TABLE 2. The 20 most variable intergenic spacers conserved by both *B. henselae* and *B. quintana* and primers used for amplification and sequencing

Spacer name[a]	Spacer position on the genome[b]	Spacer size (bp)[b]	PCR product size (bp)[b]	Forward primer	Reverse primer
tRNA-Ala/GCA-tRNA-Ile/AUC (S1)[c]	1412349–1412683	335	414	TTGCAAAGCAGGTGCTCTCC	TAAGCGTGAGGTCGGAGGTT
BH2865724-*dut* (S2)	1685859–1686289	431	602	GGTTTTTGCCACGGGTATTT	GGAAGTTCTAAACCTTGTCCATGG
dnaJ related protein-*cobS* (S3)	1828960–1829320	361	490	CAATGGAGGCAACCGTTCTT	GTGATATCGGGTACATTTTCAACTG
pssA-oxidoreductase (S4)	609654–610228	575	709	GATTTTTCTTCCGTGTAGCTTTGT	TGTGCGTAAAAATCGATTCATG
carB-cold shock protein (S5)	1292681–1293066	386	509	AGAAGCTATCGAAGCACTCACAAA	TGAATGAACCCGAAACCTTTAGT
alr-gcvP (S6)	1431110–1431442	333	540	TCAAAGAGGTGATTGGGTAGAGC	CTGTTTCACGTATTGATAATGTTGC
fisK-oxidoreductase (S7)	1799482–1799984	503	594	GCGAACCTTGAGAACTCTGCA	GGGTTTTACACCTTCATTGAGATCA
BH2864883-BH2864884 (S8)	1594026–1594377	352	524	TAACCACATCATCCCCTCCTCT	GAAATAATCATGAAACGCATAAGC
acpP2-malate oxidoreductase (S9)	853898–854063	166	296	CAACTTCACTGATTTCTGCGATAA	CGAGGAGTGGTTAATATGACAGCT
BH16140-BH16150 (S10)	1864960–1865467	508	508	CTCATTACAGAGCAAAACGGATATC	TTATCAAGGTTTGCTTCTACAGCG
dapE-hemN (S11)	76032–76228	197	395	ATGCATATGGTGGATGAGTGTGT	GATTTACAACAACAAGGGCTGGT
phoH-BH02260 (S12)	302238–302400	163	327	CTTATTTTCTCTTTAACGCGCTTT	TCACCTTGGCTTTTACCTGTTGT
Glutathione *S*-transferase–*dapB* (S13)	1383473–1383792	320	441	CTTCTTTTCGCCCTCTTTTAACA	TCGCGTCCCATTCTTCCAT
rpmF-ispA (S14)	1751167–1751490	324	394	GATGGAGAGGTTTTTCGTTTAGG	TGGGCGTGTTTTGCAAGAA
asd-BH12900 (S 15)	1441922–1442299	378	636	TACGCGATGCACCAGGCT	CCGTGTTGTGACCTATCCTGCT
recO-panC (S16)	596596–596744	149	438	TTGTGCAAAGAACTGTTCGTCC	ACCAAACCAATCGAAAATCCTAA
BH16010-*rpsP* (S17)	1846327–1846669	343	461	AGACTGGGAAATTAAAGGCCG	CGTATAGCAGCAGCAAAGCAAG
pgk-gap (S18)	1729282–1729787	506	590	GAACACGTTTTCCTGTGACATCA	GTGATACGGCTGTGGCTTTTG
uvrC-BH05560 (S19)	653261–653650	390	532	AGCTTTTCTTGCTCATTTTCGG	AGCTCAGTCCCTTTCTTATCGC
trwL4-trwL5 (S20)	1805508–1805660	153	280	AGATACATTCGTACGGTGGGGA	CCTGTTGTTATTTTTTGATTGGAG

[a] Intergenic spacer names consist of the name of the 5′-flanking gene combined (-) with the name of the 3′-flanking gene. Flanking open reading frames encoding putative proteins of unknown function are named after their open reading frame number within the *B. henselae* genome (GenBank accession number BX897699).
[b] The positions of the spacers on the genome, the spacer size, and the PCR product size were deduced from *B. henselae* (BX897699).
[c] Spacers S1 to S9 were numbered in descending order of variability.

B. henselae (39 MST types among 126 *B. henselae* isolates; $P < 0.01$).

Phylogenetic classification of MST types. Phylogenetic trees obtained from concatenated spacer sequences using the neighbor-joining (Fig. 3) and maximum parsimony methods showed similar phylogenetic classifications. The 126 tested isolates were grouped into four clusters. Asian isolates were grouped into cluster 1. European isolates were grouped into clusters 2 to 4. In contrast, American isolates did not form a coherent cluster but were spread among the four clusters.

DISCUSSION

In this study, we demonstrated that MST is a highly efficient method for genotyping *B. henselae* at the strain level, with 39 genotypes identified among 126 studied isolates using a combination of nine intergenic spacer sequences. Prior to our study, the most discriminatory genotyping method for *B. henselae*, i.e., MLST using nine genes, had identified seven genotypes among cat and human isolates of *B. henselae* (21). Therefore, MST was more discriminatory than MLST for typing *B. henselae*.

We found *B. henselae* to be significantly more genotypically variable than *B. quintana*, a human pathogen previously identified to be mostly clonal (13) ($P < 0.01$). Such a higher genetic diversity of *B. henselae* is as yet unexplained, despite the studies conducted on the relationship between cat and human isolates. In Germany and The Netherlands, a majority of human isolates were of 16S rRNA gene type I whereas cat isolates mostly belonged to type II (3, 4, 10, 28, 30). In contrast, in Switzerland, France, and the United States, investigators have demonstrated that most of the human isolates of *B. henselae* belonged to 16S rRNA gene type II (5, 8, 17). Iredell et al., using MLST identifying seven genotypes, found that human infection is caused by a limited number of genotypes (21). Therefore, the relationship between human and cat isolates of *B. henselae* remains a puzzling problem. We believe that MST may also be a suitable tool for investigating the dynamics of *B. henselae* populations in humans.

Among the 126 isolates analyzed in this study, we found a significantly higher genotypic heterogeneity among Asian isolates than among European ($P < 0.01$) and American ($P = 0.03$) isolates. This may be explained by the fact that most European isolates originate from only two neighboring coun-

```
1    CAATCTTTTTAGAAG----------------------------------------------------------  (106)
2    CAATCTTTTTAGAAGCAATCTTTTTAGAAG-------------------------------------------  (5)
3    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG----------------------------  (11)
4    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG------------  (3)
5    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG  (1)
```

FIG. 1. Description of the 15-bp repeated sequences within the tRNA-Ala/GCA-tRNA-Ile/AUC spacer. The first column contains the copy number of repeats. Numbers in parentheses indicate the numbers of strains that have the corresponding repeat numbers.

TABLE 3. Polymorphism characteristics of the nine variable intergenic spacers

Spacer name	No. of nucleotide variations	No. of genotypes	Spacer polymorphism, with reference to Houston-1 strain[a]
tRNA-Ala/GCA-tRNA-Ile/AUC (S1)	5	9	G9A, C49T, 203inserT, C256T, 294VNTR
BH2865724-*dut* (S2)	14	7	T19C, G31A, C92T, C103T, C113T, C142T, A156G, A162G, C169T, G237T, A289G, C310T, T332dele, T339C
dnaJ-related protein-*cobS* (S3)	8	6	A3G, G12A, A25G, G46A, C84T, T203C, T255C, T264dele
pssA-oxidoreductase (S4)	9	5	A49G, 51inserA, 93inser,[b] G159T, A274G, A306G, T322C, A362G, T484C
carB-cold shock protein (S5)	5	5	51inser,[e] C83A, G145C, T157C, T240C
alr-gcvP (S6)	8	4	C4T, G10A, C60A, G242A, T256G or C, 296dele,[d] A305G, A306G
ftsK-oxidoreductase (S7)	8	4	C324A, G326A, 362inser,[c] G370A, A390C, C432T, A436G, C480T
BH2864883-BH2864884 (S8)	8	3	G19C, A60G, A61G, 69inserT, A88G, C102A, C249A, C282T
acpP2-malate oxidoreductase (S9)	4	3	C28T, A40C, G96A, C114T
Total (9 spacers)	69	39	

[a] The numbers show each variable nucleotide position in reference to the Houston-1 strain. The locus before the number is that within Houston-1, and the locus after the number is a possible variable nucleotide within other strains. inser, insertion; dele, deletion; VNTR, variable number of tandem repeats.
[b] Insertion of CCAGAGTGCTATTCATTAAATAAGTTTGCTTTTAAAAAATATTTCTTG.
[c] Insertion of TTCACCTGTTTCATA.
[d] Deletion of TTTTTG.
[e] Insertion of GTAGGGCA.

tries, France and Germany, and American isolates were mostly obtained from only two states, California and Florida, whereas Asian isolates originate from three countries. However, the phylogenic analysis built by concatenating the nine spacers (Fig. 3) revealed that Asian isolates, despite their apparent genotypic heterogeneity, were phylogenetically homogeneous and were grouped into a single cluster, without any overlap with European isolates. This may suggest that Asian isolates have a more recent common origin. American isolates appeared to be phylogenetically more heterogeneous than other isolates. None of the 39 MST types identified was represented in European, American, and Asian isolates together. Thus, we did not identify any pandemic isolate. However, our data may be updated by future studies incorporating isolates from other geographic origins.

To limit the number of spacers to be sequenced, we propose specific guidelines that facilitate their selection (Fig. 2). In addition, to facilitate usage of MST for genotyping of *B. henselae*, we created an MST-dedicated, free-access online database, i.e., MST-Rick, to which any investigators

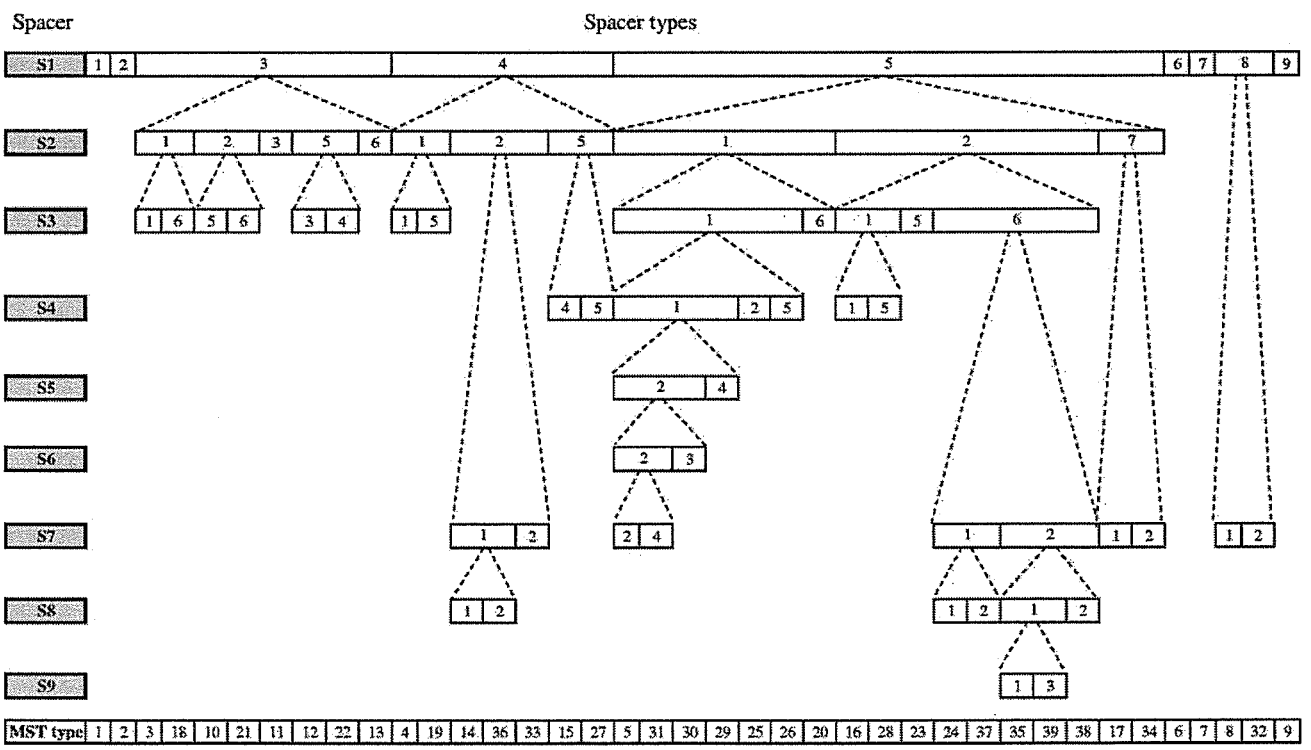

FIG. 2. Guidelines for selection of spacers for MST genotyping of *B. henselae* isolates.

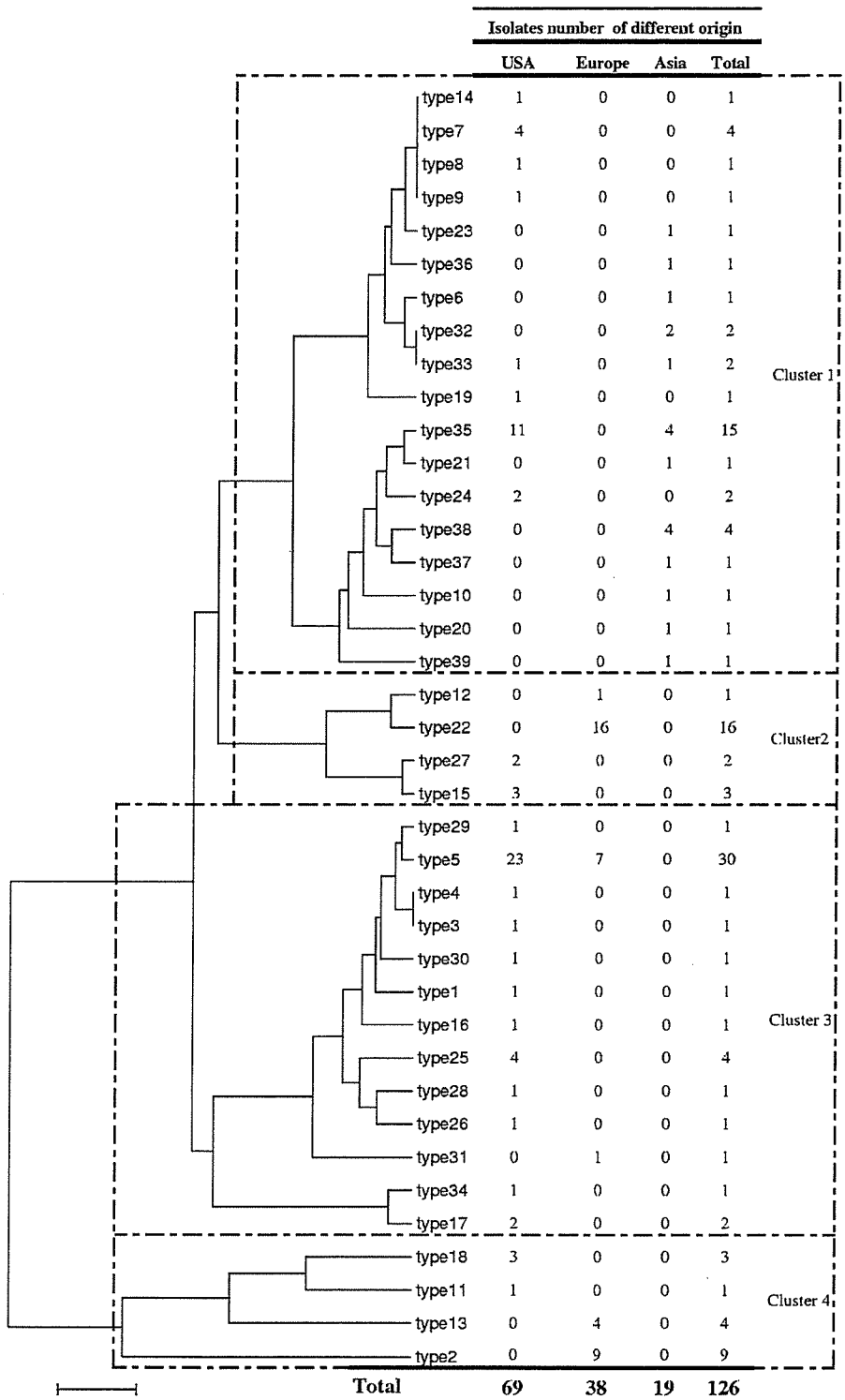

FIG. 3. Dendrogram showing the phylogenetic organization of the 39 MST genotypes, constructed using the neighbor-joining method. Sequences from the nine spacers were concatenated. The scale bar represents a 1% nucleotide sequence variation.

may compare their own spacer sequences (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst). Although our study is preliminary and includes a limited number of strains, we hope that our method and database will be used and implemented by other investigators, which would allow frequent updating of the data.

In conclusion, MST using nine variable intergenic spacers identified 39 genotypes among 126 *B. henselae* cat isolates. As

such, MST is the most discriminatory genotyping method for *B. henselae* isolates to date and may be used to investigate the relationships between human and cat isolates of *B. henselae*. Recently, we successfully used MST for genotyping *B. henselae* isolates within lymph node biopsy samples from patients with cat scratch disease (unpublished data). As *B. henselae* is extremely difficult to grow from human specimens, MST might thus serve as both a detection and a genotyping tool.

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All authors have read and approved the final version of the manuscript and do not have any conflict of interest related to this research.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25**:3389–3402.
2. **Anderson, B., K. Sims, and R. Regnery.** 1994. Detection of the *Rochalimaea henselae* DNA in specimens from cat-scratch disease patients by polymerase chain reaction. J. Clin. Microbiol. **32**:942–948.
3. **Bergmans, A. M., C. M. de Jong, G. Van Amerongen, C. S. Schot, and L. M. Schouls.** 1997. Prevalence of *Bartonella* species in domestic cats in The Netherlands. J. Clin. Microbiol. **35**:2256–2261.
4. **Bergmans, A. M., J. F. Schellekens, J. D. van Embden, and L. M. Schouls.** 1996. Predominance of two *Bartonella henselae* variants among cat-scratch disease patients in the Netherlands. J. Clin. Microbiol. **34**:254–260.
5. **Box, A. T. A., A. Sander, I. Perschil, D. L. Goldenberg, and M. Altwegg.** 2000. Cats are probably not the only reservoir for infections due to *Bartonella henselae*. J. Microbiol. Methods **27**:101–102.
6. **Chomel, B. B.** 2000. Cat-scratch disease. Rev. Sci. Tech. **19**:136–150.
7. **Chomel, B. B., R. W. Kasten, K. Floyd-Hawkins, B. Chi, K. Yamamoto, J. Roberts-Wilson, A. N. Gurfield, R. C. Abbott, N. C. Pedersen, and J. E. Koehler.** 1996. Experimental transmission of *Bartonella henselae* by the cat flea. J. Clin. Microbiol. **34**:1952–1956.
8. **Dauga, C., I. Miras, and P. A. D. Grimont.** 1996. Identification of *Bartonella henselae* and *B. quintana* 16S rDNA sequences by branch-, genus- and species-specific amplification. J. Med. Microbiol. **45**:192–199.
9. **De Lamballerie, X., C. Zandotti, C. Vignoli, C. Bollet, and P. de Micco.** 1992. A rare step microbial DNA extraction method using Chelex 100 suitable for gene amplification. Res. Microbiol. **143**:785–790.
10. **Dillon, B., J. Valenzuela, R. Don, D. Blanckenberg, D. I. Wigney, R. Malik, A. J. Morris, J. M. Robson, and J. Iredell.** 2002. Limited diversity among human isolates of *Bartonella henselae*. J. Clin. Microbiol. **40**:4691–4699.
11. **Drancourt, M., V. Roux, L. V. Dang, T. H. C. D. Lam, V. Chenal-Francisque, H. Ogata, P. E. Fournier, E. Crubezy, and D. Raoult.** 2004. Genotyping, Orientalis-like *Yersinia pestis*, and plague pandemics. Emerg. Infect. Dis. **10**:1585–1592.
12. **Ehrenborg, C., L. Wesslen, A. Jakobson, G. Friman, and M. Holmberg.** 2000. Sequence variation in the *ftsZ* gene of *Bartonella henselae* isolates and clinical samples. J. Clin. Microbiol. **38**:682–687.
13. **Foucault, C., B. La Scola, H. Lindroos, S. G. Andersson, and D. Raoult.** 2005. Multispacer typing technique for sequence-based typing of *Bartonella quintana*. J. Clin. Microbiol. **43**:41–48.
14. **Fournier, P. E., and D. Raoult.** 1998. Cat scratch disease and an overview of other *Bartonella henselae* related infections, p. 32–62. *In* A. Schmidt (ed.), *Bartonella* and *Afipia* species emphasizing *Bartonella henselae*. Karger, Basel, Switzerland.
15. **Fournier, P. E., Y. Zhu, H. Ogata, and D. Raoult.** 2004. Use of highly variable intergenic spacer sequences for multispacer typing of *Rickettsia conorii* strains. J. Clin. Microbiol. **42**:5757–5766.
16. **Glazunova, O., V. Roux, O. Freylikman, Z. Sekeyova, G. Fournous, J. Tyczka, N. Tokarevich, E. Kovacava, T. J. Marrie, and D. Raoult.** 2005. *Coxiella burnetii* genotyping. Emerg. Infect. Dis. **11**:1211–1217.
17. **Gurfield, A. N., H. J. Boulouis, B. B. Chomel, R. Heller, R. W. Kasten, K. Yamamoto, and Y. Piemont.** 1997. Coinfection with *Bartonella clarridgeiae* and *Bartonella henselae* and with different *Bartonella henselae* strains in domestic cats. J. Clin. Microbiol. **35**:2120–2123.
18. **Hadfield, T. L., R. Warren, M. Kass, E. Brun, and C. Levy.** 1993. Endocarditis caused by *Rochalimaea henselae*. Hum. Pathol. **24**:1140–1141.
19. **Handley, S. A., and R. L. Regnery.** 2000. Differentiation of pathogenic *Bartonella* species by infrequent restriction site PCR. J. Clin. Microbiol. **38**:3010–3015.
20. **Houpikian, P., and D. Raoult.** 2001. 16S/23S rRNA intergenic spacer regions for phylogenetic analysis, identification, and subtyping of. *Bartonella* species. J. Clin. Microbiol. **39**:2768–2778.
21. **Iredell, J., D. Blanckenberg, M. Arvand, S. Grauling, E. J. Feil, and R. J. Birtles.** 2003. Characterization of the natural population of *Bartonella henselae* by multilocus sequence typing. J. Clin. Microbiol. **41**:5071–5079.
22. **Koehler, J. E.** 1996. *Bartonella* infections. Adv. Pediatr. Infect. Dis. **11**:1–27.
23. **Koehler, J. E., C. A. Glaser, and J. W. Tappero.** 1994. *Rochalimaea henselae* infection: a new zoonosis with the domestic cat as a reservoir. JAMA **271**: 531–535.
24. **Kordick, D. L., K. H. Wilson, D. J. Sexton, T. L. Hadfield, H. A. Berkhoff, and E. B. Breitschwerdt.** 1995. Prolonged *Bartonella* bacteremia in cats associated with cat-scratch disease patients. J. Clin. Microbiol. **33**:3245–3251.
25. **Kumar, S., K. Tamura, and M. Nei.** 2004. MEGA 3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform. **5**:150–163.
26. **La Scola, B., Z. X. Liang, Z. Zeaiter, P. Houpikian, P. A. D. Grimont, and D. Raoult.** 2002. Genotypic characteristics of two serotypes of *Bartonella henselae*. J. Clin. Microbiol. **40**:2002–2008.
27. **La Scola, B., Z. Zeaiter, A. Khamis, and D. Raoult.** 2003. Gene-sequence-based criteria for species definition in bacteriology: the *Bartonella* paradigm. Trends Microbiol. **11**:318–321.
28. **Sander, A., M. Posselt, N. Böhm, M. Ruess, and M. Altwegg.** 1999. Detection of *Bartonella henselae* DNA by two different PCR assays and determination of the genotypes of strains involved in histologically defined cat scratch disease. J. Clin. Microbiol. **37**:993–997.
29. **Sander, A., M. Ruess, S. Bereswill, M. Schuppler, and B. Steinbrueckner.** 1998. Comparison of different DNA fingerprinting techniques for molecular typing of *Bartonella henselae* isolates. J. Clin. Microbiol. **36**:2973–2981.
30. **Sander, A., M. Ruess, K. Deichmann, N. Böhm, and W. Bredt.** 1998. Two different genotypes of *Bartonella henselae* in children with cat-scratch disease and their pet cats. Scand. J. Infect. Dis. **30**:387–391.
31. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673–4680.
32. **Welch, D. F., D. A. Pickett, L. N. Slater, A. G. Steigerwalt, and D. J. Brenner.** 1992. *Rochalimaea henselae* sp. nov., a cause of septicemia, bacillary angiomatosis, and parenchymal bacillary peliosis. J. Clin. Microbiol. **30**:275–280.
33. **Yang, J., J. Wang, Z. J. Yao, Q. Jin, Y. Shen, and R. Chen.** 2003. GenomeComp: a visualization tool for microbial genome comparison. J. Microbiol. Methods **54**:423–426.
34. **Zeaiter, Z., P. E. Fournier, and D. Raoult.** 2002. Genomic variation of *Bartonella henselae* strains detected in lymph nodes of patients with cat scratch disease. J. Clin. Microbiol. **40**:1023–1030.
35. **Zhu, Y., P. E. Fournier, H. Ogata, and D. Raoult.** 2005. Multispacer typing of *Rickettsia prowazekii* enabling epidemiological studies of epidemic typhus. J. Clin. Microbiol. **43**:4708–4712.

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Short communication

Serological survey of *Ehrlichia* and *Anaplasma* infection of feral raccoons (*Procyon lotor*) in Kanagawa Prefecture, Japan

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Abstract

Numbers of feral raccoon; the possible reservoir animal of *Ehrlichia* and *Anaplasma*, are increasing in Japan. Thus serological methods were utilized to examine *Ehrlichia* and *Anaplasma* infection in raccoons from Kanagawa Prefecture, Japan. By using an indirect immunofluorescence assay, among 187 feral raccoons examined, 1 (0.5%) serologically reacted with *Ehrlichia canis*, 3 (1.6%) with *Ehrlichia chaffeensis* and 1 (0.5%) with *Anaplasma phagocytophilum* with the titers of 1:40 or more. Although screening PCR for *Ehrlichia* and *Anaplasma* species failed to detect the presence of ehrlichial DNA in serum samples, results of the serological tests suggested that the feral raccoons might be infected with some species of *Ehrlichia* and *Anaplasma*.

Keywords: *Anaplasma*; *Ehrlichia*; Raccoon

1. Introduction

The raccoon (*Procyon lotor* (Linnaeus, 1758)) is widely distributed throughout regions ranging from Canada to Central America. However, a large number of raccoons have been imported from the U.S.A. as pet animals into Japan since the 1970s. The intentional release and escape of pet raccoons has resulted in a naturalized population in most parts of Japan. One of the strongest concerns about the establishment of this animal in Japan is the possible transmission of pathogens to both human and domestic animals, because these animals were imported without sufficient quarantine until a new regulatory law was passed recently to control imported animals. Indeed, some emerging pathogens have been detected, including a *Babesia microti*-like parasite in Hokkaido (Kawabuchi et al., 2005) and gastrointestinal helminthes in Wakayama Prefecture (Sato and Suzuki, 2006).

Both *Ehrlichia* and *Anaplasma* are important tick-borne bacteria of both humans and animals. Especially *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* cause two major human infections, human monocytic ehrlichiosis and human granulocytic anaplasmosis, respectively (Anderson et al., 1991; Bakken et al., 1994). Because the feral raccoon is one of the reservoir animals of both *E. chaffeensis* and *A. phagocytophilum*

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in the U.S.A. (Comer et al., 2000; Levin et al., 2002), these ehrlichial pathogens might have been introduced into Japan by the imported animals. But little information is available on *Ehrlichia* and *Anaplasma* infection of feral raccoons in Japan. Thus the aim of this study was to examine the sero-prevalence of antibodies against *Ehrlichia* and *Anaplasma* in raccoons in Japan.

2. Materials and methods

2.1. Raccoon sera

From October 2001 to September 2002, a total of 187 raccoons (145 adults and 42 juveniles) were captured by cage traps in Kamakura, Fujisawa, Zushi, Sagamihara, Odawara and Shiroyama areas in Kanagawa Prefecture, Japan. Before sample collection, the general body condition of raccoons was examined thoroughly. Raccoons were immobilized by administering an intramuscular injection of ketamine hydrochloride and xylazine. After immobilization, the sex of the individual was noted and the animals were differentiated to two age groups such as adults and juveniles by the general appearance of the animals and the condition of the teeth. Blood samples were collected from the jugular or saphenous veins of raccoons. The blood samples were clotted for 1–2 h at room temperature and then centrifuged at 500 × *g* for 15 min. The separated sera were stored at −20 °C until analysis.

2.2. Indirect immunofluorescence assay (IFA)

A modified method of IFA was carried out to detect antibodies against *E. chaffeensis*, *Ehrlichia canis*, and *A. phagocytophilum*. IFA antigen slides were prepared using standard methods (Brouqui et al., 1994) using DH82 cells infected with *E. chaffeensis* (Arkansas strain, supplied by J. Dawson) and *E. canis* (Israel strain, supplied by Dr. Harrus, The Hebrew University of Jerusalem), and HL60 cells infected with *A. phagocytophila* (HGE agent Webster strain, supplied by J.S. Dumler). The raccoon sera samples were screened at a 1:20 dilution in phosphate-buffered saline (pH 7.2) Tween 0.5% (PBST). The antibody against the raccoon sera was prepared as follows: raccoon immunoglobulin was inoculated into a rabbit, and then the sera purified from the rabbit were used as second antibody. This anti-raccoon rabbit serum was kindly provided by the Department of Veterinary Science, National Institute of Infectious Diseases, Japan. Fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG conjugate (Cappel Co. Ltd., USA) was also used as the third antibody of the IFA. Reactive antibodies were then detected using a fluorescence light microscope. Those samples that reacted with any one of the antigens at the screening dilution were then titrated to endpoint. Because the positive and negative controls of raccoon sera were unavailable, serum from mice that were experimentally infected with *E. chaffeensis* and *A. phagocytophilum* were used as positive controls. Serum from a dog naturally infected with *E. canis* was also used as a positive control. FITC-labeled rabbit anti-mouse IgG conjugate and FITC-labeled rabbit anti-dog IgG conjugate were used as the second antibodies for the positive controls of *E. chaffeensis* and *A. phagocytophilum*, and *E. canis*, respectively.

When the serum showed a positive result with any one of the antigens at the dilution of 1:20, the reactivity of the serum with mouse spleen infected with *Ehrlichia muris* (Hyogo strain, supplied by Dr. Masayoshi Tsuji, Rakuno Gakuen University, Japan) and *Ehrlichia* from *Ixodes ovatus* (EIO) (HF639) were also examined by the method previously described (Watanabe et al., 2004).

2.3. PCR screening

When the serum showed a positive result with any one of the antigens at the dilution of 1:20, DNA was also extracted from the serum samples that showed any positive result, by using a QIAmp DNA Mini Kit (Qiagen, Hilden, Germany). Screening PCR for *Ehrlichia* and *Anaplasma* was performed by using the primer pair of EHR16SD and EHR16SR, which can amplify the 16S rRNA gene of genus *Ehrlichia*, *Anaplasma* and *Neorickettsia* (Parola et al., 2000).

3. Results and discussion

Among 187 feral raccoons examined, 9 (4.8%) were reactive to *E. chaffeensis*, *E. canis* or *A. phagocytophilum* at the screening level. All the positive samples were from adult individuals; the percent of positivity among 145 adult raccoons was 6.2%. The results of the titration of these nine samples are shown in Table 1. All the nine samples that reacted serologically with *E. chaffeensis*, *E. canis* or *A. phagocytophilum* were negative in the PCR to detect *Ehrlichia* or *Anaplasma*.

Of these nine samples, three (Nos. 13-18, 13-94, 13-180) showed the highest titers (1:40) with *E. chaffeensis*, but these samples also reacted with *E. canis*, *E. muris* or EIO at similar titers of 1:20. It has been previously demonstrated that cross-reactivity between the different *Ehrlichia* species may occur (Brouqui et al., 1992, 1994; Dumler et al., 1995). The

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Table 1
Serological titers of individual raccoons that showed IFA seroreactivity with at least one antigen with a titer of 1:20 or higher

ID number	Serological titers				
	E. chaffeensis	*E. canis*	*E. muris*	EIO[a]	*A. phagocytophilum*
13-18	40	<20	20	<20	20
13-94	40	20	20	<20	<20
13-180	40	20	20	<20	20
14-14	20	640	20	20	20
14-38	20	<20	<20	<20	40
13-16	20	20	20	<20	20
13-97	20	<20	<20	<20	<20
14-135	20	20	<20	<20	20
14-173	<20	<20	<20	<20	20

[a] *Ehrlichia* species detected from *Ixodes ovatus*.

identification of antibodies against *E. chaffeensis* antigens suggests that *E. chaffeensis*, *E. canis*, or other serologically related pathogens such as *E. muris* and EIO, both endemic pathogens in Japan, are potentially infectious agents (Kawahara et al., 1999; Shibata et al., 2000).

Another sample (No. 14-14) reacted with *E. canis*, with a titer of 1:640. This sample also reacted marginally with the other four antigens. This higher titer of antibody only against *E. canis* suggests that the feral raccoon had been infected with the agent. The negative result of PCR for *Ehrlichia* and *Anaplasma* does not rule out the possibility of *E. canis* infection of the feral raccoon. *E. canis* can be transmitted by the ticks *Rhipicephalus sanguineus* and *Dermacentor variabilis* (Groves et al., 1975; Johnson et al., 1998). As feral raccoons are frequently parasitized by adult *D. variabilis* in the USA (Kollars, 1993; Kollars and Ladine, 1999), it is possible that the raccoon had been infected with *E. canis* before being introduced into Japan. Although *R. sanguineus* is mainly distributed in Okinawa Prefecture in Japan, this tick is occasionally found in dogs in the mainland of Japan (Shimada et al., 2003). It is also possible that the feral raccoon had been infected with *E. canis* in Japan by the vector ticks.

The other sample (No. 14-38) showed highest titers with *A. phagocytophilum* at the titer of 1:40, and the same sample showed marginal titers for the other species. This result suggests the possibility that this feral raccoon was infected with *A. phagocytophilum*. Feral raccoons are important hosts for *Ixodes scapularis*, a vector of *A. phagocytophilum* in the north-eastern USA (Fish and Daniels, 1990; Maneli et al., 1993; Vignes and Fish, 1997). More than 10% of blood samples of raccoons in the north-eastern USA showed positive PCR for *A. phagocytophilum* (Levin et al., 2002). It is possible that the feral raccoons examined in this study were infected with *A. phagocytophilum* before being introduced into Japan. Recently, *A. phagocytophilum* DNA was detected from *Ixodes persulcatus* ticks and deer in Japan (Kawahara et al., 2006; Ohashi et al., 2005). Thus, the other possibility is that the infection with *A. phagocytophilum* might have occurred via tick infestation in Japan.

The remaining four samples showed marginal titers (1:20 or less) with at least any one of the antigens; however, it was not possible to precisely evaluate these lower titers of antibodies quantitatively. Because all nine samples that reacted serologically with ehrlichial antigens were negative in the PCR assay, it was not possible to determine whether raccoons can be reservoir animals of these organisms or not. A specific PCR for *Ehrlichia* was also failed to detect DNA from raccoon serum in the previous study (Comer et al., 2000). Further epidemiological studies, including examination and analysis of the peripheral blood and spleen of feral raccoons will be necessary to clarify the role of this animal in human infection with *Ehrlichia* and *Anaplasma*.

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References

Anderson, B.E., Dawson, J.E., Jones, D.C., Wilson, K.H., 1991. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J. Clin. Microbiol. 29, 2838–2842.

Bakken, J.S., Dumler, J.S., Chen, S.M., Eckman, M.R., Van Etta, L.L., Walker, D.H., 1994. Human granurocytic ehrlichiosis in the upper Midwest United States: a new species emerging? J. Am. Med. Assoc. 272, 212–218.

Please cite this article in press as: Inokuma, H. et al., Serological survey of *Ehrlichia* and *Anaplasma* infection of feral raccoons (*Procyon lotor*) in Kanagawa Prefecture, Japan, Vet. Parasitol. (2006), doi:10.1016/j.vetpar.2006.11.002

Brouqui, P., Dumler, J.S., Raoult, D., Walker, D.H., 1992. Antigenic characterization of Ehrlichiae; protein immunoblotting of *Ehrlichia canis*, *Ehrlichia sennetsu*, and *Ehrlichia risticii*. J. Clin. Microbiol. 30, 1062–1066.

Brouqui, P., Lecam, C., Olson, J., Raoult, D., 1994. Serological diagnosis of human monocytic ehrlichiosis by immunoblot analysis. Clin. Diag. Lab. Immunol. 1, 645–649.

Comer, J.A., Nicholson, W.L., Paddock, C.D., Sumner, J.W., Childs, J.E., 2000. Detection of antibodies reactive with *Ehrlichia chaffeensis* in the raccoon. J. Wildl. Dis. 36, 705–712.

Dumler, D.H., Asanovich, K.M., Bakken, J.S., Richter, P., Kimsey, R., Madigan, J.E., 1995. Serological cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granurocytic ehrlichia. J. Clin. Microbiol. 33, 1098–1103.

Fish, D., Daniels, T.J., 1990. The role of medium-sized mammals as reservoirs of *Borrelia burgdorferi* in southern New York. J. Wildl. Dis. 26, 339–345.

Groves, M.G., Dennis, G.L., Amyx, H.L., Hursll, D.L., 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. 36, 937–940.

Johnson, E.M., Ewing, S.A., Barker, R.W., Fox, J.C., Crow, D.W., Kocan, K.M., 1998. Experimental transmission of *Ehrlichia canis* (Rickettsiales: Ehrlichieae) by *Dermacentor variabilis* (Acari: Ixodidae). Vet. Parasitol. 31, 277–288.

Kawabuchi, T., Tsuji, M., Sado, A., Matoba, Y., Asakawa, M., Ishihara, C., 2005. *Babesia microti*-like parasites detected in feral raccoons (*Procyon lotor*) captured in Hokkaido, Japan. J. Vet. Med. Sci. 67, 825–827.

Kawahara, M., Ito, T., Suto, C., Shibata, S., Rikihisa, Y., Hata, K., Hirai, K., 1999. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serological survey of humans and animals with *E. muris* as antigen. J. Clin. Microbiol. 37, 1123–1129.

Kawahara, M., Rikihisa, Y., Lin, Q., Isogai, E., Tahara, K., Itagaki, A., Giramiysu, Y., Tajima, T., 2006. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Appl. Environ. Microbiol. 72, 1102–1109.

Kollars Jr., T.M., 1993. Ticks (Acari: Ixodidae) infesting medium-sized wild mammals in south-western Tennessee. J. Med. Entomol. 30, 896–900.

Kollars Jr., T.M., Ladine, T.A., 1999. Patterns of infestation by adult *Dermacentor variabilis* (Acari: Ixodidae) in a mark-recapture study of raccoons (Mammalia: Carnivora) and *Virginia opossums* (Mammalia: Didelphimorphia) in Tennessee. J. Med. Entomol. 36, 263–267.

Levin, M.L., Nicholson, W.L., Massung, R.F., Sumner, J.W., Fish, D., 2002. Comparison of the reservoir competence of medium-sized mammals and *Peromyscus leucopus* for *Anaplasma phagocytophilum* in Connecticut. Vector Bor. Zoon. Dis. 2, 125–136.

Maneli, A., Kitron, U., Jones, C.J., Slajchert, T.J., 1993. *Ixodes* infestation on medium-sized mammals and blue jays in northwestern Illinois. J. Med. Entomol. 30, 950–952.

Ohashi, N., Inayoshim, M., Kitamura, K., Kawamori, F., Kawaguchi, D., Nishimura, Y., Naitou, H., Hiroi, M., Masuzawa, T., 2005. *Anaplasma phagocytophilum* infected ticks, Japan. Emerg. Infect. Dis. 11, 1780–1783.

Parola, P., Roux, V., Camicas, J.-L., Baradji, I., Brouqui, P., Raoult, D., 2000. Detection of ehrlichiae in African ticks by PCR. Trans. Roy. Soc. Trop. Med. Hyg. 94, 707–708.

Sato, H., Suzuki, K., 2006. Gastrointestinal helminthes of feral raccoons (*Procyon lotor*) in Wakayama Prefecture, Japan. J. Vet. Med. Sci. 68, 311–318.

Shibata, S.-I., Kawahara, M., Rikihisa, Y., Fujita, H., Watanabe, Y., Suto, C., Ito, T., 2000. New *Ehrlichia* species related to *Ehrlichia chaffeensis* isolated from *Ixodes ovatus* ticks in Japan. J. Clin. Microbiol. 38, 1331–1338.

Shimada, Y., Beppu, T., Inokuma, H.T., Okuda, M., Onishi, T., 2003. Ixodid tick species recovered from domestic dogs in Japan. Med. Vet. Entomol. 17, 38–45.

Vignes, F., Fish, D., 1997. Transmission of the agent of human granulocytic ehrlichiosis by host-seeking *Ixodes scapularis* (Acari: Ixodidae) in southern New York state. J. Med. Entomol. 34, 379–382.

Watanabe, M., Okuda, M., Tsuji, M., Inokuma, H., 2004. Seroepidemiological study of canine ehrlichial infections in Yamaguchi Prefecture and surrounding areas of Japan. Vet. Parasitol. 124, 101–107.

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サルから分離された *Yersinia pseudotuberculosis* の病原性状

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特集　人と動物の共通感染症最前線3

サルから分離された *Yersinia pseudotuberculosis* の病原性状

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◎要　約◎

2001～2005年の間に，我が国のサル飼育施設11か所において発生した，延べ15回の *Yersinia pseudotuberculosis* 感染症のサル感染致死例から分離された15株について，血清型および病原性状などの特徴を整理した。その結果，我が国でリスザルなどの外来のサル類が本菌感染により極めて重篤な症状を示すことが多い理由として，本菌の産生するスーパー抗原（YPM）が関与している可能性が示唆された。また，血清型7型によるサルの感染致死事例が初めて確認された。

1．はじめに

Yersinia pseudotuberculosis は，代表的な人獣共通感染症原因菌として知られている。本菌が動物に感染した場合，多くは不顕性に推移するが，サル類，特にリスザルなどの外来のサル類は，本菌に対して感受性が高く，毎年のように動物園などのサル飼育施設で本菌による感染死亡例が発生しており，サルの飼育管理のみならず公衆衛生の面からも大きな問題となっている。本研究では，我が国のサル飼育施設における *Y. pseudotuberculosis* 感染症の実態を把握し，その防除を図るための研究の一環として，サルの感染致死個体から分離された菌について血清型や病原性状などの特徴を整理した。

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エルシニア，サルモネラ，豚丹毒などの細菌性人獣共通感染症に関する研究を行っています。国内の動物園などのサル飼育施設におけるエルシニア症の調査を始めて，想像以上にサルにエルシニア症が頻発していることを知り，現在，ワクチンによる効果的な予防法を研究中です。

2．調査方法

供試菌株として2001年4月～2005年3月の間に，我が国のサル飼育施設11か所（関東地方3か所，近畿地方1か所，中国地方1か所，四国地方1か所および九州地方5か所）において，延べ15回発生した *Y. pseudotuberculosis* 感染症によるサルの感染致死個体（リスザル12頭，オランウータン1頭，マントヒヒ1頭およびシロガオサキ1頭）から分離された *Y. pseudotuberculosis* 15株を用いた。また，供試菌株の血清型別は，抗血清によるスライド凝集反応と，近年Bogdanovichらにより報告されたPCR法により行った[1)]。病原因子としては，病原性プラスミド（pYV）DNA上に存在するものとして *virF* 遺伝子を，染色体DNA上に存在するものとして，本菌の侵襲性に関与する *inv* 遺伝子，スーパー抗原である *Yersinia pseudotuberculosis* - Derived Mitogen（YPMs）のサブタイプYPMa，YPMbおよびYPMcをそれぞれコードする *ypmA*，*ypmB* および *ypmC* 遺伝子，ならびにHigh - pathogenicity island（HPI）に存在し，取り込み蛋白をコードしている *irp2* 遺伝子を選び，PCR法により検索を行った。

3．調査成績

供試した *Y. pseudotuberculosis* 15株の血清型は，4b型が7株(46.7%)で最も多く，次いで1b型が5株(33.3%)，3，6および7型がそれぞれ1株（6.7%）であった（表1）。このうち，1bおよび4b型は，我が国では人，動物ならびに環境から高頻度に分離される血清型で，今回，サル感染致死個体から分離された本菌の血清型の分布は，これらの姿とよく一致するものであった。また，血清型7型に

表1　我が国の飼育サルから分離された *Y. pseudotuberculosis* の由来と性状

菌株 No.	施設名	施設所在地	発生年	由来	病原遺伝子						血清型
					virF	*inv*	スーパー抗原			*irp2*	
							ypmA	*ypmB*	*ypmC*		
1	A	関東地方	2002	リスザル	＋[a]	＋	＋	－[b]	－	－	4b
2	B	関東地方	2003	オランウータン	＋	＋	＋	－	－	－	4b
3	C	関東地方	2003	リスザル	＋	＋	＋	－	－	－	4b
4	C	関東地方	2005	リスザル	＋	＋	＋	－	－	－	4b
5	D	近畿地方	2003	リスザル	＋	＋	＋	－	－	－	4b
6	E	中国地方	2004	リスザル	＋	＋	＋	－	－	－	4b
7	F	四国地方	2001	リスザル	＋	＋	＋	－	－	－	1b
8	F	四国地方	2003	リスザル	＋	＋	＋	－	－	－	6
9	F	四国地方	2005	リスザル	＋	＋	＋	－	－	－	1b
10	G	九州地方	2002	リスザル	＋	＋	－	－	－	－	4b
11	G	九州地方	2003	リスザル	＋	＋	＋	－	－	－	7
12	H	九州地方	2003	リスザル	＋	＋	＋	－	－	－	1b
13	I	九州地方	2005	マントヒヒ	＋	＋	－	－	－	－	3
14	J	九州地方	2005	リスザル	＋	＋	＋	－	－	－	1b
15	K	九州地方	2005	シロガオサキ	＋	＋	＋	－	－	－	1b

[a] ＋：PCR 陽性　[b] －：PCR 陰性

ついては，これまで非病原性の血清型とされていたが，本調査により 7 型によるリスザルの感染致死事例が確認され，本血清型も病原性を有することが明らかになった。本症例は 7 型による世界で初めての動物感染致死例である。さらに，供試菌株について，PCR 法により病原遺伝子の保有状況を調べた結果，15 株いずれもが *inv* と *virF* 遺伝子を，また，血清型 7 型の株を含む 13 株（86.7%）が，スーパー抗原である YPMa をコードする *ypmA* 遺伝子を保有していた。一方，*ypmB*，*ypmC* および *irp2* 遺伝子は検出されなかった（表 1）。すなわち，サル由来株は全てが病原性プラスミドを保有する病原性株で，また，その多くがスーパー抗原活性を有する株であった。YPMa は日本を含むアジア極東地域で分離される *Y. pseudotuberculosis* に限局して分布することが知られている[2]。我が国において，南米，東南アジアおよびアフリカを原産とするサルが本菌に感染した場合，極めて重篤な症状を示すことが多い理由として，これらのサルの原産地には YPM を保有する *Y. pseudotuberculosis* が分布しておらず，これまでに病原性の強い *Y. pseudotuberculosis* の感作を受けたことがないため，YPM を保有する強毒な菌株に対する抵抗性を持たないことによると思われる。実際，動物園には日本原産のニホンザルがサル山などの形で数多く飼育されているが，これらニホンザルにおける *Y. pseudotuberculosis* 感染症の発生はまれである。

おわりに

動物園などのサル飼育施設において多発する *Y. pseudotuberculosis* 感染症は，これらの施設で飼育されているサル類の多くが国際自然保護連合により絶滅危惧種に指定されるような希少動物であり，物理的，経済的にも入手が困難であることから，サル飼育上の重要な問題となっており，早急にその感染予防対策を立てる必要がある。現在，本菌感染予防のためのワクチンはなく，簡便でかつ効果的な経口ワクチンの開発が望まれる。

現在，*Y. pseudotuberculosis* 感染症は，感染症法や食品衛生法の対象疾病に規定されていないため，人での発生実態は明らかではないが，西日本を中心に散発的に患者発生が報告されている。また，動物園ではリスザルなどのサル類だけでなく，鳥類や齧歯類など他の飼育動物にも本菌による感染致死事例が多発しており，動物飼育上のみならず公衆衛生上からも看過できない問題となっている。したがって，本菌の動向については今後とも十分な注意を払っていくべきであり，将来的には感染症法の対象疾病にすることを検討する必要があろう。

引用文献

1) Bogdanovich,T., Carniel, E., Fukushima,H. et al. (2003)：*J. Clin. Microbiol.* 41, 5103-5112.

2) Fukushima,H., Matsuda,Y., Seki,R. et al. (2001)：*J. Clin. Microbiol.* 39, 3541-3547.

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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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.*

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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/m*l*)	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/m*l*, *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/m*l*.

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/m*l*, and two out of 4 samples inoculated at a level of 10 CFU/m*l* (Table 5). Moreover, the organism was isolated from only one out of 4 samples inoculated at a level of 1 CFU/m*l* 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/m*l*, and from two out of 4 samples inoculated at a level of 10^3 CFU/m*l*. However, the organism was not isolated from any sample inoculated with injured cells at a level of 10 CFU/m*l* 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/m*l*)	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.

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REFERENCES

1. Andrews, W. H. and Ray, B. 1989. Importance and regulatory implication of the recovery of injured microorganisms from foods and water. pp. 217–223. *In*: Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds (Ray, B. ed.), CRC Press Inc., Boca Raton, Fla.
2. Aulisio, C. C. G., Mehlman, I. J. and Sanders, A. C. 1980. Alkali method for rapid recovery of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from food. *Appl. Environ. Microbiol.* **39**: 135–140.
3. Christensen, B., Torsvik, T. and Lien, T. 1992. Immunomagnetically captured thermophilic sulfate-reducing bacteria from North Sea oil field waters. *Appl. Environ. Microbiol.* **58**: 1244–1248.
4. Fukushima, H. 1985. Direct isolation of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from meat. *Appl. Environ. Microbiol.* **50**: 710–712.
5. Fukushima, H. 1987. New selective agar medium for isolation of virulent *Yersinia enterocolitica*. *J. Clin. Microbiol.* **25**: 1068–1073.
6. Fukushima, H. 1992. Direct isolation of *Yersinia pseudotuberculosis* from fresh water in Japan. *Appl. Environ. Microbiol.* **58**: 2688–2690.
7. Hara-Kudo, Y., Ikedo, M., Kodaka, H., Goto, K., Masuda, T., Konuma, H., Kojima, T. and Kumagai, S. 2000. Selective enrichment with a resuscitation step for isolation of freeze-injured *Escherichia coli* O157:H7 from foods. *Appl. Environ. Microbiol.* **66**: 2866–2872.
8. Hayashidani, H., Ohtomo, Y., Toyokawa, Y., Saito, M., Kaneko, S., Kosuge, J., Kato, M., Ogawa, M. and Kapperud, G. 1995. Potential source of sporadic human infection with *Yersinia enterocolitica* serovar O:8 in Aomori prefecture, Japan. *J. Clin. Microbiol.* 33: 1253–1257.
9. Hayashidani, H., Ishiyama, Y., Okatani, A. T., Yoshida, S., Ishikawa, M., Kato, Y., Ohtomo, Y., Saito, M., Horisaka, T., Kaneko, K. and Ogawa, M. 2003. Molecular genetic typing of *Yersinia enterocolitica* serovar O:8 isolated in Japan. *Adv. Exp. Med. Biol.* **529**: 363–365.
10. Hornes, E., Wasteson, Y. and Olsvik, Ø. 1991. Detection of *Escherichia coli* heat-stable enterotoxin genes in pig stool speciments by an immobilized, colorimetric, nested polymerase chain reaction. *J. Clin. Microbiol.* **29**: 2375–2379.
11. Hurst, A. 1977. Bacterial injury: a review. *Can. J. Microbiol.* **23**: 936–944.
12. Ichinohe, H., Yoshioka, M., Fukushima, H., Kaneko, S. and Maruyama, T. 1991. First isolation of *Yersinia enterocolitica* serotype O:8 in Japan. *J. Clin. Microbiol.* **29**: 846–847.
13. Iinuma, Y., Hayashidani, H., Kaneko, K., Ogawa, M. and Hamasaki, S. 1992. Isolation of *Yersinia enterocolitica* serovar O8 from free-living small rodents in Japan. *J. Clin. Microbiol.* **30**: 240–242.
14. Kang, D. H. and Fung, D. Y. C. 1999. Thin agar layer method for recovery of heat-injured *Listeria monocytogenes*. *J. Food. Prot.* **62**: 1346–1349.
15. Kang, D. H. and Sirragusa, G. R. 1999. Agar underlay method for recovery of sublethally heat-injured bacteria. *Appl. Environ. Microbiol.* **65**: 5334–5337.
16. Kapperud, G., Vardund, T., Skjerve, E., Hornes, E. and Michaelsen, T. E. 1993. Detection of pathogenic *Yersinia enterocolitica* in foods and water by immunomagnetic separation, nested polymerase chain reactions, and colorimetric detection of amplified DNA. *Appl. Environ. Microbiol.* **59**: 2938–2944.
17. Kay, B. A., Wachsmuth, K., Gemski, P., Feeley, J. C., Quan, T. J. and Brenner, D. J. 1983. Virulence and phenotypic characterization of *Yersinia enterocolitica* isolated from humans in the United States. *J. Clin. Microbiol.* **17**: 128–138.
18. Lund, A., Hellemann, A. L. and Vartdal, F. 1988. Rapid isolation of K88$^+$ *Escherichia coli* by using immnomagnetic particles. *J. Clin. Microbiol.* **26**: 2572–2575.
19. McCleery, D. R. and Rowe, M. T. 1995. Development of a selective plating technique for the recovery of *Escherichia coli*

O157:H7 after heat stress. *Lett. Appl. Microbiol.* 21: 252–256.
20. Morgan, J. A., Winstanley, C., Pickup, R. W. and Saunders, J. R. 1991. Rapid immunocapture of *Pseudomonas putida* cells from lake water by using bacterial flagella. *Appl. Environ. Microbiol.* 57: 503–509.
21. Ohtomo, Y., Toyokawa, Y., Saito, M., Yamaguchi, M., Kaneko, S. and Maruyama, T. 1995. Epidemiology of *Yersinia enterocolitica* serovar O:8 infection in the Tsugaru area in Japan. *Contrib. Microbiol. Immunol.* **13**: 48–50.
22. Saito, M., Yamaguchi, M., Toyokawa, Y., Ohtomo, Y., Kaneko, S. and Maruyama, T. 1994. *Yersinia enterocolitica* serotype O:8 infection in the Hirosaki district in Aomori Prefecture from 1984 to 1991. *J. Jpn. Assoc. Infect. Dis.* **68**: 960–965.
23. Schiemann, D. A. 1979. Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Can. J. Microbiol.* **25**: 1298–1304.
24. Schiemann, D. A. 1989. *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. pp. 601–672. *In*: Foodborne Bacterial Pathogens (Doyle, M. P. ed.), Marcel Dekker, New York, NY.
25. Ueda, S., Maruyama, T. and Kuwabara, Y. 2003. A method combining immunomagnetic separation followed by plating and polymerase chain reaction assay for the detection of pathogenic *Yersinia enterocolitica* from food and fecal samples. *Biocontrol Sci.* **8**: 77–86.

FULL PAPER *Public Health*

Babesia microti-Like Parasites Detected in Eurasian Red Squirrels (*Sciurus vulgaris orientis*) in Hokkaido, Japan

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ABSTRACT. Six Eurasian red squirrels (*Sciurus vulgaris orientis*), victims of road traffic found during 2002 and 2004 near the Noppro Forest Park in Ebetsu, Hokkaido, Japan, were examined for the presence of *Babesia* parasites. Three of the six squirrels exhibited positive signals by nested PCRs targeting both the 18S rRNA and β-tubulin genes. Three squirrels proved to be infected with a *B. microti*-like parasite as evidenced by sequencing the amplified DNAs and by the morphology of the intraerythrocytic parasites. Genotypically, however, the parasite appeared to be of a new type, as it was clearly distinguishable from any of the known types that have previously been reported in various wild animals. This is the first report showing molecular evidence for the presence of *B. microti*-like parasites in Sciuridae.

KEY WORDS: *Babesia microti*, Eurasian red squirrel, Hokkaido, *Sciurus vulgaris*, Zoonosis.

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Since the recent discovery of the first human babesiosis case in Japan [3, 4], we have been conducting epizootiologic surveys on small wild mammals in the country that may serve as reservoirs for the human babesiosis agent. So far, three types of *Babesia microti*-like parasites, referred to as Kobe, Hobetsu, and U.S. types, have been found in Japan from various small wild rodent species, as well as from shrews [9, 10, 13]. The Kobe type has been isolated from Japanese large field mice (*Apodemus speciosus*) captured only in Awaji Island; this type of parasite was proven to be the causative agent in the Japanese index case patient of human babesiosis [4, 10]. The Hobetsu type is the most predominant type and is distributed throughout the major Japanese islands; this type is also mainly isolated from *A. speciosus* [9, 13] and is suggested to be infectious to human [1]. The U.S. type has very recently been found in Japan [13], and its distribution appears to be confined within a narrow area in the eastern part of Hokkaido. While both the Kobe and Hobetsu types have so far been reported only in Japan, the U.S. type has been found ubiquitously distributed in the temperate zones not only of North American but also of Eurasian Continents [11].

In 1973, Takahashi and Yamashita [6] reported on *Babesia* sp. in Eurasian red squirrels (*Sciurus vulgaris orientis*) that were captured or found dead near the Nopporo Forest Park, which is located adjacent to Rakuno Gakuen University in Ebetsu, Hokkaido, Japan. Although they described the morphology of the intraerythrocytic parasites in detail, precise species identification could not be made because the parasite did not morphologically match any of the other *Babesia* spp. previously described in squirrels [6, 7].

In the present study, we were given the opportunity to investigate Eurasian red squirrels found dead due to traffic accidents near the Nopporo Forest Park. As some of the squirrels had *Babesia* parasites very similar to those described by Takahashi and Yamashita [6], attempts were made to isolate the parasite and to obtain molecular evidence for species identification.

MATERIALS AND METHODS

Field collections: In the years from 2002 to 2004, six Eurasian red squirrels that died from road traffic were found near the Nopporo Forest Park and brought into Rakuno Gakuen University. Blood specimens were collected from the ventricles of the hearts of two subjects (nos. 3222 and 3360) which were relatively in a fresh state (found in winter, probably within 24 or 48 hr of death); while the other four (nos. 0203, 0204, 3738, and 3739) were somewhat decomposed.

DNA analyses: DNA extraction and nested PCR were carried out according to the method described previously [11, 13]. For detection of *Babesia* parasites in the blood samples, nested PCR was carried out targeting both the 18S rRNA and β-tubulin genes. The PCR primers for the 18S rRNA gene, which are broadly specific for most of the hemoprotozoa in Piroprasmida, consisted of Piro0F (5 -GCC AGT AGT CAT ATG CTT GTG TTA-3) and Piro6R (5 -CTC CTT CCT YTA AGT GAT AAG GTT CAC-3) for the first round; and Piro1F (5 -CCA TGC ATG TCT WAG TAY AAR CTT TTA-3) and Piro5.5R (5 -CCT YTA AGT GAT AAG GTT CAC AAA ACT T-3) for the second round. The primers for the β-tubulin gene, which are highly specific for *B. microti*-like parasites, consisted of BmTubu93F (5 -GAY AGY CCC TTR CAA CTA GAA AGA GC-3) and BmTubu897R (5 -CGR TCG AAC ATT TGT TGH GTC ART TC-3) for the first round; and

BmTubu192F (5 -ACH ATG GAT TCT GTT AGA TCY GGC-3) and BmTubu782R (5 -GGG AAD GGD ATR AGA TTC ACA GC-3) for the second round. Amplified DNAs were sequenced according to the method described elsewhere [4, 11].

Phylogenetic analysis: Phylogenetic relationships were analyzed with the sequences of the 18S rRNA and β-tubulin genes using MacVector software version 8.0 (Accelrys Inc., San Diego, CA, U.S.A.). The parasites included for the analyses of the 18S rRNA and β-tubulin genes, and their sequence accession numbers in GenBank were as follows: Gray strain of U.S.-type *B. microti*, AY693840 and AB083377; Ko524 strain of Kobe-type *B. microti*-like parasite, AB032434 and AB083440; Ho234 strain of Hobetsu-type *B. microti*-like parasite, AB050732 and AB083441; Munich strains of *B. microti*, AB071177 and AB124587; *Babesia* sp. from Alaskan voles, AY144687 and AY144710; *Babesia* sp. from a skunk in the United States, AY144698 and AF546902; *Babesia* sp. from a raccoon in the United States, AY144701 and AY144708; *Babesia* sp. from Spanish dogs (=*Theileria annae* [12]), AY144700 and AY144709; and *B. rodhaini*, AB049999 and AB083442. Sequences corresponding to regions 464 to 1718 of AY693840 and 304 to 1205 of AB083377 were used for the analyses of the 18S rRNA and β-tubulin genes, respectively. They were aligned with the program CLUSTAL W Alignment [8], and a phylogenetic tree was constructed by the neighbor-joining method [5] from the aligned sequences using the program Phylogenetic Analysis in the MacVector software. Support for tree nodes was calculated with 1,000 bootstrap replicates using the bootstrap tree algorithm.

Laboratory animals: Isolation of *Babesia* parasites was attempted using three laboratory animals that had been splenectomized prior to use: a golden Syrian hamster (8-week-old male Std:Syrian, Japan SLC Inc., Hamamatsu, Japan), a Mongolian gerbil (10-week-old female MON/Jms/Gbs, Japan SLC Inc.), and a SCID mouse (9-week-old female NOD/shi-*scid*, maintained in the Laboratory Animal Facility in Rakuno Gakuen University [4, 10]). Blood specimen from squirrel no. 3360 was intraperitoneally inoculated into these animals (approximately 200 μl for each animal). Blood samples of the animals were examined once a week for 2 months to determine the presence or absence of *Babesia* parasites by both microscopy and nested PCR.

Nucleotide sequence accession numbers: The nucleotide sequences determined in this paper have been deposited in DDBJ under accession numbers AB219802 and AB219803.

RESULTS

Nested PCR targeting the 18S rRNA gene gave rise to positive signals in three (nos. 0203, 3222 and 3360) of the six squirrels. The three amplified DNAs had identical sequences showing a high degree of similarity to those of *B. microti*-like parasites (Fig. 1A). The three squirrels also exhibited positive signals by nested PCR targeting the β-

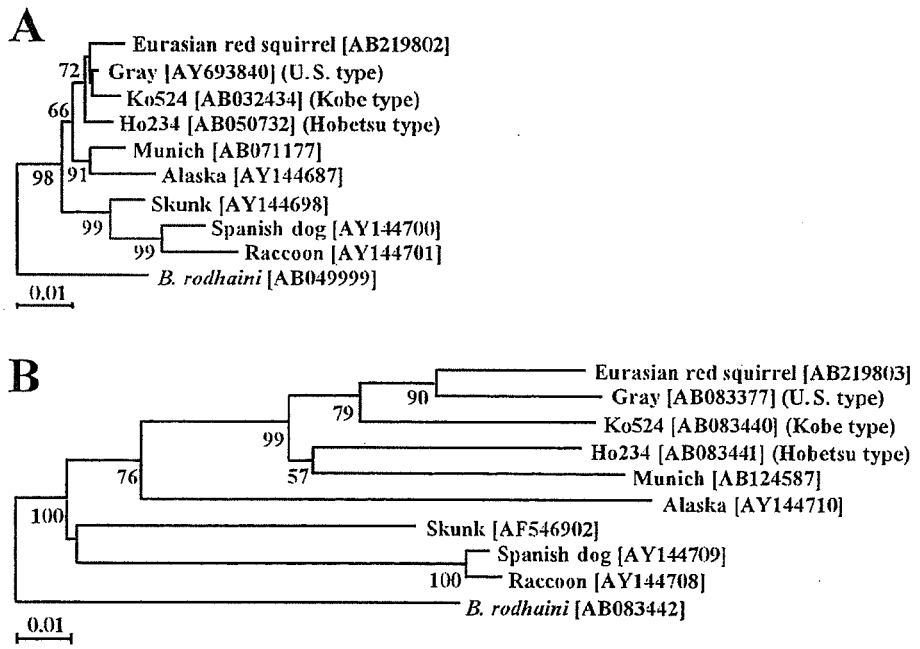

Fig. 1. Phylogenetic trees inferred from the sequences of 18S rRNA (A) and β-tubulin (B) genes. *B. microti*-like parasite in the Eurasian red squirrels and its close relatives were included in the analyses. The GenBank accession number for each DNA sequence is given in parenthesis. The number on each branch indicates the percent occurrence in 1,000 bootstrap replicates (numbers less than 50 are not shown). The scale bars in both A and B represent 0.01 substitutions per site.

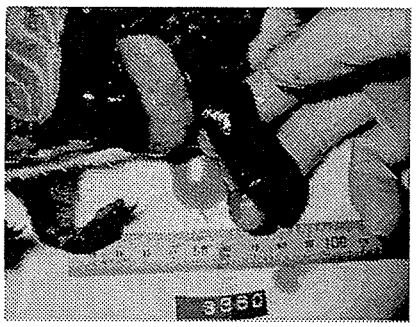

Fig. 2. A splenomegaly observed in the Eurasian red squirrel no. 3360.

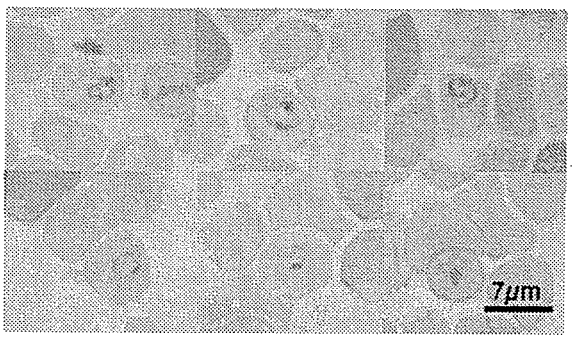

Fig. 3. Morphology of the *B. microti*-like parasites in blood smears from squirrels nos. 3222 (upper panels) and 3360 (lower panels).

tubulin gene; their amplified DNA sequences were identical, indicating that all three were infected by the same *B. microti*-like parasite. The sequences of the 18S rRNA and β-tubulin genes, together with those from closely related *B. microti*-group parasites, were used to construct phylogenetic trees, respectively (Fig. 1A and B). Although branching patterns in the two trees were similar, the interrelationships among the parasites inferred from the 18S rRNA tree were unclear. In contrast, the tree based on the β-tubulin gene sequences gave rise to better resolution, because sequence diversity in the β-tubulin gene is much greater than that in the 18S rRNA gene.

The two squirrels (nos. 3222 and 3360) whose bodies were in a relatively fresh condition enabled us to conduct some biological examinations. Squirrel no. 3360 had obvious splenomegaly (Fig. 2), a major clinical manifestation of babesiosis. Unclotted blood remaining in the hearts of both allowed us to prepare Giemsa-stainied thin-smear blood films, that clearly showed the presence of intraerythrocytic parasites (Fig. 3) morphologically very similar to the *Babesia* sp. described by Takahashi and Yamashita [6]. Since a relatively large amount of unclotted blood could be obtained from squirrel no. 3360, attempts were made for parasite isolation by intraperitoneal inoculations of the blood into three species of splenectomized laboratory animals. Development of parasitemia, however, was not detected in any of them by either microscopy or PCR.

DISCUSSION

In this study, we presented the first molecular evidence for the presence of a *B. microti*-like parasite in Sciuridae. Phylogenetically, the parasite in the Eurasian red squirrels (*Sciurus vulgaris orientis*) is most closely related to the U.S.-type *B. microti* (=*Babesia microti sensu stricto*, which is regarded as the major causative agent of human babesiosis [2, 7]). Our finding, therefore, implies that the squirrels may serve as an additional reservoir for the human babesiosis agent, although the zoonotic potential of this newly identified parasite has yet to be proven.

In our earlier studies [9 11, 13], we conducted field surveys at various places in Japan, and also in some regions in the northeastern Eurasian Continent. Investigations of a large number of small wild mammals revealed that various species of the family Muridae (*Apodemus agrarius, A. peninsulae, A. speciosus, Clethrionomys rufocanus, C. rutilus, Eothenomys smithii, Lagurus luteus*, and *Microtus montebelli*) and some shrews (*Sorex unguiculatus* and *S. caecutiens*) carried *B. microti*-like parasites. Based on their 18S rRNA sequences, they were classified into three genotypes, designated as the Kobe, Hobetsu, and U.S. types [9, 11, 13]. Furthermore, regardless of the host species and place of collection, there were virtually no intragenotypic sequence variations. The *Babesia* sp. from squirrels differed from Kobe-, Hobetsu-, and U.S-type parasites in their 18S rRNA sequences, 1.47%, 1.52%, and 1.24%, respectively. The distinctive sequence detected in the present study from squirrels seems to, therefore, indicate a new variant different from either one of the three genotypes reported earlier [9, 11, 13].

Three decades ago, Takahashi and Yamashita [6] had reported infection of *S. vulgaris orientis* with a *Babesia* parasite. It is not sure whether the *Babesia* sp. described by them is identical to that shown in this study. However, we believe that this is probably the case, based on the parasite's morphology and place of sample collection, which is the Noppro Forest Park comprising 2,051 hectares, and surrounded by the urban areas of Sapporo, Ebetsu, and Kitahiroshima. The natural environment within the park has been relatively well preserved with most of the animal populations having only slight chances for interaction with populations in other areas. Thus, it is highly likely that the *Babesia* sp. infection in squirrels in the park has also been maintained for many years. *Sciurus vulgaris orientis* in Hokkaido Island is regarded as a subspecies of *S. vulgaris*, which is widely distributed in the northeastern Eurasian Continent. Although the prevalence of *Babesia* infection in squirrels was consistently high in both this and previous studies [6], whether or not this is also the case in other regions in Hokkaido Island and in Eurasian Continent need to be investigated.

Unfortunately, our attempt to isolate the *Babesia* parasite

from a dead squirrel using three species of splenectomized laboratory animals that were presumed to be susceptible to *B. microti* was unsuccessful. A low level of parasitemia (approximately 0.02%) may have accounted for this. However, recording of negative results may be important inasmuch as encountering such an opportunity is extremely rare. Our earlier request for capturing live squirrels in the park to obtain a sufficient amount of blood for parasite isolation was disapproved owing to a possible high risk of fatal injury to the animals, whose number in the park was estimated to be decreasing (probably less than 50; T. Kataoka *et al.*, unpublished data). Nevertheless, further efforts should be made towards achieving parasite isolation and determination of tick vector(s).

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REFERENCES

1. Arai, S., Tsuji, M., Kaiho, I., Murayama, H., Zamoto, A., Wei, Q., Okabe, N., Kamiyama, T. and Ishihara, C. 2003. Retrospective seroepidemiological survey for human babesiosis in an area in Japan where a tick-borne disease is endemic. *J. Vet. Med. Sci.* **65**: 335 340.
2. Goethert, H. K. and Telford, S. R., III. 2003. What is *Babesia microti*? *Parasitology* **127**: 301 309.
3. Matsui, T., Inoue, R., Kajimoto, K., Tamekane, A., Okamura, A., Katayama, Y., Shimoyama, M., Chihara, K., Saito-Ito, A. and Tsuji, M. 2000. First documentation of transfusion-associated babesiosis in Japan. *Rinsyo Ketsueki* **41**: 628 634 (in Japanese with English summary).
4. Saito-Ito, A., Tsuji, M., Wei, Q., He, S., Matsui, T., Kohsaki, M., Arai, S., Kamiyama, T., Hioki, K. and Ishihara, C. 2000. Transfusion-acquired, autochthonous human babesiosis in Japan: isolation of *Babesia microti*-like parasites with hu-RBC-SCID mice. *J. Clin. Microbiol.* **38**: 4511 4516.
5. Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4**: 406 425.
6. Takahashi, K. and Yamashita, S. 1973. *Babesia* sp. from *Sciurus vulgaris orientis* Thomas in Hokkaido. *J. Coll. Dairying.* **5**: 1 6 (in Japanese with English summary).
7. Telford, S. R., III, Gorenflot, A., Brasseur, P. and Spielman, A. 1993. Babesial infections in humans and wildlife. pp. 1 47. *In*: Parasitic Protozoa, 2nd ed., vol. 5 (Kreier, J. P. ed.), Academic Press. Inc., New York.
8. Thompson, J. D., Higgins, D. G. and Gison, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**: 4673 4680.
9. Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H. and Ishihara, C. 2001. Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasites. *J. Clin. Microbiol.* **39**: 4316 4322.
10. Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Teleford, S. R. III and Ishihara, C. 2001. Human babesiosis in Japan: isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. *J. Clin. Microbiol.* **39**: 2178 2183.
11. Zamoto, A., Tsuji, M., Wei, Q., Cho, S.-H., Shin, E.-H., Kim, T.-S., Leonova, G. N., Hagiwara, K., Asakawa, M., Kariwa, H., Takashima, I. and Ishihara, C. 2003. Epizootiologic survey for *Babesia microti* among small wild mammals in Eastern Eurasia with finding of divergence of β-tubulin genes. *J. Vet. Med. Sci.* **66**: 785 792.
12. Zahler, M., Rinder, H., Schein, E. and Gothe, R. 2000. Detection of a new *Babesia microti*-like species in dogs. *Vet. Parasitol.* **89**: 241 248.
13. Zamoto, A., Tsuji, M., Kawabuchi, T., Wei, Q., Asakawa, M. and Ishihara, C. 2004. U.S.-type *Babesia microti* isolated from small wild rodents in eastern Hokkaido, Japan. *J. Vet. Med. Sci.* **66**: 919 926.