the monkeys infected by *Y. pseudotuberculosis* and was negative for the other samples, even after 60 min of incubation (Fig. 3). Thus, this result shows the high specificity of this method for detection of *Y. pseudotuberculosis* in clinical specimens.

Furthermore, as complicated thermoregulators are not needed to carry out the reactions and LAMP amplicons can be detected by visually confirming a white precipitate of magnesium pyrophosphate, this method might also be a useful and powerful tool for the screening and detection of *Y. pseudotuberculosis* in the field. Thus, further studies applying this LAMP method to detect this bacterium in food and environmental samples should be carried out.

We thank Hiroshi Fukushima (The Shimane Prefectural Institute of Public Health and Environmental Science, Shimane, Japan) for kindly providing us with *Y. pseudotuberculosis* strains. We also thank Keiko Watanabe (Eiken Chemical Co., Ltd.) for technical assistance.

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Microbiol. Immunol., 49(1), 1–7, 2005

Yersinia enterocolitica Serovar O:8 Infection in Breeding Monkeys in Japan

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Received May 12, 2004; in revised form, October 21, 2004. Accepted November 1, 2004

***Abstract*: In the period from December 2002 to January 2003, 5 of 50 squirrel monkeys (*Saimiri sciureus*) housed at a Zoological Garden in the Kanto region of Japan died following a few days' history of diarrhea. After this outbreak had ended in the squirrel monkeys, 1 of 2 dark-handed gibbons (*Hylobates agilis*) died in April of 2003, showing similar clinical signs. We examined the organs of 3 of the dead squirrel monkeys and of the dark-handed gibbon, and *Yersinia enterocolitica* serovar O:8, which is the most pathogenic serovar of *Y. enterocolitica*, was isolated. In order to determine the source and the transmission route of infection, 98 fecal samples (45 from squirrel monkeys, 20 from other monkeys of 18 different species, and 33 from black rats captured around the monkey houses) and 7 water samples were collected in the Zoological Garden, and were examined for the prevalence of *Y. enterocolitica* serovar O:8. Serovar O:8 was isolated from 21 of 65 monkeys (32.3%) and 5 of 33 (15.2%) black rats (*Rattus rattus*). Furthermore, we examined the 30 isolates using molecular typing methods, pulsed field gel electrophoresis (PFGE), ribotyping using the RiboPrinter system, and restriction endonuclease analysis of virulence plasmid DNA (REAP), and compared the isolates in this outbreak with Japanese O:8 isolates previously identified. Genotyping showed that almost all the isolates were identical, and the genotype of the isolates was highly similar to that from wild rodents captured in Niigata Prefecture. This is the first report of fatal cases of *Y. enterocolitica* serovar O:8 infection in monkeys anywhere in the world.**

***Key words*: *Yersinia enterocolitica* serovar O:8, Pulsed field gel electrophoresis (PFGE), Ribotyping, Breeding monkey**

The term yersiniosis refers to infections caused by either *Yersinia enterocolitica* or *Yersinia pseudotuberculosis*, which appear as enteritis and sometimes septicemia in humans and animals (17, 26). Monkey species, especially New World monkeys such as the squirrel monkey (*Saimiri sciureus*), seem to be sensitive to *Y. pseudotuberculosis*. Many cases of yersiniosis in breeding monkeys have been reported, and *Y. pseudotuberculosis* in particular frequently causes fatal infection (6, 15, 21, 25, 27, 29, 32, 34, 37, 39). There have also been some reports of monkey infection with pathogenic *Y. enterocolitica* (3, 7, 28, 31, 36); however, no such infection has yet been reported in Japan. We report here on an outbreak of *Y. enterocolitica* serovar O:8, the most pathogenic serovar of this bacterium (4, 12, 23), in breeding monkeys at a Zoological Garden in the Kanto region of Japan, which we observed in the process of investigating occurrences of the *Yersinia* infection in breeding monkeys.

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Materials and Methods

Case history. Between December 2002 and January

Abbreviations: CFU, colony forming unit; IN, irgasan-novobiocin; LB, Luria-Bertani, NT, not tested; PBS, phosphate-buffered saline; PFGE, pulsed field gel electrophoresis; REAP, restriction endonuclease analysis of virulence plasmid DNA; TSA, trypticase soy agar; *Y. enterocolitica*, *Yersinia enterocolitica*; *Y. pseudotuberculosis*, *Yersinia pseudotuberculosis*.

2003, 5 of 50 squirrel monkeys housed at a Zoological Garden in the Kanto region located in central Honshu island, Japan, died following a few days' history of diarrhea. This outbreak was ended by treatment with antibiotics; however, despite this treatment, 1 of 2 dark-handed gibbons (*Hylobates agilis*) also died in April 2003, showing similar clinical signs. All of the dead monkeys showed severe enteritis, swelling of Peyer's patch and multiple abscesses in the spleen and liver.

This Zoological Garden keeps many species of monkeys. Squirrel monkeys are housed in an indoor-outdoor enclosure located on the northern edge of the Garden, and many small wild animals, such as small rodents, have easy access to the outdoor area. Additionally, the other monkeys are housed in outdoor cages which are about 50 m from the squirrel monkey enclosure.

Specimens. After 3 of the squirrel monkeys and the dark-handed gibbon died, their bodies were immediately transported to the laboratory, where they were dissected and their organs aseptically removed. Fecal samples were collected from 45 squirrel monkeys, from 20 other monkeys of 18 different species and from 33 black rats (*Rattus rattus*) captured around the monkey houses. Water samples were also collected at 7 points, drains and pools, in the monkey houses. All samples were immediately transported to the laboratory under cool conditions and examined for the presence of *Yersinia* spp.

Isolation and identification of Yersinia *spp.* The organs (liver, spleen, lung, small intestine, and intestinal content) of the dead monkeys were homogenized in phosphate-buffered saline (PBS; pH 7.2) and 10-fold serial dilutions of the suspension were plated on irgasan-novobiocin (IN) agar plates (10). All PBS suspensions were incubated at 4 C for 3 weeks as a cold enrichment. Thereafter, the suspensions were submitted to alkali treatment by mixing 0.1 ml of the suspensions with 0.9 ml of 0.4% KOH for 20 sec, and plated on IN agar (2). Feces (about 1 g) were suspended in 9 ml of PBS, and the PBS suspensions were treated as described above. Water samples were centrifuged for 15 min at 8,000 rpm, and the sediments were resuspended in PBS for obtaining 200-fold concentrations of the components of the original suspension, following the method of Fukushima (9). These concentrations were then plated on an IN agar plate after alkali treatment.

The plates were incubated at 25 C for 48 hr. Colonies morphologically similar to those of *Yersinia* spp. were subcultured with trypticase soy agar (TSA; BBL, Sparks, Md., U.S.A.) for biochemical examination. In brief, biochemical characteristics were examined on triple sugar iron medium (Eiken Chemical Co., Ltd., Tokyo), lysine indole motility medium (Nissui Pharmaceutical Co., Ltd., Tokyo), and urea broth (Eiken). If the following typical reactions of *Yersinia* spp., glucose and urease positive, gas and lysine decarboxylase and H_2S negative, were seen, additional biochemical tests were performed with the methods of Wauters et al. (40). Serotyping of *Y. enterocolitica* was accomplished by slide agglutination with a commercial rabbit anti-*Y. enterocolitica* sera set (Denka-Seiken, Co., Tokyo). To evaluate potential pathogenicity, *Y. enterocolitica* serovar O:8 isolates were examined for temperature-dependent calcium requirement by the method of Gemski et al. (11) and for temperature-dependent autoagglutination by the method of Laird and Cavanaugh (24).

Pulsed field gel electrophoresis (PFGE). Chromosomal DNAs from strains cultured overnight at 25 C in 10 ml of Luria-Bertani (LB) broth (Difco, Detroit, Mich., U.S.A.) were prepared using a CHEF Bacterial Genomic DNA Plug Kit (Bio-Rad Laboratories, Inc., Hercules, Calif., U.S.A.) according to the manufacturer's instructions. The DNAs were digested by the restriction enzyme *Not*I (TaKaRa, Shiga, Japan) in the reaction mixture. The DNA fragments were separated in 1.2% agarose NA (Amersham Pharmacia Biotech, Uppsala, Sweden) that was prepared in 0.5× Tris-borate-EDTA buffer (50 mM Tris base, 50 mM boric acid, 2 mM EDTA) on a CHEF-DRIII Pulsed Field Electrophoresis System (Bio-Rad). Electrophoresis was carried out for 24 hr at 14 C and 200 V with pulse times of 1 to 25 sec following the method of Buchrieser et al. (5). A CHEF DNA Size Standard Lambda Ladder (Bio-Rad) was used as a DNA size marker. The gels were stained with ethidium bromide for 2 hr, destained in distilled water, and photographed under UV light. Relatedness among PFGE patterns was analyzed based on the guidelines described by Tenover et al. (38). Criteria for interpreting PFGE patterns described by Tenover et al. is as follows. Isolates might be considered identical when PFGE patterns contain the same number and sizes of fragments; closely related when they differ by one to three bands; possibly related when they differ by four to six bands; and different when they differ by seven or more bands.

Ribotyping. Ribotyping was performed using the RiboPrinter System (Qualicon, Inc., Wilmington, Del., U.S.A.), and proprietary reagents (Qualicon), according to the manufacturer's instructions. Bacterial strains were incubated at 25 C for 48 hr on TSA and suspended in 200 μl of sample buffer. Thirty microliters of the suspension were transferred to the sample carrier and heated at 80 C for 10 min. After adding lytic enzymes and loading the bacterial cells and all the consumables into the system, the bacterial cells were automatically lysed, and the released DNA was digested with a restriction endonuclease *Eco*RI. The DNA restriction

fragments were size-separated by electrophoresis on an agarose gel, transferred to a nylon membrane, denaturated and hybridized with a labeled rRNA operon probe. After the addition of a chemiluminescent substrate, the light intensity of the obtained targeted DNA fragments composing DNA fragment patterns, namely ribopatterns, were captured by a customized CCD camera, converted to digital information, and stored in the system's computer data base. The ribopatterns with a similarity coefficient higher than 0.93 were considered identical by the RiboPrinter software and were grouped together in a same ribotype.

Restriction endonuclease analysis of plasmid DNA (REAP). Plasmid DNA was prepared following the method described by Kado and Liu (20) with some modifications. Briefly, REAP was performed with the enzymes *Eco*RI (TaKaRa) and *Bam*HI (TaKaRa) as described by Nesbakken et al. (30). Electrophoresis was performed for 105 min at 50 V in a 1.2% agarose NA gel using a Mupid-2 (Advance Co., Tokyo). A 1 kb PLUS DNA ladder (Invitrogen Co., Carlsbad, Calif., U.S.A.) was used as a DNA size marker. The gels were stained with ethidium bromide for 10 min, and photographed under UV light.

Table 1. Isolation of *Yersinia enterocolitica* serovar O:8 from organs of dead monkeys

No.	Monkey species	The number of bacteria in organs (log CFU/g)					
		Liver	Spleen	Lung	Small intestine	Intestinal content	Mandibular abscess [c]
1	Common squirrel monkey (*Saimiri sciureus*)	6.7	7.8	6.7	8.6	7.7	NT[b]
2	Common squirrel monkey (*Saimiri sciureus*)	+[a]	+[a]	3.9	2.5	+[a]	NT[b]
3	Common squirrel monkey (*Saimiri sciureus*)	NT[b]	NT[b]	NT[b]	NT[b]	NT[b]	+[a]
4	Dark-handed gibbon (*Hylobates agilis*)	8.0	7.9	6.1	NT[b]	6.3	NT[b]

[a] Detected after cold enrichment (the case of mandibular abscess, detected with smear culture).
[b] Not tested.
[c] Smeared culture.

Table 2. Isolation of *Yersinia enterocolitica* serovar O:8 from breeding monkeys and environmental materials in the Zoological Garden

	Source	Number of animals examined	Number of serovar O:8 isolates (%)
Breeding monkeys	Common squirrel monkey (*Saimiri sciureus*)	45	17 (37.8)
	Common chimpanzee (*Pan troglodytes*)	2	1 (50.0)
	Crab-eating macaque (*Macaca fascicularis*)	1	1 (100.0)
	De Brazza's monkey (*Cercopithecus neglectus*)	1	1 (100.0)
	Vervet monkey (*Cercopithecus aethiops*)	1	1 (100.0)
	Geoffroy's spider monkey (*Ateles geoffroyi*)	2	0 (0.0)
	Abyssinian colobus (*Colobus guereza*)	1	0 (0.0)
	Black-capped capuchin (*Cebus apella*)	1	0 (0.0)
	Black spider monkey (*Ateles paniscus*)	1	0 (0.0)
	Dark-handed gibbon (*Hylobates agilis*)	1	0 (0.0)
	Hamadryas baboon (*Papio hamadryas*)	1	0 (0.0)
	Japanese macaque (*Macaca fuscata*)	1	0 (0.0)
	Lesser spot-nosed monkey (*Cercopithecus petaurista*)	1	0 (0.0)
	Lion-tailed macaque (*Macaca silenus*)	1	0 (0.0)
	Mandrill (*Mandrillus sphinx*)	1	0 (0.0)
	Patas monkey (*Erythrocebus patas*)	1	0 (0.0)
	Ruffed lemur (*Varecia variegata*)	1	0 (0.0)
	White-handed gibbon (*Hylobates lar*)	1	0 (0.0)
	White-throated capuchin (*Cebus capucinus*)	1	0 (0.0)
	subtotal	65	21 (32.3)
Environmental materials	Black rat (*Rattus rattus*)	33	5 (15.2)
	Water	7	0 (0.0)
	subtotal	40	5 (12.5)
	Total	83	26 (31.3)

Results

Isolation of Y. enterocolitica *Serovar O:8 from Monkeys and Black Rats*

The numbers of *Y. enterocolitica* serovar O:8 (log CFU/g) isolated from each organ are summarized in Table 1. This pathogen was isolated from all tested organs of the dead monkeys. From No. 1 and 4 especially, high numbers of viable bacteria (log CFU/g) were isolated from livers (6.7 and 8.0), spleens (7.8 and 7.9), lungs (6.7 and 6.1), small intestine (8.6 in No. 1) and intestinal contents (7.7 and 6.3). From the other fecal samples examined, serovar O:8 was isolated from 21 of 65 monkeys (32.3%; 17 squirrel monkeys, 1 chimpanzee, 1 velvet monkey, 1 De Brazza's monkey, and 1 crab-eating macaque) and 5 of 33 (15.2%) black rats, but it was not isolated from any water samples (Table 2).

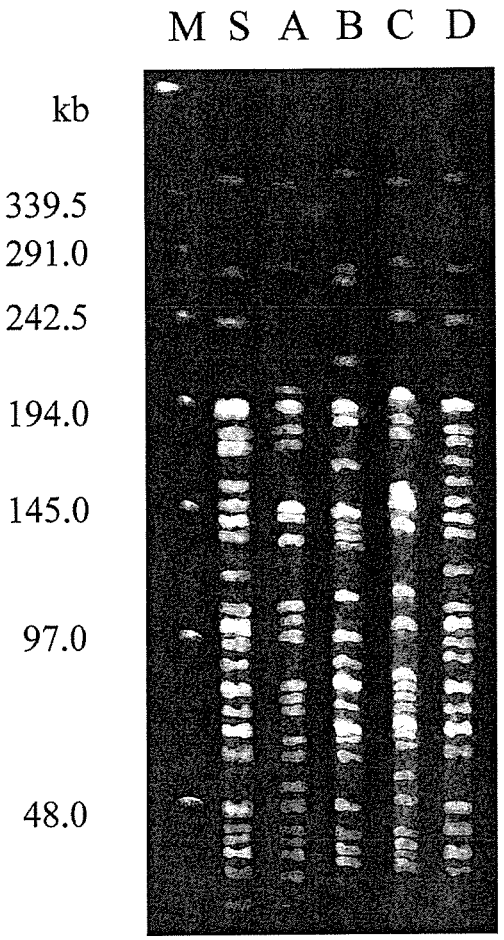

Fig. 1. Comparison of the PFGE patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes A to D). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); A, YE89023 (human patient); B, NY936005 (wild rodent); C, NY9504002 (wild rodent); D, YE9809001 (wild rodent).

All isolates showed a positive reaction for virulence-associated properties such as calcium dependency and autoagglutination, and harbored a 70-kb virulence plasmid (data not shown). These isolates were therefore identified as pathogenic serovar O:8 strains.

Molecular Genotyping of Y. enterocolitica *Serovar O:8 Isolates*

The 30 isolates, consisting of 4 isolates from the organs of the dead monkeys (1 from each monkey), and 26 isolates from the other samples, were analyzed using molecular genotyping methods and showed the same PFGE pattern S (Fig. 1). Japanese O:8 isolates were previously identified and classified into four patterns designated as A to D (14). Compared with those patterns, our pattern S was almost identical to pattern D, differing by only two bands; according to the proposal made by Tenover et al. (38), these strains can thus be considered closely related.

The same strains were grouped into two ribotypes by the RiboPrinter (Fig. 2): 29 isolates were identified as S1, and 1 isolate from a black rat was identified as S2. Comparing these two ribotypes with the four ribotypes (R1 to R4) obtained from the same Japanese isolates submitted to PFGE, the ribotype S1 strains were assigned to the same R1 ribotype group, while S2 was assigned to ribotype R2 (14).

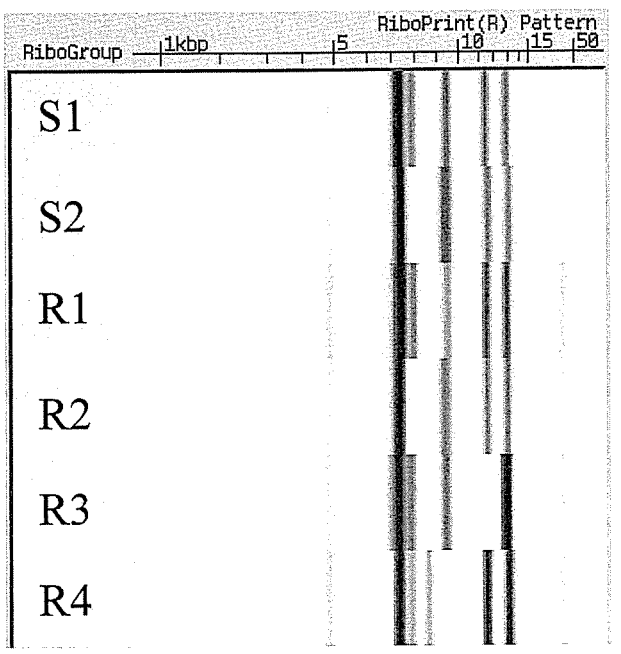

Fig. 2. Comparison of the ribotypes of representative outbreak isolates (lanes S1 and S2) and Japanese isolates (lanes R1 to R4). Lanes: S1, NY0212001 (squirrel monkey); S2, NY0304008 (black rat); R1, NY936005 (wild rodent); R2, YE9809001 (wild rodent); R3, YE87069 (human patient); R4, YE89023 (human patient).

A comparison of the REAP patterns obtained from the outbreak isolates and other Japanese isolates is shown in Fig. 3. All outbreak isolates showed the same REAP pattern, designated as pattern S, while the patterns of the other Japanese isolates have been identified as P1 to P3 (14). REAP pattern S corresponds to pattern P3.

Japanese O:8 isolates have been classified into seven genotypes (I–VII) based on the combination of the results of PFGE by *Not*I and ribotyping by *Eco*RI (14). Our 30 outbreak isolates were classified into two genotypes, with 29 belonging to a genotype similar to the genotype VI of the isolates from wild rodents captured in Niigata Prefecture, and 1 outbreak isolate from a black rat belonging to a genotype similar to the genotype VII of the isolate from wild rodent captured in Yamagata Prefecture (Table 3).

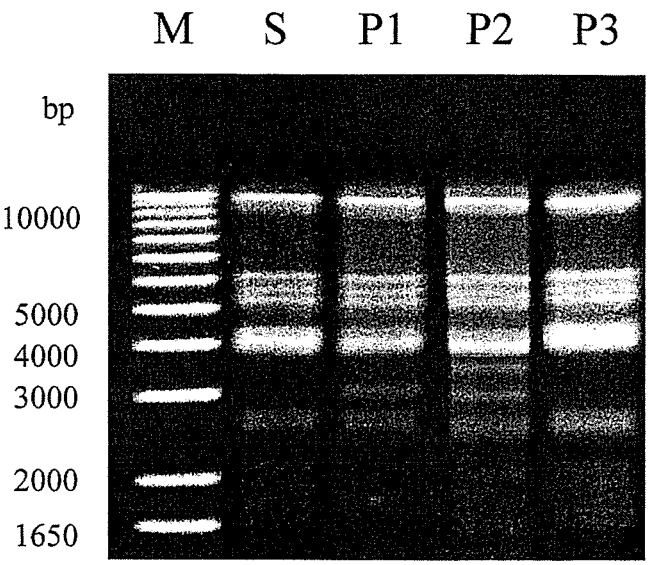

Fig. 3. Comparison of the REAP patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes P1 to P3). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); P1, NY9306089 (wild rodent); P2, NY936005 (wild rodent); P3, YE9809001 (wild rodent).

Discussion

To the best of our knowledge, this is the first report of fatal cases of *Y. enterocolitica* serovar O:8 infection in monkeys anywhere in the world. There have been some reports of *Yersinia* spp. outbreaks in monkeys all over the world showing that monkeys are sensitive to bacteria of this genus (6, 15, 21, 31, 39). However, the majority of these outbreaks, which usually affect a large number of monkeys and show high mortality, are caused by *Y. pseudotuberculosis*. *Yersinia* outbreaks in breeding monkeys have also been reported in Japan, but all reported outbreaks were of *Y. pseudotuberculosis* (15, 21, 27, 29, 34, 39). Therefore, the present study indicates the need for more attention to the possibility of the occurrence of *Y. enterocolitica* serovar O:8 outbreaks, especially in countries such as the United States and Japan, where reservoirs of this serovar are found in nature (1, 8, 13, 18, 19, 22, 33, 35).

Moreover, although a human case of *Y. enterocolitica* serovar O:8 infection was reported in 1997, in Kanagawa Prefecture (16), located at the south of the Kanto region that lies in the central part of Honshu island, isolation of this pathogen from rodents has been reported only in wild rodents of the north area of Honshu island (13, 19). This study showed the presence of this pathogen in black rats living in the Kanto region, indicating that serovar O:8 strains are also present in this area.

Molecular genetic analysis of the 30 isolates from the analyzed samples showed that all but one had the same molecular genotype, suggesting that these isolates

Table 3. Typing results for *Yersinia enterocolitica* serovar O:8 isolated in Japan using PFGE and ribotyping

Genotype[a)]	PFGE pattern	Ribopattern	REAP pattern	Strain	Region	Source
I	A	R1	P1	YE93009	Aomori	Patient
II	A	R4	P1	YE89023	Aomori	Patient
III	B	R1	P2	APCC Y9314	Aomori	Patient
IV	B	R3	P2	YE87069	Aomori	Patient
V	C	R1	P1	NY9504002	Aomori	Wild rodent
VI	D	R1	P3	NY891001	Niigata	Wild rodent
VII	D	R2	P3	YE9809001	Yamagata	Wild rodent
VI′	D′	R1	P3	NY0212001	Saitama	Squirrel monkey
VII′	D′	R2	P3	NY0304008	Saitama	Black rat

[a)] Genotype was produced by combining the results obtaining using PFGE with *Not*I and ribotyping.
The prime (′) denotes a closely related type or pattern.

originated from a common source. Nevertheless, it is unlikely that the bacterium spread by direct transmission from the squirrel monkeys to the dark-handed gibbon because the enclosure for these two monkey species are separated and the people responsible for the squirrel monkeys do not work with the dark-handed gibbons or vice-versa. Moreover, the historical order in which the infection occurred in the different monkey species in the Zoological Garden, with the initial outbreak among the squirrel monkeys (December 2002 to January 2003) followed by the case of the dark-handed gibbon (April 2003), together with the results of molecular genetical analysis, suggests that O:8 infection occurred first in the colony of squirrel monkeys, and was then transmitted to the dark-handed gibbon. Since the isolates from black rats had the same molecular genotypes of the two monkey species, these rats might be the vector between the two species. Moreover, given that the prevalence of *Y. enterocolitica* serovar O:8 in the black rats captured in this area was relatively high, and considering the time lag between the infection of the two colonies of monkeys, the black rats might be considered a reservoir of strains of this serovar.

A comparison of the molecular genotypes of the isolates of the present study with other Japanese isolates analyzed by Hayashidani et al. (14) shows that the molecular genotype of 29 of the present isolates was highly similar to that of the strains isolated from wild rodents captured in Niigata Prefecture, which borders the Kanto region in the northwest, and that the genotype of the 1 isolate that differed from the other 29 showed a molecular genotype similar to that of an isolate from a wild rodent in Yamagata Prefecture, located in the northeast of Japan. It is tempting to speculate that the strains isolated in the present study might have originated in wild rodents. Future detailed epidemiological studies are necessary to elucidate the origin or the route of transmission of these strains.

Pathogenic *Yersinia* including *Y. enterocolitica* serovar O:8 are a cause of zoonotic disease, and we cannot deny the possibility of human infection from monkeys, especially when an outbreak occurs throughout a colony. Therefore, from the point of view of public health, it is important to develop preventive methods, such as effective vaccines, to prevent pathogenic *Yersinia* spp. infection in monkeys.

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FULL PAPER *Public Health*

Immuno-Magnetic Separation and Agar Layer Methods for the Isolation of Freeze-Injured *Yersinia enterocolitica* O:8 from Water

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(Received 14 June 2005/Accepted 8 November 2005)

ABSTRACT. To develop an effective method to isolate an injured pathogenic *Yersinia enterocolitica* O:8 organism from environmental samples, we compared the isolation of freeze-injured and non-injured *Y. enterocolitica* O:8 and found that the isolation was more successful when immuno-magnetic separation (IMS) with anti-*Y. enterocolitica* O:8 antibody was used. Plating onto cefsulodin-irgasan-novobiocin (CIN) agar and Virulent *Yersinia enterocolitica* (VYE) agar by means of the agar layer method was found to be effective in isolating the injured cells. The alkali treatment which is generally used for selective detection of *Yersinia* organism failed to isolate freeze-injured pathogenic *Y. enterocolitica* O:8 cells. Recovery methods without using the alkali treatment were superior for detecting freeze-injured *Y. enterocolitica* O:8. Our results demonstrate that the IMS and the agar layer methods should be used to isolate injured pathogenic *Yersinia* organisms from environmental samples such as water.

KEY WORDS: agar layer, immuno-magnetic separation, injured, isolation, *Yersinia enterocolitica*.

J. Vet. Med. Sci. 68(3): 195–199, 2006

Out of 60 *Yersinia enterocolitica* serovars, the pathogenic serovars O:3, O:5,27, O:8, and O:9 cause food- and water-borne infections. The pathogenic *Y. enterocolitica* serovars O:3, O:5,27 and O:9 are distributed in most parts of the world [12, 13, 17, 21, 22, 24]. The main source of O:3 and O:5,27 infection is meat. On the other hand, infection with *Y. enterocolitica* O:8 results from water contaminated with the micro-organism [9]. It is suspected that soil and water become contaminated with these bacteria via wild rodents, which are an important natural reservoir of these pathogenic serovars [8].

In this study, the methods for isolating O:8 from water were studied. Alkali treatment that kills a large number of competing bacteria but not alkali-tolerant *Yersinia* organisms has generally been used as an effective method for the selective isolation of *Yersinia* organisms [2, 4, 6]. However, bacteria including *Yersinia* are injured by a variety of inimical processes, such as acidification, heating, and freezing [1, 19] and other various environmental stresses. As a result of decreased tolerance to these stresses, the injured bacteria can not form colonies on selective agars on which non-injured cells can grow [7, 11]. Alkali treatment is likely to affect *Y. enterocolitica* that are injured in environmental waters and thus have their alkali tolerance compromised.

It is known that immuno-magnetic separation (IMS) with a specific antibody is effective for isolating non-injured *Y. enterocolitica* O:3 and O:8 [16, 25]. This process has been observed in other bacteria as well [3, 10, 18, 20]. Furthermore, the agar layer method can resuscitate injured food-borne pathogens in foods [14, 15]. In this study, we found that recovery methods without using the alkali treatment were superior for detecting freeze-injured *Y. enterocolitica* O:8 in environmental samples.

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MATERIALS AND METHODS

Preparation of immuno-magnetic beads: Pathogenic *Y. enterocolitica* O:8 strain (biotype 1B, YE92012) was cultured in 500 m*l* of trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD) at 25°C for 24 hr with shaking. To heat-kill the bacteria, the culture was heated for 1 hr at 105°C in an autoclave. The bacterial cells were washed three times with saline by centrifuging at 2,000 × g for 30 min, and re-suspending in saline at 2 mg/m*l*. Specific O antisera against *Y. enterocolitica* O:8 (with a titer of 1:1026) was obtained by immunizing rabbits with a suspension of the heat-killed bacterial cells. Superparamagnetic polystyrene particles with covalently linked sheep anti-rabbit IgG (Dynabeads® M-280 Sheep anti-Rabbit IgG) (Dynal, Oslo, Norway) were coated with our prepared rabbit IgG antibodies against *Y. enterocolitica* O:8 by following the manufacturer's instructions so as to prepare the immuno-magnetic bead solution.

Sensitivity and specificity of IMS: *Y. enterocolitica* O:8 (strain YE92012) colonies on TSA were suspended in sterile saline at approximately 10^9 CFU/m*l* and diluted with saline to 10^1–10^3 CFU/m*l*. One m*l* of each dilution was added to 25 μ*l* of the immuno-magnetic bead solution. The IMS procedure was performed according to the manufacturer's instructions. The immuno-magnetic beads were finally sus-

pended in 1 m*l* of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin, and 0.1 m*l* of the final solution was spread onto trypticase soy agar (TSA) (BBL Microbiology Systems). After a 48 hr-incubation at 25°C, the number of colonies on the TSA was counted.

Recovery of Y. enterocolitica O:8 from artificially inoculated river water using the IMS method and direct KOH treatment: Fresh river water was collected from five rivers around Tokyo, Japan, and examined on the same day. The means of the aerobic plate counts and coliform counts in the fresh water samples were 4.1×10^3 and 27.5 CFU/m*l*, respectively. The number of *Yersinia* spp. in the five rivers was $<10^2$ CFU/m*l*. Ten liters of the water sample was filtered using a 0.45 μm membrane filter. Precipitates on the filter were suspended in sterile saline. After centrifugation at 5,000 × g for 30 min, the precipitate was suspended in 4.5 m*l* of saline. This suspension was the prepared concentrated river water. *Y. enterocolitica* O:8 colonies (strain YE92012) on TSA were suspended in sterile saline (approximately 10^9 CFU/m*l*), and diluted to 10^1–10^4 CFU/m*l* in 10-fold in saline. To prepare river water that was artificially inoculated with *Y. enterocolitica* O:8, each bacterial dilution was added to nine times the volume of concentrated river water. The river water inoculated with *Y. enterocolitica* O:8 at 0.5 m*l* was diluted with an equal volume of saline and mixed with 25 μ*l* of immuno-magnetic bead solution. IMS was carried out following the manufacturer's instructions. For KOH treatment, an equal volume of 0.72% KOH in saline was added to 0.5 m*l* of each bacterial solution and mixed using a vortex mixer (Automatic Labo-Mixer, Iuchiseieido Co., Ltd.) for 30 s. After either the IMS method or the KOH treatment, 0.1 m*l* of each final solution was spread onto cefsulodin-irgasan-novobiocin (CIN) agar [23] and Virulent *Yersinia enterocolitica* (VYE) agar [5], both of which were supplemented with cefsulodin (15.0 mg/liter), irgasan (4.0 mg/liter) and novobiocin (2.5 mg/liter) (YERSINIA Selective Supplement) (Oxoid, Ltd., Hampshire, UK). After incubation for 48 hr at 25°C, the numbers of typical colonies on the agar media were counted, and the colonies were confirmed to be *Y. enterocolitica* O:8 using the slide agglutination method.

Comparison of freeze-injury among four Y. enterocolitica O:8 strains: Colonies of four *Y. enterocolitica* O:8 strains, YE92012, YE91009, YE92009, and WA, were grown on TSA and suspended to a turbidity equivalent to a No.4 McFarland standard in 5 m*l* of chilled sterilized reagent grade water obtained with a Milli-Q Plus filter (Nihon Millipore Ltd., Tokyo, Japan) and were sedimented by centrifugation at 2,000 × g for 30 min. The cells were washed three times with the reagent grade water and finally were re-suspended in the reagent grade water at a density of 10^0–10^4 CFU/m*l*. The cell suspensions were kept in a freezer at –20°C for 24 hr, thawed, and then 0.1 m*l* of each suspension was spread onto TSA and CIN. The number of freeze-injured *Y. enterocolitica* O:8 cells was estimated by subtracting the number of CFU on CIN as a selective medium from the number of CFU on TSA as a non-selective medium. After incubation for 48 hr at 25°C, the numbers of colonies on the media were counted.

Comparison of the selective media for recovery of freeze-injured Y. enterocolitica O:8: A portion (0.1 m*l*) of freeze-injured cells (approximately 10^3 CFU/m*l*) of strain YE92012 was spread directly onto TSA, CIN, and VYE. For the agar layer method, 0.1 m*l* of freeze-injured cell suspension was spread onto CIN and VYE agar, on which CIN and VYE agar without the supplements was overlaid thinly, respectively. After incubation for 48 hr at 25°C, the numbers of colonies on the media were counted.

Detection of freeze-injured Y. enterocolitica O:8 cells in inoculated fresh water by IMS and direct KOH treatment: Fresh water was collected from a river in Tokyo, Japan, and examined on the same day. The means of the aerobic plate count and viable coliform count in the fresh river water samples were 6.6×10^2 and 15 log CFU/m*l*, respectively. The number of *Yersinia* spp. in the five rivers was $<10^2$ CFU/m*l*. The river water was concentrated using the method described above. Freeze-injured *Y. enterocolitica* O:8 cells (strain YE92012) and dilutions in PBS were inoculated into the concentrated river water. One m*l* of the inoculated river water was used for IMS and direct KOH treatment to recover *Y. enterocolitica* O:8. Each final solution (0.1 m*l*) was spread directly onto CIN, VYE, and an agar layer plate with VYE. After incubation for 48 hr at 25°C, the numbers of typical colonies on the media were counted, and the colonies were confirmed to *Y. enterocolitica* O:8 using the slide agglutination method.

Statistical analysis: For statistical analysis of the results, the software package SPSS for Windows (SPSS Japan Inc., Tokyo) was used. Significant differences in the percentage of recovery of *Y. enterocolitica* O:8 among the selective agars were tested with *t* tests. Significant differences in the rate of recovery of *Y. enterocolitica* O:8 among the selective agars were tested with the Fisher exact test. For all tests, a *P* value of <0.05 was used for significance.

RESULTS

Y. enterocolitica O:8 was recovered at 20% of the ratio from artificially inoculated saline at $6.1 \times 10 - 6.1 \times 10^3$ CFU/m*l* (Table 1). On the other hand, the recovery ratios of serogroups O:3, O:5,27 and O:9 were less than 1.0% using the 0:8 antisera.

At an inoculation level of 100 CFU/m*l*, *Y. enterocolitica* O:8 was isolated from all samples using IMS with both agar

Table 1. Recovery of *Y. enterocolitica* O:8 from artificially inoculated saline by the IMS method

Bacteria concentration in saline (CFU/m*l*)	Recovered bacterial number (CFU/m*l*)	Recovery (%)
6,100	1,288 ± 768[a)]	21.1 ± 8.4[a)]
610	135 ± 83	22.2 ± 6.3
61	15	24.6

a) Mean ± SD in triplet.

Table 2. Recovery of *Y. enterocolitica* O:8 from inoculated river water by IMS method and with direct KOH treatment

Bacterial concentration in river water (CFU/ml)	IMS		Direct KOH treatment	
	CIN	VYE	CIN	VYE
1,000	5/5[a)]	5/5	5/5	5/5
100	5/5	5/5	5/5	3/5
10	3/5	1/5	1/5	1/5
1	0/5	0/5	0/5	0/5

a) *Y. enterocolitica* isolated sample number/Total tested sample number.

media and by direct KOH treatment with CIN; whereas *Y. enterocolitica* O:8 was isolated from three out of five samples by direct KOH treatment with VYE (Table 2). At an inoculation level of 10 CFU/ml, *Y. enterocolitica* O:8 was isolated from three out of 5 samples using the IMS method with CIN, whereas it was isolated from only one out of 5 samples using IMS with VYE and using the direct KOH treatment with both agar media. However, the micro-organism was not isolated from any sample inoculated at a concentration below 1 CFU/ml.

As a result, more than 90% of the population in each strain was injured by freezing (Table 3). YE92012, an easily injured strain, was used in the following experiments.

The recovery ratios on CIN, VYE, the agar layer plate with CIN, and the agar layer plate with VYE were 5.6, 8.4, 8.9, and 16.5%, respectively (Table 4). The agar layer plate with VYE resulted in a recovery of more *Y. enterocolitica* O:8 than any other media. However, significant difference was not observed among them.

Using IMS with CIN and an agar layer plate with VYE, *Y. enterocolitica* O:8 was isolated from all samples inoculated at a level of 100 CFU/ml, and two out of 4 samples inoculated at a level of 10 CFU/ml (Table 5). Moreover, the organism was isolated from only one out of 4 samples inoculated at a level of 1 CFU/ml using IMS with an agar layer plate with VYE. By the direct KOH treatment with CIN and an agar layer plate with VYE, *Y. enterocolitica* O:8 was isolated from all samples inoculated at a level of 10^4 CFU/ml, and from two out of 4 samples inoculated at a level of 10^3 CFU/ml. However, the organism was not isolated from any sample inoculated with injured cells at a level of 10 CFU/ml by direct KOH treatment. IMS seemed to be more effective than direct KOH treatment for recovering freeze-injured *Y. enterocolitica* O:8 in river water inoculated with the micro-organism although no significant differences in the rate of recovery of *Y. enterocolitica* O:8 among the selective agars in the same bacterial concentration was obserbed. It is suspected that the alkali treatment kills or damages injured *Y. enterocolitica* O:8.

Table 3. Freeze injured *Y. enterocolitica* O:8 strains caused by frozen storage

Strain	Ratio of freeze-injured cells after freezing (%)[a)]
YE92012	94.7
YE91009	91.8
YE92009	89.8
WA	94.8

a) {[(Number of bacteria on TSA)–(Number of bacteria on CIN)]/(Number of bacteria on TSA)} × 100.

Table 4. Recovery of freeze-injured *Y. enterocolitica* O:8 by plating

Agar medium	Recovery (%)[a)]
CIN	5.6 ± 5.0[b)]
VYE	8.4 ± 8.2
CIN, agar layer	8.9 ± 6.1
VYE, agar layer	16.5 ± 13.8

a) (Number of bacteria on a selective medium)/(Number of bacteria on TSA) × 100.
b) Mean ± SD in triplet.

Table 5. Recovery of freeze-injured *Y. enterocolitica* O:8 from inoculated river water by IMS method and with direct KOH treatment

Bacterial concentration in river (CFU/ml)	IMS			Direct KOH treatment		
	CIN	VYE	VYE, agar overlay	CIN	VYE	VYE, agar overlay
100,000	4/4[a)]	4/4	4/4	4/4	4/4	4/4
10,000	4/4	4/4	4/4	4/4	3/4	4/4
1,000	4/4	4/4	4/4	2/4	2/4	2/4
100	4/4	3/4	4/4	1/4	1/4	1/4
10	2/4	2/4	2/4	0/4	0/4	0/4
1	0/4	0/4	1/4	0/4	0/4	0/4

a) *Y. enterocolitica* isolated sample number/Total tested sample number.

DISCUSSION

This study demonstrates that IMS was more effective than direct KOH treatment in recovering freeze-injured *Y. enterocolitica* O:8 from river water inoculated with the injured cells. This strongly suggests that IMS should be preferred over the conventional direct KOH treatment for recovering injured pathogenic *Y. enterocolitica* from frozen water. It was demonstrated that *Y. enterocolitica* O:8 was easily injured in water during frozen storage because high numbers of all tested strains were injured by freezing for a short period of time (Table 3). *Y. enterocolitica* O:8 cells in water may be detected more successfully using IMS.

It is known that VYE agar is a useful medium for the isolation of pathogenic *Y. enterocolitica* strains from environmental samples that are highly contaminated with environmental *Yersinia* spp. [5]. Virulent *Y. enterocolitica* forms a red colony on VYE agar and is easily differentiated from most environmental *Yersinia* spp. and other gram-negative bacteria. CIN is generally used for the isolation of *Y. enterocolitica*, although virulent *Y. enterocolitica* is not distinguished from non-virulent *Yersinia* spp. on CIN. An agar layer method [14] was used to recover injured *Y. enterocolitica* in this study. During the resuscitation of injured cells on the top agar layer of CIN or VYE, the selective agents in selective plating medium diffused to a non-selective medium to inhibit the other microorganisms. As a result, the recovery of *Y. enterocolitica* by the agar layer method with VYE was higher than the recovery achieved by the method with CIN and direct plating on VYE and CIN (Table 4). These data indicate that the agar layer plate with VYE was the most effective medium for the recovery of injured *Y. enterocolitica* O:8 when competitive growth in environmental samples is inhibited by antibiotics. On an agar layer medium with VYE, pathogenic *Y. enterocolitica* O:8 colonies could be easily differentiated from the non-pathogenic colonies in this study.

In conclusion, this study shows that IMS is an efficient method for isolating injured pathogenic *Y. enterocolitica* from environmental samples, and that an agar layer medium with VYE is effective for achieving this isolation. These results also suggest that immuno-magnetic beads for several serovars of pathogenic *Y. enterocolitica* may also be useful.

ACKNOWLEDGMENT. This work was supported by a Health Sciences Research Grant from the Ministry of Health, Labor and Welfare, Japan.

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NOTE *Parasitology*

Babesia microti-Like Parasites Detected in Feral Raccoons (*Procyon lotor*) Captured in Hokkaido, Japan

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(Received 10 February 2005/Accepted 14 April 2005)

ABSTRACT. Raccoons (*Procyon lotor*), which have recently become feral in Japan, were examined for the presence of *Babesia microti*-like parasites. Out of 372 raccoons captured in the west-central part of Hokkaido, 24 animals with splenomegaly were selected and tested by nested PCR targeting the babesial 18S rRNA gene. *B. microti*-like parasites were detected in two of the 24 individuals, and their DNA sequences were identical to that of the *B. microti*-like parasite reported from raccoons in the United States, suggesting that the parasites were probably imported into Japan and that the life cycle of the parasite has already been established in the country. The potential risk of this *B. microti*-like parasite spreading among dogs and foxes in Japan will need to be carefully monitored, as parasitization by phylogenetically very close parasites has been reported from such animals.

KEY WORDS: *Babesia microti*, Hokkaido, Raccoon.

J. Vet. Med. Sci. 67(8): 825 827, 2005

The raccoon is a Carnivora species native to North America [9]. A large number of raccoons, however, have been imported as pets into Japan since the 1970s. In Hokkaido, the northernmost island of Japan, the accidental release and subsequent escape of these pet raccoons has resulted in a feral population, distributed mainly in the suburban areas of Sapporo, the biggest City on the island [1]. In 1997, the Hokkaido government approved of the control killing of feral raccoons only locally in response to agricultural and aquiculture damage. From 1999, the Hokkaido government started a feral raccoon management program, which enabled scientists in various fields to conduct collaborative investigations on captured raccoons [1, 7].

Parasites phylogenetically closely related to *Babesia microti*, referred to as *Babesia microti sensu lato*, have recently been reported from several Carnivoras including raccoons, foxes and skunks in the United States ("clade 2 in the reference [6]). *Babesia microti sensu stricto* ("clade 1 in the reference [6]) is most frequently found in small wild rodents and occasionally appears as a causative agent of human babesiosis [11]. Recently, a fulminating disease in dogs has emerged in Spain [2, 4, 5, 14], and the causative agent was named *Theileria annae* [14]. Phylogenetic studies, however, have subsequently made it clear that this parasite belong to the *B. microti* group [13], and that its closest relative is the one in raccoons [6], which have recently become feral in Europe also [9]. In the present study, we had an opportunity to obtain blood samples from raccoons captured in Hokkaido, and investigated them to determine whether they carried babesial infections.

Raccoons were captured from June to November in 2004 at 9 places in the west-central part of Hokkaido (Fig. 1) under the feral raccoon management program directed by the Hokkaido government. The number of captured raccoons in each region is shown in Table 1. The animals were euthanized according to the procedure approved by the program, and examined for sex, body weight, pregnancy, spleen size, and tick infestation, followed by tissue and blood collections. DNA samples were prepared from the blood samples with a DNA Extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan), and partial sequences within the nuclear small subunit ribosomal RNA gene (18S rRNA gene) were amplified using 2 sets of nested PCR primers (Table 2), according to the procedure described in our previous studies [8, 12, 15]. One set of primers consisted of Bab1A and Bab4A for the 1st round and Bab2A and Bab3A for the 2nd-round PCR; a set that is highly specific for *B. microti*-like parasites and useful for their detec-

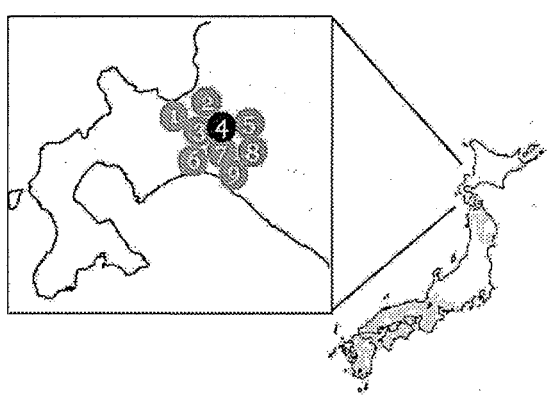

Fig. 1. Location of the study area for feral raccoons in the west-central parts of Hokkaido, Japan. Trapping reagion; 1, Sapporo; 2, Ebetsu; 3, Kitahiroshima; 4, Maoi; 5, Yubari; 6, Tomakomai; 7, Oiwake; 8, Hobetsu; 9, Mukawa.

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Table 1. Summary of the survey on raccoons captured in the west-central parts of Hokkaido, Japan

Region	No.of raccoon captured	Splenomegaly[a] /no. of raccoon	PCR positive[b] /splenomegaly
Sapporo	90	4/90	0/4
Ebetsu	42	4/42	0/4
Kitahiroshima	77	5/77	0/5
Maoi	34	6/34	2/6
Yubari	8	0/8	
Tomakomai	45	4/45	0/4
Oiwake	12	1/12	0/1
Hobetsu	38	0/38	
Mukawa	26	0/26	
Total	372	24/372	2/24

a) Splenomegaly was judged by spleen size (in length) being more than 10 cm.
b) Nested PCR specific for *B. microti*-like parasites was carried out using Bab1A and Bab4A for the first round, and Bab2A and Bab3A for the second round.

Table 2. PCR primers used to amplify babesial 18S rRNA gene sequence

Primer	Sequence (5 to 3)	Orientation
Bab1A	5 -GTCTTAGTATAAGCTTTTATACAGCG-3	Forward
Bab2A	5 -CAGTTATAGTTTATTTGATGTTCGTTTTAC-3	Forward
Bab3A	5 -CGGCAAAGCCATGCGATTCGCTAAT-3	Reverse
Bab4A	5 -GATAGGTCAGAAACTTGAATGATACATCG-3	Reverse
Piro0F	5 -GCCAGTAGTCATATGCTTGTGTTA-3	Forward
Piro1F	5 -CCATGCATGTCTWAGTAYAARCTTTTA-3	Forward
Piro5.5R	5 -CCTYTAAGTGATAAGGTTCACAAAACTT-3	Reverse
Piro6R	5 -CTCCTTCCTYTAAGTGATAAGGTTCAC-3	Reverse

tion [8, 12, 15]. The other set of primers consisted of Piro0F and Piro6R for the 1st round, and Piro1F and Piro5.5R for the 2nd-round PCR; a set that is not highly specific for *B. microti*-like parasites but useful to obtain a near full-length 18S rRNA gene sequence. The DNA fragments amplified with the latter set of primers were cloned in a plasmid vector for sequencing as described previously [10]. At least 3 plasmid clones were sequenced for both strands to obtain a consensus sequence.

Out of 372 raccoons captured, 24 animals were found to have splenomegaly (Table 1). DNA samples were extracted from those 24 animals, and tested by the nested PCR highly specific for *B. microti*-like parasites (see above). A positive signal was obtained from 2 of the 24 samples (Table 1). Near full-length 18S rRNA gene sequences, 1684 bp in size, were amplified from those two samples, and their sequences determined (accession number AB197940). The sequences from the 2 samples were the same, and bases 428 to 1684 were identical to the sequence reported for a *B. microti*-like parasite from a raccoon in the United States (accession number AY144701). The 2 PCR-positive animals were both trapped within the Maoi region (Fig. 1). Ticks were not found on the 2 animals, but *Ixodes ovatus, I. persulcatus*, and *I. tanuki* were found on the other raccoons captured in this region. Although the level of parasitemia was very low (less than 0.01%), *Babesia* parasites were microscopically observed in a blood smear prepared from one of the two PCR-positive raccoons (Fig. 2).

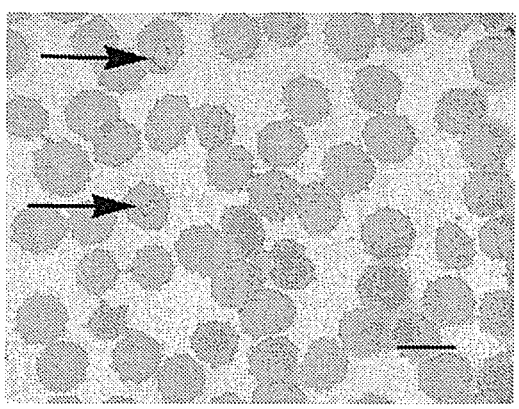

Fig. 2. Light micrograph of Giemsa-stained thin-smear blood film showing *Babesia microti*-like parasites. Bar, 10 μm.

Our study represents the first detection of *B. microti*-like parasites in Japanese raccoons. As evidenced by the sequence identity of the 18S rRNA gene, the *B. microti*-like parasite found in Japan is probably the same as that in the United States. The fact that raccoons became feral in Japan only very recently suggests two possibilities: namely, that

the parasite was also recently introduced into Japan, and that its life cycle has already been established within the detection area. In the present study, we could not identify the vector tick involved in the life cycle, but one of the three *Ixodes* ticks obtained from captured raccoons, namely, *I. ovatus*, *I. persulcatus*, and *I. tanuki*, may well be the candidate. Phylogenetically, the parasite found in this study is very closely related to the *B. microti*-like parasite causing newly emerging babesiosis in Spanish dogs [2, 4, 5, 14], for which *I. hexagonus* has been indicated to serve as the vector tick [3]. Speculating from the phylogenetic relationship, it would be interesting to investigate the hypothesis that the *B. microti*-like parasite in Spanish dogs might be a dog-adapted variant that was derived from the parasite in raccoons or foxes in the United States (personal communication with S. R. Telford).

The results presented in this study are still preliminary because only 24 out of the 372 samples were examined. These 24 raccoons, however, had splenomegaly, a major sign of babesiosis. Further studies are now underway to obtain more precise information about parasite prevalence and geographical distribution. Careful monitoring of not only raccoons but also dogs and foxes will be needed for assessment of the potential risk of this *B. microti*-like parasite spreading among these animals; such a field survey may also provide us with an unique opportunity for testing the hypothesis described above.

ACKNOWLEDGMENTS. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan, by Health Science Grants for Research on Emerging and Re-emerging Infectious Diseases from the Ministry of Health, Labor and Welfare of Japan, and by Gakujutsu Frontier Cooperative Research and High Technological Research Centers at Rakuno-Gakuen University. We wish to thank the Nature Preservation Division of Hokkaido Government and Hokkaido Forest Management Corporation for providing us with the raccoon blood samples.

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FULL PAPER *Public Health*

U.S.-Type *Babesia microti* Isolated from Small Wild Mammals in Eastern Hokkaido, Japan

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(Received 16 December 2003/Accepted 23 March 2004)

ABSTRACT. Our previous report demonstrated that small wild rodents in Japan harbored two types of novel *Babesia microti*-like parasites (Kobe and Hobetsu types), but not the type widely distributed throughout the temperate zones of North American and Eurasian Continents (U.S. type). In this study, we surveyed small wild mammals collected at various places in the northern part of Japan, seeking for U.S.-type *B. microti*. A total of 197 small mammals comprising 10 species, *Apodemus speciosus, A. argenteus, Clethrionomys rufocanus, C. rutilus, Eothenomys andersoni, Microtus montebelli, Tamias sibiricus, Sorex unguiculatus, S. caecutiens,* and *Urotrichus talpoides*, were examined. *Babesia* parasites were detected in *A. speciosus, C. rufocanus, C. rutilus, M. montebelli, S. unguiculatus,* and *S. caecutiens* by microscopy of blood smears and by PCR targeting babesial nuclear small-subunit rRNA (rDNA) and β-tubulin genes. Inoculation of their bloods into experimental animals gave rise to 23 parasite isolates, which included 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus, M. montebelli* and *S. unguiculatus*. Sequencing analyses of their rDNA and β-tubulin genes revealed that, of the 23 isolates, 20 and 3 were of Hobetsu and U.S. types, respectively. The U.S.-type *B. microti* strains isolated in Japan, however, were distinguishable from the isolates in the United States when their β-tubulin gene sequences and antigen profiles in Western blots were compared. We conclude that U.S.-type *B. microti* exists in Japan although it has been genetically and antigenically diversified from that distributed in the United States. The results also suggest that not only rodents, but also some insectivo res may serve as reservoirs for the agent of human babesiosis.

KEY WORDS: *Babesia microti*, Japan, reservoir, U.S. type, wild animal.

J. Vet. Med. Sci. 66(8): 919–926, 2004

Babesia microti is an erythroparasitic protozoon frequently seen in small wild rodents. This parasite is known to be the causative agent of human babesiosis, an emerging tick-borne zoonosis which has increasingly been recognized in the Northeastern and upper-Midwestern United States [8, 11, 20]. Parasitization of *B. microti* in various rodent species has been reported in North America [3, 7, 26], Europe [9, 10, 26], and East Asia [18, 24, 29]. Curiously, however, despite such worldwide distribution of *B. microti* throughout the northern temperate zone, symptomatic human infections have been reported almost exclusively from the United States [11, 20]. It is well established that white-footed mouse, *Peromyscus leucopus*, and deer tick, *Ixodes dammini* (also known as *I. scapularis*), serve as the rodent reservoir and the tick vector, respectively, for *B. microti* in the northeastern United States [7, 19]. The parasite was also found in short tailed shrews, *Blarina brevicauda*, indicating the role of Insectivora as additional reservoir hosts [21].

Recently, the first case of transfusion-acquired, symptomatic human babesiosis has been reported in Japan [13, 16]. In order to identify wild animals that serve as the reservoirs for the agents of human babesiosis, we conducted epizootiologic surveys at various places in the country [23]. The results reveal that there are two types of *B. microti*-like parasites (designated as the Hobetsu and Kobe types) in Japan, and that the large Japanese field mouse, *Apodemus speciosus*, serves as the major reservoir host for both of the two types. The nuclear small-subunit rRNA gene (rDNA) sequences of these two types (GenBank accession nos. AB032434 and AB050732 for the Kobe and Hobetsu types, respectively) clearly differed from that of the United States *B. microti* (the U.S. type; GenBank accession no. U09833). Therefore, although human babesiosis in Japan may be able to be categorized as an emerging disease because of its sudden appearance, it became apparent that *B. microti*-like parasites in the country were not imported from the regions in the United States where human babesiosis is endemic. The U.S.-type *B. microti* has not yet been found in any places surveyed in our previous study [23].

More recently, we have extended our epizootiologic surveys to regions surrounding Japan, including South Korea, Far East Russia, and the northwestern China [29]. Animals harboring *B. microti* were detected in these three regions, and all of the parasites were identified to be of U.S. type, based on their rDNA sequences being identical to that of the United States *B. microti* [29]. Several European strains of *B. microti* have also been reported to have the U.S.-type rDNA sequence [6, 28]. These lines of epidemiological evidence suggest that the U.S.-type *B. microti* is probably distributed throughout the temperate zones of not only North American but also Eurasian Continents, whereas that the Hobetsu and Kobe types may be the parasites unique to Japan [23, 29]. Phylogenetically, the Hobetsu-, Kobe-, and U.S.-type parasites are quite closely related to each other,

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but clearly distinguishable from each other antigenically [23].

In our previous study [29], *A. peninsulae*, *A. agrarius*, and *Clethrionomys rufocanus* are identified to be the rodent species harboring the U.S.-type *B. microti* in northeastern Eurasia. Both *A. peninsulae* and *C. rufocanus* are also known to exist on Hokkaido Island, the far northernmost part of Japan, because this island had been connected with Eurasian Continent via Sakhalin Island during the latest ice age until approximately ten thousand years ago. Hence, despite the current unavailableness of U.S.-type *B. microti* in Japan, it may be possible to find this type in that region of the country. In the present study, therefore, we conducted epizootiologic surveys at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan, seeking for U.S.-type *B. microti* in small wild rodents.

MATERIALS AND METHODS

Field collections: Small wild mammals examined in the present survey were collected during 1999 and 2001 at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan. The locations of the field collections are shown in Fig. 1. Identification of animal species was done according to the key characteristics described by Abe *et al.* [1]. We collected a total of 197 small mammals comprising 10 species, which included 104 of *A. speciosus*, 41 of *A. argenteus*, 25 of *C. rufocanus*, 12 of *C. rutilus*, 1 of *Eothenomys andersoni*, 1 of *Microtus montebelli*, 1 of *Tamias sibiricus*, 4 of *Sorex unguiculatus*, 5 of *S. caecutiens*, and 3 of *Urotrichus talpoides*. Their blood samples were processed according to the methods described previously [23] for preparations of blood smear and DNA, which were used for microscopic examination and for PCR targeting babesial rDNA and β-tubulin gene, respectively [27, 29].

Detection of Babesia parasites: Nested PCRs targeting the babesial rDNA and β-tubulin gene were carried out for detection of *Babesia* parasites in the blood specimens from the field collections according to previously published protocols [27, 29]. DNA samples were prepared from 100 μl of blood samples with a DNA Extractor WB kit (Wako Pure Chemical Industries, Osaka). Approximately 1/30 of the final DNA preparation was used for the first round of PCR, followed by the second round of PCR with 1 μl of the first-round PCR product. Type-specific PCRs based on the sequences of β-tubulin gene for genotypic classification were carried out as described previously [29].

Experimental animals: Syrian hamsters (Std:Syrian), Mongolian gerbils (MON/Jms/Gbs) were obtained from SLC Inc. (Shizuoka). House musk shrews, *Suncus murinus* (Jic:Sun-Her), were purchased from CLEA Japan, Inc. (Tokyo). NOD/shi-*scid* mice [12] had been provided from the Central Institute of Experimental Animals, Kawasaki, and have been maintained in the laboratory animal facility in Rakuno-Gakuen University. Animals were housed in

Fig. 1. Map of the northern part of Japan, showing the locations of field collections (●).

vinyl-film isolators at a temperature of between 22 and 25°C, and were provided with γ-ray-irradiated pellet diet and autoclaved tap water. All hamsters and gerbils were splenectomized, and were used for experiments after the surgical wounds had healed completely. Animal experimentation was carried out according to the Laboratory Animal Control Guidelines of Rakuno-Gakuen University.

Isolation of parasites: Red blood cell (RBC) samples from the field collections were inoculated into splenectomized hamsters for isolation of parasites. Other experimental animals, including splenectomized gerbils, house musk shrews, and splenectomized NOD/shi-*scid* mice, were also used in attempts to isolate *Babesia* parasites from shrews (*Sorex unguiculatus*, and *S. caecutiens*). Blood samples were collected periodically from the tail vein of the inoculated animals, and Giemsa stained thin-smeared blood films were prepared for microscopic detection of parasitemia. When the level of parasitemia reached 20 to 40%, blood was harvested by cardiocentesis from anesthetized animals, washed in phosphate buffered saline (PBS), resuspended in a cell freezing solution (Cell Banker; Nippon Zenyaku Co., Ltd., Fukushima), and cryopreserved in liquid nitrogen. The isolates were further propagated by subpassage into new splenectomized hamsters, and their RBCs (parasitemia level, 30 to 50%) were washed in PBS and stored at –80 °C without cryopreservatives for subsequent use to prepare parasite DNAs and antigens for Western blot analysis. For production of antibodies, hamsters with intact spleens were infected with parasites, and serum samples were collected from the animals when they had high antibody titers.

Sequencing analyses: For each parasite isolate, genomic

DNA was prepared from the frozen parasitized RBC stock described above using a Whole-blood DNA extraction kit (GenTLE; TaKaRa Biochemical, Shiga). Sequences of the rDNA and β-tubulin gene were amplified from the DNA samples by PCR with the primer sets described in previous studies [14, 29]. Specifically amplified products were cloned in a plasmid vector and sequenced as described elsewhere [16].

Phylogenetic analysis: Phylogenetic relationship of β-tubulin gene sequences was analyzed by using the MacVector software package, version 7.0 (Genetic Computer Group Inc., Madison, Wis, U.S.A.). The β-tubulin gene sequences (GenBank accession numbers are given in parentheses) included in phylogenetic analysis were of U.S.-type *B. microti* collected from Vladivostok in Russia (AB083379), from South Korea (AB083380), and from Xinjiang in China (AB083378), of the Hobetsu- and Kobe-type *B. microti*-like parasites (AB083441 and AB083440 for the strains Ho234 and Ko524, respectively), and of *B. rodhaini* (AB083442). The sequences were aligned with the program CLUSTAL W Alignment [22], and a phylogenetic tree was constructed by neighbor-joining method [17] from the aligned sequences with the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates by use of the bootstrap tree algorithm.

Antigenic analyses: The indirect immunofluorescent antibody test (IFAT) was carried out by the method described previously [16]. A mixture of fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G (IgG) plus IgM, anti-rat IgG plus IgM and anti-Syrian hamster IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pa, U.S.A.) was used for the detection of antibodies in the wild animals collected from the fields. Western blot analysis was performed as described previously [27]. Frozen stocks of *Babesia*-infected RBCs were thawed and washed five times at 4°C in TE (10 mM Tris-HCl and 10 mM EDTA, pH 7.5) by centrifugation at 10,000 × *g* for 10 min. The resulting pellets were dissolved in 125 mM Tris-HCl (pH 6.5) containing 5% β-mercaptoethanol, 2% sodium dodecylsulfate, 10% glycerol, and 0.1% bromophenol blue, heated at 98°C for 5 min, and vigorously vortexed. The samples were diluted such that each contained material from equivalent numbers of parasitized RBCs and were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis, followed by blotting onto Fluorotrans membranes (Pall Bio Support, Port Washington, N.Y., U.S.A.). After blocking was done with PBS containing 0.5% casein, the membranes were reacted with appropriately diluted antisera and subsequently with secondary antibodies (alkaline phosphatase-conjugated affinity purified goat anti mouse IgG heavy and light chains or anti-Syrian hamster IgG heavy and light chains; Jackson ImmunoResearch Laboratories). Immunoreactive antigens were detected with 5 bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium alkaline phosphatase substrates kit IV (Vector Laboratories, Inc., Burlingame, Calif., U.S.A.).

Reference parasite strains: The Gray and GI strains have been described in our previous study [16], and were used as the *B. microti* isolates obtained in the Northeastern United States. The strains Ho234 [23] and Ko524 [27] were used as the type strains of the Hobetsu- and Kobe-type *B. microti*-like parasites, respectively. The Australian strain of *B. rodhaini* was kindly provided from the National Institute of Animal Health, Tsukuba. These parasites were propagated in hamsters or NOD/shi-*scid* mice as described previously [23].

Nucleotide sequence accession number: All three U.S.-type *B. microti* isolates obtained in Japan had identical β-tubulin gene sequences. The sequence from the strain NM69, as a representative, was submitted to DNA Data Bank of Japan (DDBJ) and has been given the accession number AB085813.

RESULTS

Field surveys: Small wild mammals examined in the present survey were collected at various places on Hokkaido Island and the northern part of Honshu Island (the mainland) of Japan (Fig. 1). A total of 197 small mammals comprising 10 species were collected. Their blood samples were examined by microscopy of blood smears and by PCR targeting babesial rDNA and β-tubulin gene. The results of survey are summarized in Table 1. *Babesia* parasites were detected in 24 animals, consisting of 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus*, *M. montebelli*, *S. unguiculatus* and *S. caecutiens*.

Parasite isolation: The 24 blood specimens, in which, *Babesia* parasites had been detected, were inoculated into experimental animals for isolation of parasites. A single splenectomized hamster (*Mesocricetus auratus*) was used for each of the 24 samples. In addition, *Suncus murinus* and NOD/shi-*scid* mice were used for the sample from *Sorex caecutiens* collected at Nemuro, and Mongolian gerbils (*Meriones unguiculatus*) for the samples from *Sorex unguiculatus* and *Microtus montebelli* collected at Kiyosato and Shizukuishi, respectively. We were able to finally obtain a total of 23 parasite isolates, which included 16 from *A. speciosus*, 4 from *C. rufocanus*, and 1 each from *C. rutilus*, *M. montebelli* and *S. unguiculatus* (Table 2). However, we failed to isolate a parasite from the sample of *S. caecutiens*, in which Hobetsu-type *B. microti*-like parasites had been detected by PCR.

Genotypic classification: On the 24 DNA samples from the field collections, in which the rDNA sequence of *B. microti* had been detected by PCR, we carried out the type-specific PCR based on the β-tubulin gene sequence [29] for genotypic classification. Of the 24 samples, 21 and 2 were identified to be of Hobetsu and U.S. types, respectively (Table 1), and another one from *C. rutilus* trapped in Nemuro were positive for both Hobetsu and U.S. types. We also carried out sequencing analysis for the rDNA of the 23 parasite isolates to determine their genotype (Table 2). Of the 23 isolates, 20 and 3 had the rDNA sequences which were

Table 1. Summary of field surveys of *B. microti*-like parasites among small wild mammals in the nothern part of Japan

Site	Species	No. of animals captured	No. of postive/no. of tested by: Nested PCR targeting the following genes: Microscopy[a]	rDNA[b]	β-tubulin[c] Universal	U.S.	Hobetsu	Kobe	No. of isolates[d]
Nemuro	*Clethrionomys rufocanus*	4	2/4	2/4	2/4	1/2	1/2	0/2	2/2
	Clethrionomys rutilus	10	1/10	1/10	1/10	1/1	1/1	0/1	1/1
	Sorex caecutiens	3	1/3	1/3	1/3	0/1	1/1	0/1	0/1
Akkeshi	*Apodemus speciosus*	4	0/4	1/4	1/4	1/1	0/1	0/1	1/1
	Clethrionomys rufocanus	7	0/7	1/7	1/7	0/1	1/1	0/1	1/1
	Clethrionomys rutilus	2	0/2	0/2	0/2	–	–	–	–
	Tamias sibiricus	1	0/1	0/1	0/1	–	–	–	–
Horonobe	*Apodemus speciosus*	2	0/2	0/2	0/2	–	–	–	–
	Apodemus argenteus	3	0/3	0/3	0/3	–	–	–	–
	Clethrionomys rufocanus	1	0/1	0/1	0/1	–	–	–	–
	Sorex unguiculatus	2	0/2	0/2	0/2	–	–	–	–
Kiyosato	*Apodemus speciosus*	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
	Apodemus argenteus	2	0/2	0/2	0/2	–	–	–	–
	Clethrionomys rufocanus	2	0/2	0/2	0/2	–	–	–	–
	Sorex unguiculatus	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
Hobetsu[e]	*Apodemus speciosus*	10	7/10	7/10	7/10	0/7	7/7	0/7	7/7
	Apodemus argenteus	2	0/2	0/2	0/2	–	–	–	–
	Clethrionomys rufocanus	3	0/3	1/3	1/3	0/1	1/1	0/1	1/1
Ebetsu[e]	*Apodemus speciosus*	22	0/22	0/22	0/22	–	–	–	–
	Apodemus argenteus	15	0/15	0/15	0/15	–	–	–	–
	Clethrionomys rufocanus	7	0/7	0/7	0/7	–	–	–	–
	Sorex unguiculatus	1	0/1	0/1	0/1	–	–	–	–
Setana	*Apodemus speciosus*	11	0/11	0/11	0/11	–	–	–	–
	Apodemus argenteus	8	0/8	0/8	0/8	–	–	–	–
	Clethrionomys rufocanus	1	0/1	0/1	0/1	–	–	–	–
	Sorex caecutiens	2	0/2	0/2	0/2	–	–	–	–
Okushiri	*Apodemus speciosus*	14	0/14	0/14	0/14	–	–	–	–
Shizukuishi	*Apodemus speciosus*	23	0/23	0/23	0/23	–	–	–	–
	Apodemus argenteus	10	0/10	0/10	0/10	–	–	–	–
	Eothenomys andersoni	1	0/1	0/1	0/1	–	–	–	–
	Microtus montebelli	1	1/1	1/1	1/1	0/1	1/1	0/1	1/1
	Urotrichus talpoides	2	0/2	0/2	0/2	–	–	–	–
Takanosu	*Apodemus speciosus*	17	7/17	7/17	7/17	0/7	7/7	0/7	7/7
	Apodemus argenteus	1	0/1	0/1	0/1	–	–	–	–
	Urotrichus talpoides	1	0/1	0/1	0/1	–	–	–	–
Total		197	21/197	24/197	24/197	3/24	22/24	0/24	23/24

a) Detection of parasitezed RBCs by microscopy of thin-smear blood films.
b) Detection of rDNA sequence by nested PCR with primers specific for *B. microti* [27].
c) Detection of β-tubulin gene sequence by nested PCR with primers universal for three types of *B. microti*-like parasites, and genotypic classification with primers specific for the U. S., Hobetsu and Kobe types [29].
d) *Babesia* parasites were isolated by inoculation of the blood samples into experimental animals as shown in Table 2.
e) The samples of Hobetsu and Ebetsu were obtained in our previous study [27].

identical to those of the Hobetsu type (GenBank accession no. AB050732) and the U.S. type (GenBank accession no. U09833), respectively. All of the three *B. microti* isolates identified to be of U.S. type were derived from animals trapped in an eastern area of Hokkaido Island (Nemuro and Akkeshi). The results of genotypic classification determined by the type-specific PCR targeting the β-tubulin gene and by sequencing of rDNA were in good agreement, except for the sample from *C. rutilus* trapped in Nemuro. Whereas the blood from this vole appeared to have contained both Hobetsu- and U.S.-type parasites, the parasites isolated after inoculation into a hamster were of U.S. type. To dissolve this discrepancy, we attempted to pass the isolated parasites through NOD/shi-*scid* mice three times. Since U.S.-type *B. microti* generally grew only very poorly in mice, this passage gave rise to isolation of Hobetsu-type parasite, con-