day 10 p.i. by lavage with Eagle's minimum essential medium containing heparin (50 IU) and by centrifugation at 800 × *g* for 10 min. The pellets were fixed overnight at 4°C in a mixture containing 5% glutaraldehyde, 2.5% paraformaldehyde, and 0.03% trinitrophenol in 0.1 M sodium cacodylate buffer (pH 7.4). The cells were washed twice with 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide in 1.5% potassium ferrocyanide for 1 h, block stained with 1% uranyl acetate, dehydrated in a graded ethanol-propylene oxide series, and embedded in Poly/Bed 812 resin (Polysciences, Inc., Warrington, Pa.). Ultrathin sections were stained with uranyl acetate and lead citrate, and the stained sections were examined with a Philips model 300 electron microscope at 60 kV.

Extraction of DNA. Spleen homogenates of mice inoculated with strain I-268 isolated from *A. speciosus* and strain NA-1 isolated from a tick on day 10 p.i. were aseptically prepared at a rate of 10% (wt/vol) in phosphate-buffered saline. The homogenates were centrifuged at 500 × *g* at 4°C, and the supernatants were centrifuged at 8,000 × *g* for 20 min at 4°C. TE buffer (10 mM Tris, 1 mM EDTA, pH 8) containing 1% sodium dodecyl sulfate and 20 mg/ml proteinase K (Wako, Osaka, Japan) was added to the pellets, and these were incubated at 50°C for 2 h. The resulting lysed suspensions were extracted twice with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and with an equal volume of chloroform. The top layers, the aqueous phases, were transferred to fresh tubes, and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold 95% ethanol were added, mixed, and cooled at −70°C for 1 h to precipitate DNA. The tubes were centrifuged at 8,000 × *g* for 20 min, and the pellets were washed in 500 µl of ice-cold 70% ethanol and again centrifuged at 8,000 × *g* for 20 min at 4°C. The ethanol was completely discarded, and the DNA pellets were dissolved in 100 µl of TE buffer.

Analysis of the 16S rRNA gene sequences of isolates. 16S rRNA gene sequences were amplified with primer EC9 (5′-AAGGATCCTACCTTGTTACG ACTT-3′) and EC12 (5′-AATCTAGAGTTTGATCMTGG-3′), which are universal primers for the 16S rRNA gene sequences of prokaryotic cells. PCR was performed as described previously (1). The amplified DNA fragment that was the expected size (approximately 1.5 kb) was purified from a 1% low-melting-temperature agarose gel by using a PCR DNA purification system (Promega, Madison, Wis.). The purified double-stranded DNA was kept at −20°C. The 16S rRNA PCR product was cloned by using the pGEM-T Vector System (Promega), and the insert was sequenced with the ABI PRISM Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Norwalk, Conn.). The primers used for sequencing were EC9, EC10 (5′-AATCTAGATTAGATACCCTDGT AGTCC-3′, where D is A, T, or G), EC11 (5′-AAGGATCCGGACTACHAG GGTATCTAAT-3′, where H is C, T, or A), EC12, U396 (5′-GAAGGCCTTC GGGTTGTA-3′), U1124 (5′-GATAAACTGGAGGAAGGTGGG-3′), L297 (5′-AGACCGTATCTCAGTTCCAGTG-3′), L696 (5′-CAGTGTCAGTATCG AACCAGA-3′), and L1148 (5′-GGGCCGTGCTGACTTGACATC-3′). The sequences of three clones each were determined on both DNA strands.

Computer analysis of DNA sequences. The sequence data obtained were prepared for analysis by using AutoAssembler Version 1.4 (Perkin-Elmer). The corrected levels of nucleotide divergence of 16S rRNA genes were calculated by using Genetic Information Processing Software GENETYX-MAC Version 8.0 (Software Development Co., Tokyo, Japan) on a Power Mac 8500. A phylogenetic tree was constructed by the unweighted pair group method analysis using arithmetic averages and data from the distance matrix.

Serum specimens from humans and animals. Serum samples collected from 24 wild mice used for the isolation of *Ehrlichia*-like organisms from Hinohara Village, Nishitama County in 1992; 122 samples collected from wild mice in Hinode Town in 1992; 17 samples collected from wild mice in Akikawa City in 1990; 78 samples collected from wild mice in Hachioji City in 1992; and 31 samples collected from wild mice in Oume City in 1992 were used (Table 1). Forty *R. norvegicus* serum samples from Shikine Island collected in 1990 and 1,487 human serum samples collected in Itsukaichi Town and 316 human serum samples collected in Oume City from 1991 through 1995 were examined. Serum samples collected from 976 humans with various symptoms in the Hospital of Gifu University in 1995; 699 serum samples collected from dogs in Gifu Prefecture and in Tsukuba City Ibaragi Prefecture, from 1981 through 1989; 48 serum samples collected from bears, 70 collected from monkeys, 20 collected from deer, and 18 collected from wild boars in Gifu or a nearby prefecture from 1991 through 1992; and 221 serum samples collected from wild mice and 327 serum samples collected from *R. norvegicus* in Nagoya City in 1993 were examined.

Serological test using *E. muris* antigen. Titers of antibody against *E. muris* in the sera were measured by IFA (9). Fluorescein isothiocyanate-conjugated anti-human, -dog, -monkey, -mouse, and -rat antibodies were obtained from Organon Teknika (Durham, N.C.) and used at 1:40 (human, dog, and monkey) and 1:30 (mouse and rat) dilutions for the respective serum samples, and fluorescein isothiocyanate-protein G (Zymed Laboratories, Inc., South San Francisco, Calif.) was used at a 1:30 dilution against bear, deer, and wild-boar specimens as the secondary probe. The positive control sera were mouse anti-*E. muris* serum, dog anti-*E. canis* serum, and human anti-*E. chaffeensis* serum (kindly provided by the

FIG. 2. Morulae (arrows) of strain I-268 (*A. speciosus*, Tokyo isolate) in the cytoplasm of murine peritoneal cells stained with Diff-Quik. Magnification, ×2,200.

Centers for Disease Control and Prevention, Atlanta, Ga.). A cutoff titer of 1:10 was chosen based on the result obtained with negative control sera from laboratory-reared mice, rats, and dogs and our previously obtained data on mice and rats (9, 10). However, for human, monkey, bear, wild boar, and deer specimens, such negative control sera were not available.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for the 16S rRNAs used for comparison in this study are as follows: *E. chaffeensis*, M73222; *E. canis*, M73221; *E. sennetsu*, M73225; *E. muris*, U15527. The nucleotide sequences of the 16S rRNAs of strains I-268 and NA-1 have been deposited in the GenBank data library under accession no. AB013008 and AB013009.

RESULTS

Isolation from wild mice and ticks. Of 24 wild mice caught in Hinohara Village, Nishitama County, Tokyo, Japan (Table 1), 15 were seropositive for *E. muris*, having antibody titers of 1:80 to 1:2,560 (Tables 1; see Table 3). Of 18 *A. speciosus* and 6 *A. argenteus* isolates, 9 and 1, respectively, induced splenomegaly. Mice were caught outside the residential area of metropolitan Tokyo. However, not only villagers but also 1 million hikers visit the area annually. Also, five isolates inducing splenomegaly were obtained from 10 *H. flava* ticks collected in Asuke Town, Aichi Prefecture, where the *E. muris* type strain had been isolated (9). Ten control mice without attached ticks in the same environment did not develop splenomegaly. These isolation rates were greater than that in our previous study. The agent which induces splenomegaly in laboratory mice was isolated from only one of six wild-mice (*E. kageus*) caught in Asuke Town in 1983, and after several passages through lab-

oratory mice, the agent was isolated in cell culture, genetically and antigenically characterized, and identified as *E. muris* (9, 21).

Clinical signs, relative spleen size, and titers of antibody against *E. muris*. All BALB/c mice inoculated with agents inducing splenomegaly, including homogenates from wild mice (10 isolates) and *H. flava* (5 isolates), developed clinical signs including ruffled fur, inactivity, and anorexia, but no mice died. Relative spleen weights ranged from 2.4 to 3.9% on day 20 p.i., and titers of antibody against *E. muris* ranged from 1:80 to 1:320 on the same day.

Serological comparison of isolates and *E. muris*. Serologic reactivities were tested among *E. muris*, three isolates (I-268, I-289, and I-306) from *A. speciosus*, one isolate (I-269) from *A. argenteus*, one isolate (strain NA-1) from *H. flava*, and *E. sennetsu* by IFA. Antisera against the isolates reciprocally reacted with each other and with *E. muris*. No antisera against any isolate reacted with *E. sennetsu* and vice versa.

Light and electron microscopic observation. Inclusion bodies were seen in the cytoplasm of peritoneal macrophages of infected BALB/c mice infected with strains I-268 and NA-1 on day 10 p.i. when impression smears were stained with Diff-Quik (Fig. 2). The inclusion bodies (morulae) were compact (1 to 3 μm) and round and stained reddish purple, resembling those of *E. muris* (9).

Electron microscopy, on day 10 p.i., of peritoneal macrophages of BALB/c mice infected with strains I-268 and NA-1
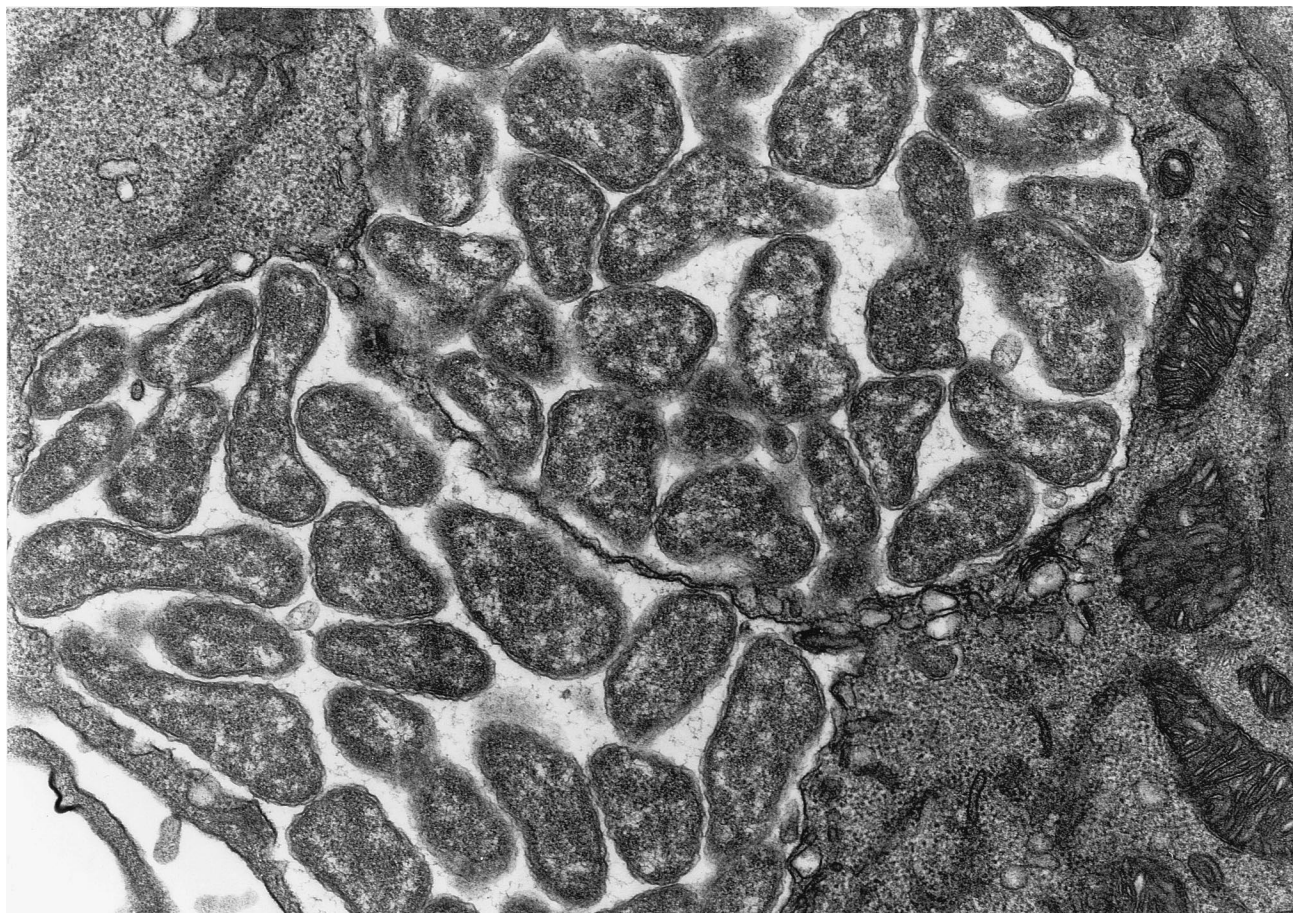
FIG. 3. Electron micrograph of morulae of *E. muris* I-268 (*A. speciosus*, Tokyo isolate) in the cytoplasm of murine peritoneal cells at day 10 postinfection. Note the numerous pleomorphic coccobacilli enveloped in two layers of membranes embedded in a fine filamentous matrix in the membrane-bound inclusion. Magnification, ×22,100.

revealed that the isolates had an ultrastructural morphology compatible with that of *E. muris* (9). Numerous tightly packed organisms were observed in membrane-lined cytoplasmic inclusions. The organisms were pleomorphic and variable in size. Each organism was surrounded by an outer membrane and an inner membrane; the outer membrane was often rippled (Fig. 3 and 4).

Analysis of the 16S rRNA gene base sequence. A single DNA fragment (1.5 kb) was amplified by PCR from the genomic DNAs of strains I-268 and NA-1 by using a pair of primers that flanked the 16S rRNA gene. These DNA fragments were sequenced, and a 1,499-base sequence was obtained. The greatest similarities were found among the sequences of *E. muris*, strain NA-1 isolated from *H. flava* (99.93%) and strain I-268 isolated from an *A. speciosus* wild mouse (99.79%) (Table 2). Comparing the 16S rRNA gene sequences of these isolates, the base G at position 331 changed to a C in strain NA-1, the base T at position 102 changed to a C, a base G was inserted at position 382, and the base C at position 829 was absent in strain I-268. The phylogenetic tree obtained from data is shown in Fig. 5. These results suggest that NA-1 and I-268 are strains of *E. muris*.

Seroepidemiological survey of humans, wild mice, and *R. norvegicus* in metropolitan Tokyo. Although *E. muris* infection of mice induces vigorous polyclonal activation, antigen-specific immune stimulation is impaired, giving low titers of immuno-globulin G and M antibodies against *E. muris* (10). There is no antigenic cross-reactivity between other related intracellular bacteria at a 1:10 dilution (9). All of the negative control sera tested were negative at this dilution. Therefore, this low cutoff value was used for this study. The rate of seropositivity for *E. muris* of wild mice from Hinohara Village was 63% (15 of 24), and the antibody titer was greater than 1:80, with the highest titer measuring 1:2,560 (Table 3). The higher the antibody titer, the greater the *E. muris* isolation rate (Table 3). On the other hand, samples from wild mice collected in other areas, such as Akikawa City, Hinode Village, and Oume City (Fig. 1), rarely contained antibodies against *E. muris*, and *R. norvegicus* rats caught on Shikine Island, off Tokyo, were seronegative. Seropositivity rates in human samples were 1.1% (16 of 1,487) in Itsukaitchi Town and 1.3% (4 of 316) in Oume City, but antibody titers were lower (1:10 to 1:160) than those of wild mice (Table 4). Clinical signs of seropositive humans are unknown but presumably absent or not serious, because these serum specimens were collected as a community service for screening the health status of residents.

Seroepidemiological survey of humans and animals for *E. muris* in Gifu prefecture and Nagoya City. The seropositivity rates of dogs for *E. muris* in Gifu and Tsukuba City were 3.6% (18 of 499) and 5.5% (11 of 200), respectively. Antibody titers were 1:10 to 1:1,280. The seropositivity rates of wild monkeys, bears, deer, and boars were 1.4% (1 of 70), 2.1% (1 of 48), 15%
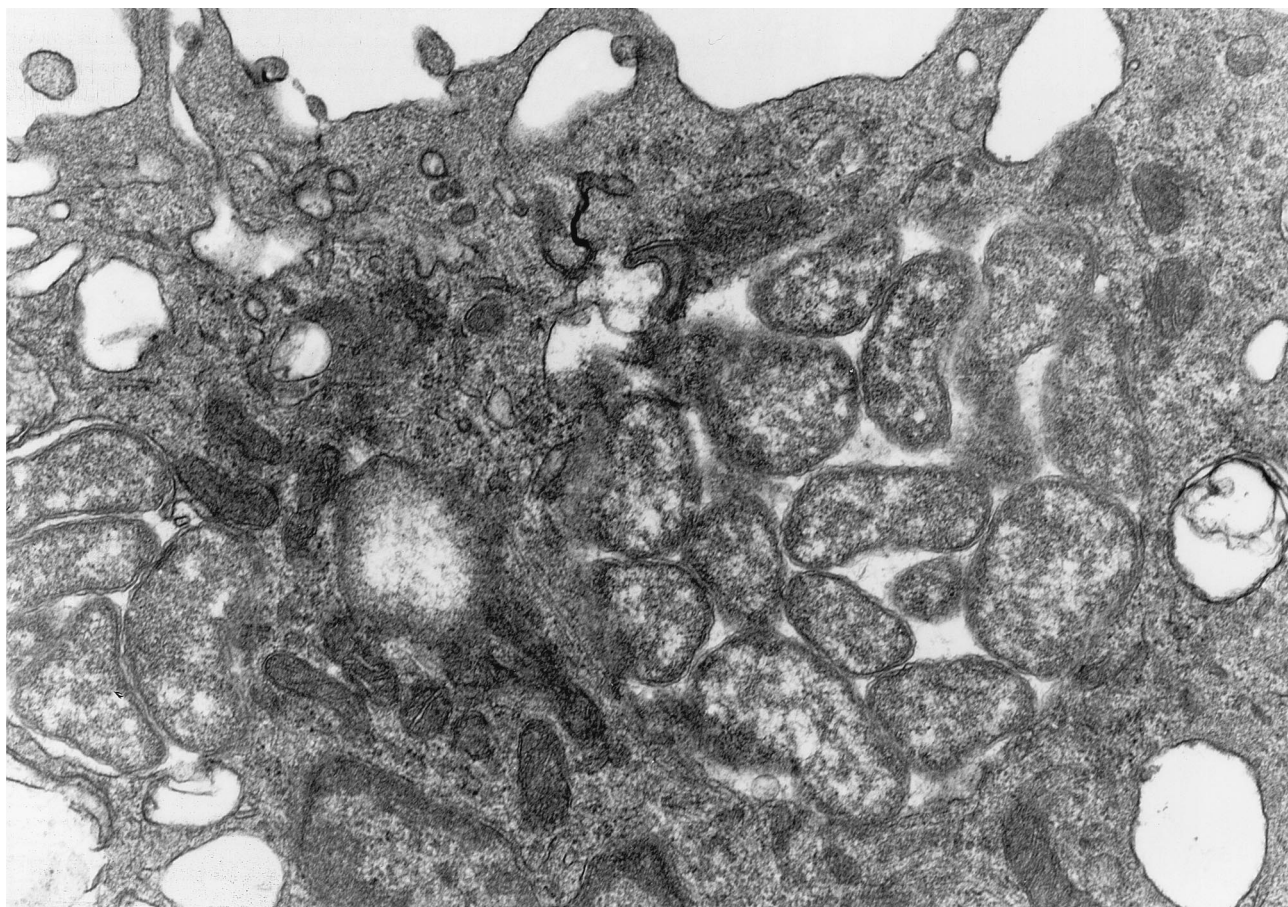
FIG. 4. Electron micrograph of morulae of *E. muris* NA-1 (*H. flava* tick, Aichi isolate) in the cytoplasm of murine peritoneal cells at day 10 postinfection. Note the several pleomorphic coccobacilli in the membrane-bound inclusion. Magnification, ×28,100.

(3 of 20), and 17% (3 of 18), respectively. The range of antibody titers was 1:20 to 1:320. In Nagaya City, the seropositivity rate of wild mice was 9.5% (21 of 221), but no antibody against *E. muris* was detected in 327 *R. norvegicus* rats. Rats in Nagoya City were caught in a park, in vegetable gardens, and in fields. Asuke Town is outside the residential area. Other than villagers, no hikers are known to visit this area. All 976 human sera collected at a hospital in Gifu Prefecture were negative (Table 4).

TABLE 2. Levels of similarity and evolutionary distances between 16S rRNA gene sequences

Organism	Nucleotide substitution distances or nucleotide sequence similarity[a]					
	E. muris	NA-1	I-268	*E. chaffeensis*	*E. canis*	*E. sennetsu*
E. muris		0.0011	0.0012	0.0103	0.0204	0.1034
NA-1	99.93		0.0021	0.0113	0.0214	0.1044
I-268	99.79	99.72		0.0104	0.0205	0.1035
E. chaffeensis	97.72	97.65	97.58		0.0155	0.0985
E. canis	96.13	96.06	95.99	97.44		0.1018
E. sennetsu	83.75	83.68	83.61	83.86	83.40	

[a] The values on the upper right are average numbers of substitutions per sequence position (evolutionary distances), adjusted as described by the Kimura two-parameter model for multiple substitutions at individual positions and calculated for 1,428 positions which could be aligned unambiguously. The values on the lower left are levels of fractional nucleotide identity between sequences.

DISCUSSION

Based on pathologic findings, electron microscopic observations, serological cross-reactivity testing, and sequencing of 16S rRNA genes, 10 isolates from wild mice (*A. speciosus* and *A. argenteus*) caught in Hinohara Village, metropolitan Tokyo, and 5 isolates from *H. flava* ticks collected in Aichi Prefecture belong to *E. muris*. The geographic distributions of *E. kageus* and *A. speciosus* overlap in Japan. *A. speciosus* is the most common mouse in mountainous areas of Japan, while *E. kageus* is rare. The isolation of *E. muris* from *A. speciosus* and *A. argenteus* wild mice caught in Tokyo, about 320 miles away
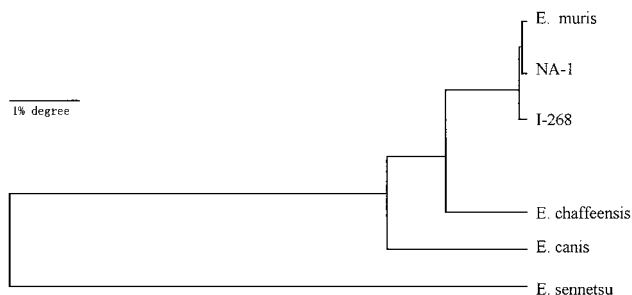

FIG. 5. Phylogenetic relationships between our isolates of strains NA-1 and I-268 and other members of the tribe *Ehrlichieae*.

TABLE 3. Presence of antibody to *E. muris* in wild mice and humans in Tokyo

Animal and area	No. of samples	No. of positive sera	% of positive sera[a]	No. of specimens with following antibody titer (no. of wild mice from which ehrlichia were isolated):									
				<10	10	20	40	80	160	320	640	1,280	2,560
Wild mice from:													
Akikawa City	16	0	0	16									
Hinohara Village	24	15	63	9				1	1 (1)	1	6 (3)	3 (3)	3 (3)
Hinode Town	122	0	0	122									
Hachiouji City	78	4	5	74	1	1		1	1				
Oume City	31	0	0	31									
R. norvegicus from: Shikine Island	40	0	0	40									
Humans from:													
Itsukaichi Town	1,487	16	1	1,471	2	7	2	2	3				
Oume City	316	4	1	312	1	2			1				

[a] A titer of >1:10 is considered positive.

from Aichi Prefecture, where the type strain of *E. muris* had been isolated from an *E. kageus* wild mouse, suggests that *E. muris* is widely distributed among different species of wild mice in Japan. The seroprevalence rate among wild mice in Hinohara Village, where 10 strains of *E. muris* were isolated, was very high (63%), whereas the seroprevalence rates in the surrounding areas were very low. Thus, this small area appears to be a hot spot for *E. muris* infection similar to the prevalence observed for *Orientia tsutsugamushi* (11).

Antibodies against *E. muris* were detected in human sera collected in Itsukaichi Town and Oume City, Tokyo. Although titers were lower than those in wild mice, this is the first report in Japan of serologic evidence of human exposure to an *Ehrlichia* sp. other than *E. sennetsu*. There is a possibility that these people were infected with an *Ehrlichia* sp. belonging to group 1, including *E. chaffeensis*, *E. canis*, or *E. muris*. Antigenic cross-reactivity is small among different groups of *Ehrlichia* spp. Since we did not detect this antibody in any of human sera collected in Gifu Prefecture, the seropositive reactions do not appear to be nonspecific. Further investigation is needed to determine whether this difference is related to geographic factors.

Dogs seropositive for *E. canis* in western Japan were previously reported, and the positivity rate was 1.9%, and the highest reported antibody titer was 1:360 (24). In the current study, the rate of seropositivity for *E. muris* was 3.6% in dogs in Gifu Prefecture and 5.5% in dogs in Tsukuba City. The highest antibody titer was 1:1,280. This result suggests that using *E. muris* as an IFA antigen is more effective for screening of dogs exposed to group 1 *Ehrlichia* spp. than using *E. canis* in Japan. Dogs were shown to be susceptible to infection with *E. chaffeensis* (6), and of 38 dogs from southeastern Virginia, 8 were positive by *E. chaffeensis*-specific PCR (5). Therefore, as in the United States, some of these dogs in Japan may be infected with *E. chaffeensis* rather than *E. canis* or *E. muris*. Again, isolation of the organisms from dogs is needed to confirm these observations and characterize the infectious agent. Furthermore, several other wild animals also had antibodies against *E. muris*, but their highest titers were lower than those in dogs.

A total of 9.5% of the wild mice caught in Nagoya City had antibodies against *E. muris*. The existence of this antibody in wild mice caught in Nagoya City indicates that *Ehrlichia* spp. exist not only in mountainous areas but also in urban areas. We did not detect antibodies against *E. muris* in any rats caught on Shikine Island, in metropolitan Tokyo, or in Nagoya City, and these results agree with our previous experiments, which found that rats are resistant to *E. muris* infection (9). In the United States, the major wild-animal reservoir of *E. chaffeensis* is white-tailed deer (13), and eight species of wild rodents from the southeastern United States, where *E. chaffeensis* infections of deer and humans have been confirmed, are negative for antibodies to *E. chaffeensis* (14). In Japan, it remains to be determined which animal species are reservoirs for potential human ehrlichial infection.

The isolation of *E. muris* from *H. flava* ticks suggests that *H. flava* is a potential vector for *E. muris* transmission among wild rodents. According to the available literature, *H. flava* is seen only in Japan and Korea (23). In Japan, *H. flava* is found on all four major islands. *H. flava* adults take blood meals on hares and dogs, but they have also been found in considerable numbers on cows, horses, wild boar, deer, and bears. Immature forms of the tick were found on the same hosts as adults, as well as on birds and small rodents. All of the rodents described above (*A. argenteus*, *A. speciosus*, and *E. kageus*) are bitten by immature stages of *H. flava*, which is one of the most common ticks found in mountainous areas of Japan. From a public health viewpoint, *H. flava* is one of the most important ticks because of its probable role in the epidemiology of tularemia on Honshu Island in Japan. Whether these *E. muris*-infected *H. flava* nymphs serve as vectors for human transmission remains to be studied. By screening 140 pools of 1,579 total ticks consisting of *Amblyomma americanum*, *Dermacentor variabilis*,

TABLE 4. Antibody to *E. muris* in animal and human sera detected by IFA

Animal (capture site or residence)	No. of samples	No. of positive samples	% positive	Range of antibody titer	Mean titer ± SEM
Dog (Gifu)	499	18	3.6	10–1,280	126 ± 38
Dog (Tsukuba)	200	11	5.5	10–640	62 ± 38
Wild monkey (Gifu)	70	1	1.4	160	160
Wild bear (Gifu)	48	1	2.1	160	160
Wild deer (Gifu)	20	3	15.0	20–80	40 ± 18
Wild boar (Gifu)	18	3	17.0	40–320	101 ± 24
Wild mouse (Nagoya)	221	21	9.5	10–640	50 ± 33
Wild *R. norvegicus* (Nagoya)	327	0	0		
Human (Gifu)	976	0	0		

and *Ixodes scapularis* by *E. chaffeensis*-specific PCR, Anderson et al. (2) found positive reactions in *A. americanum* adults but not in nymphs or in the other two species of ticks. This report suggests either that transstadial transmission is very inefficient or that it does not occur and that adult ticks which are infected as nymphs transmit *E. chaffeensis* to humans. *Rhipicephalus sanguineus* ticks infected as nymphs were shown to transmit *E. canis* to dogs (12). *Anaplasma marginale*, which belongs to *Ehrlichia* sp. group 2, is more efficiently transmitted to cattle by transstadial transmission by adult male *Dermacentor andersoni* ticks than by adult ticks infected as nymphs (7). Whether transstadial transmission also occurs in ehrlichial infection remains to be studied.

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