developed for *Yersinia pestis* (11), was also applied with success to strains of other human pathogens, including *Rickettsia conorii* (15), *Rickettsia prowazekii* (35) and *Coxiella burnetii* (16). MST was developed with the assumption that intergenic spacers are more variable than genes for genotyping bacteria at the strain level. In this study, to estimate the usefulness of MST for studying the population genetics of *B. henselae*, we applied it to a large collection of cat isolates.

MATERIALS AND METHODS

Study design. One hundred twenty-six *B. henselae* cat isolates of various geographic origins were incorporated in this study (Table 1). All 38 European isolates were grown in our laboratory. For the other 88 isolates, from the United States and Asia, we studied DNA extracted by two of the authors (B.B.C. and L.G.) from their isolates.

Bartonella henselae **culture and DNA extraction.** *B. henselae* isolates were cultivated on Columbia agar with 5% sheep blood (BioMerieux, Marcy l'Etoile, France) at 37°C in 5% CO_2 (Genbag CO_2 system; BioMerieux). Genomic DNA of *B. henselae* strains was extracted by using the Chelex procedure as previously described (9) or the QIAmp Tissue kit following the manufacturer's recommendations (QIAGEN, Hilden, Germany).

Selection of target sequences. We aligned the genomic sequences of *B. henselae* (GenBank accession number BX897699) and *B. quintana* (BX897700) by using the BLASTn (1) and GenomeComp (33) software programs to identify conserved pairs of consecutive genes. Then, intergenic sequences were aligned using the CLUSTAL W program (31). We classified intergenic spacers conserved by both genomes, with sizes ranging from 150 to 600 bp, by degree of similarity and then selected the 20 most variable spacers (detailed in Table 2).

PCR amplification and sequencing. Primers were designed to amplify the 20 most variable spacers fulfilling the above criteria using the Primer 3.0 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Primers for amplifying the 20 most variable spacers were selected within genes flanking the selected spacers and are listed in Table 2. All primers were obtained from Eurogentec (Seraing, Belgium). Their specificity was predicted by comparison with GenBank using the BLASTn software (1). PCRs were carried out in a PTC-200 automated thermal cycler (MJ Research, Waltham, Mass.). One nanomolar concentration of each DNA preparation was amplified in a 25-μl reaction mixture containing 50 pM of each primer; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Invitrogen, Gaithersburg, Md.); 1 U of eLONGase polymerase (Invitrogen); 1 μl of eLONGase buffer A; and 4 μl of eLONGase buffer B. The following conditions were used for amplification: an initial 3 min of denaturation at 94°C was followed by 40 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 52°C, and extension for 1 min at 68°C. Amplification was completed by holding the reaction mixture for 10 min at 68°C to allow complete extension of the PCR products. PCR products were purified by using the MultiScreen PCR filter plate (Millipore, Saint-Quentin en Yvelines, France) as recommended by the manufacturer. PCR products were sequenced in both directions by using the d-Rhodamine Terminator Cycle Sequencing Ready Reaction kit with Amplitaq polymerase FS (Perkin-Elmer, Coignieres, France) as described by the manufacturer. Sequencing products were resolved using an ABI 3100 automated sequencer (Perkin-Elmer). Sterile water was used as a negative control in each PCR assay. Sequences from each genotype were checked twice in both directions to ensure the reliability of the MST method.

Sequence analysis and phylogenetic analysis. Nucleotide sequences were edited using the Autoassembler package (Perkin-Elmer). For each intergenic spacer, a genotype was defined as a sequence exhibiting unique mutations. MST genotypes were defined as unique combinations of spacer genotypes. Multiple alignment of sequences was carried out using the CLUSTAL W software (31). Phylogenetic analysis of the studied isolates was obtained using the neighbor-joining and maximum parsimony methods within the MEGA 3 software (25). For this purpose, sequences of the selected spacers were concatenated. To facilitate sequence comparison with our MST sequences, we developed an online site named MST-Rick. This site contains a local BLAST to help scientists compare their sequences to our database.

Statistical tests. The genotypic variability of *B. henselae* isolates according to their geographic origin was estimated using Fisher's exact test. A difference was considered significant when P was <0.05.

Nucleotide sequence accession numbers. The different genotypes for the discriminatory spacers have been deposited in the GenBank database under accession numbers DQ383226 to DQ383270.

RESULTS

MST genotyping. One thousand four hundred thirteen intergenic spacers were found conserved by *B. henselae* and *B. quintana* genomes. Among them, 293 had a size ranging from 150 to 600 bp. We tested the 20 most variable (S1 to S20) of these 293 spacers among the 126 *B. henselae* cat isolates available. Nine of the spacers (S1 to S9) were found highly variable among these isolates (Table 2). The tRNA-Ala/GCA-tRNA-Ile/AUC spacer (S1), flanked by two tRNA genes, was found to be the most variable spacer among the nine tested, with five variable nucleotide positions and a 15-bp sequence fragment presenting either as a single copy or repeated up to five times, depending on the isolate (Fig. 1; Table 3). Sequences from the S1 spacer classified the 126 isolates into nine genotypes. The BH2865724-*dut* spacer (S2), with 14 variable nucleotide positions, was the second most variable spacer and allowed the 126 tested isolates to be classified into seven genotypes (Table 3). The *dnaJ*-related protein–*cobS* spacer (S3) held eight variable nucleotide positions and classified the 126 isolates into six genotypes (Table 3). The *pssA*-oxidoreductase (S4) and *carB*-cold shock protein (S5) spacers had nine and five variable nucleotide positions, respectively, and classified the 126 isolates into five genotypes each (Table 3). The *alr-gcvP* (S6) and *ftsK*-oxidoreductase spacers (S7) contained eight variable nucleotide positions each and classified the 126 isolates into four genotypes each (Table 3). The BH2864883-BH2864884 (S8) and *acpP2*-malate oxidoreductase (S9) spacers harbored eight and four variable nucleotide positions, respectively, and classified the 126 isolates into three genotypes each (Table 3). In total, 69 variable nucleotide positions were found within the nine intergenic spacers (Table 3). Each variable nucleotide was checked three times to ensure the reliability of MST. Only two alleles at each variable position were found, with the exception of position 256 within the *alr-gcvP* spacer. At this position, 117 isolates had a thymine (types 2 and 3), compared to a cytosine in five European isolates (type 1) and a guanine in four American isolates (type 4) (Table 3). By combining the genotypes obtained from each variable spacer, the 126 tested isolates could be classified into 39 MST genotypes (Table 1). Each of the 39 genotypes was identified based on sequence specificities from either a single spacer or a combination of a maximum of seven spacers (Fig. 2). Sequences from each genotype from the nine spacers were added to the MST-Rick database (http://ifr48.timone.univ-mrs.fr/MST_BHenselae/mst).

Among the 39 MST types, 24 MST types (types 1, 3, 4, 6, 8 to 12, 14, 16, 19 to 21, 23, 26, 28 to 31, 34, 36, 37, and 39) contained only one isolate each and five MST genotypes (types 17, 24, 27, 32, and 33) contained only two isolates each (Table 1). The 19 Asian isolates were distributed into 12 MST genotypes, compared to 6 ($P < 0.01$) and 24 ($P = 0.03$) MST types for the 38 European and 69 American isolates, respectively (Fig. 3). Among the 39 MST genotypes, 10 (types 6, 10, 20, 21, 23, 32, and 36 to 39), 21 (types 1, 3, 4, 7 to 9, 11, 14 to 19, 24 to 30, and 34), and 5 (types 4, 9, 12, 22, and 31) genotypes were specific to Asian, American, and European isolates, respectively.

A significant difference in genotypic diversity was found between *B. quintana* (4 MST types out of 71 isolates) (13), and

TABLE 1. List of *B. henselae* isolates incorporated in this study and corresponding genotypes

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
Amber	USA[b]	5	1	1	1	2	2	2	1	1	5
Aron	USA	7	2	5	4	1	2	1	1	3	7
BisQuick	USA	5	2	6	5	2	2	2	1	1	35
Budda	USA	5	1	1	1	2	2	2	1	1	5
Buster Brown	USA	5	1	1	2	2	2	2	1	1	25
Cleo	USA	5	1	1	1	2	2	2	1	1	5
Kody	USA	4	5	5	5	1	2	1	1	3	27
Earl Grey	USA	5	1	1	2	2	2	2	1	1	25
Erick	USA	5	1	1	1	2	2	2	1	1	5
Faleen	USA	5	1	1	1	2	2	2	1	1	5
Gigi	USA	4	5	5	4	1	2	1	1	3	15
Jackie	USA	5	2	1	1	2	2	2	1	1	16
Junior	USA	5	1	1	1	2	2	2	1	1	5
Kelly	USA	8	2	5	4	1	2	1	1	3	8
Kodie	USA	5	1	1	1	2	2	2	1	1	5
Lathious	USA	5	2	6	5	2	2	2	1	1	35
Levi	USA	5	1	1	1	2	2	2	1	1	5
Mew Mew	USA	7	2	5	4	1	2	1	1	3	7
Mitzi	USA	5	1	1	1	2	2	2	1	1	5
Mokka	USA	5	1	1	1	2	2	2	1	1	5
Molly	USA	5	1	1	2	2	2	2	1	1	25
Norman	USA	5	1	1	1	2	2	2	1	1	5
Patches	USA	7	2	5	4	1	2	1	1	3	7
Pyewacket	USA	5	1	1	1	2	2	2	1	1	5
Rafiki	USA	5	1	1	1	2	2	2	1	1	5
Rocket	USA	7	2	5	4	1	2	1	1	3	7
Rum Tum	USA	5	1	1	1	2	2	2	1	1	5
Sabrina	USA	5	2	6	5	2	2	2	1	1	35
Sadie	USA	4	5	5	4	1	2	1	1	3	15
Saki	USA	5	1	1	1	2	2	2	1	1	5
Sam	USA	9	2	5	4	1	2	1	1	3	9
Samantha	USA	5	1	1	1	2	2	2	1	1	5
Sassy	USA	1	1	1	1	2	2	2	1	1	1
Shannon	USA	5	1	1	5	2	2	2	1	1	26
Simba	USA	3	1	1	1	2	2	2	1	1	3
Sinbad	USA	5	1	1	1	4	2	2	1	1	29
Spaz	USA	5	1	1	1	2	2	2	1	1	5
Sunday	USA	5	2	1	5	2	2	2	1	1	28
Sweetie	USA	5	1	1	2	2	2	2	1	1	25
Tabatha	USA	5	1	1	1	2	2	2	1	1	5
Tasha	USA	3	1	6	3	5	4	4	3	2	18
Timothy	USA	5	1	1	1	2	2	2	1	1	5
Toby	USA	5	1	1	1	2	2	2	1	1	5
Tori	USA	4	5	5	5	1	2	1	1	3	27
Zipper	USA	5	2	6	5	2	2	2	1	1	35
Zoe	USA	5	1	1	1	2	3	2	1	1	30
Newmans	USA	4	2	5	4	1	2	2	1	3	33
White	USA	5	2	6	5	2	2	2	1	1	35
Lavery	USA	5	7	6	1	2	2	1	2	1	17
Rae	USA	5	7	6	1	2	2	2	2	1	34
Fairminer	USA	5	2	6	5	2	2	2	1	1	35
Shaw-Lamon	USA	5	2	6	5	2	2	1	1	1	24
Moyle	USA	5	7	6	1	2	2	1	2	1	17
Linnan	USA	5	2	6	5	2	2	1	1	1	24
Silcock	USA	5	2	6	5	2	2	2	1	1	35
Hunt	USA	5	2	6	5	2	2	2	1	1	35
Eichtais	USA	5	2	6	5	2	2	2	1	1	35
Taylor	USA	5	2	6	5	2	2	2	1	1	35
Ramm	USA	5	2	6	5	2	2	2	1	1	35
USA1	USA	5	1	1	1	2	2	2	1	1	5
USA4	USA	4	1	1	1	2	2	2	1	1	4
USA6	USA	4	5	5	4	1	2	1	1	3	15
USA7	USA	3	1	6	3	5	4	4	3	2	18
USA8	USA	5	1	1	1	2	2	2	1	1	5
USA11	USA	4	1	5	4	1	2	1	1	3	19

Continued on following page

TABLE 1—*Continued*

Isolate	Origin	No. of genotypes[a]									
		S1	S2	S3	S4	S5	S6	S7	S8	S9	MST
USA12	USA	3	3	6	3	5	4	4	1	2	11
USA15	USA	4	2	5	4	1	2	1	1	3	14
USA16	USA	3	1	6	3	5	4	4	3	2	18
USA17	USA	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.32	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.33	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.34-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.35	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.50-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.52-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.54-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.55-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 56-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.57-M	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC.58-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 59-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC.67	France	5	1	1	1	2	2	4	2	1	31
UR.BH.M.NHC.72-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 77-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.78-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.79-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 80-H	France	3	5	4	5	2	2	2	1	3	22
UR.BH.M.NHC 82-M	France	5	1	1	1	2	2	2	1	1	5
UR.BH.M.NHC.84-M	France	2	4	1	1	3	1	3	3	2	2
UR.BHM.M.NHC.128	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.129	France	3	6	2	3	5	4	4	3	2	13
UR.BHM.M.NHC.130	France	3	6	2	3	5	4	4	3	2	13
UR.BH.M.NHC 154	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 155	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 156	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 159	France	2	4	1	1	3	1	3	3	2	2
UR.BH.M.NHC 161	France	2	4	1	1	3	1	3	3	2	2
FR96/BK7	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK26II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK36	Germany	3	5	4	5	2	2	2	1	3	22
FR96/BK36II	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK75	Germany	5	1	1	1	2	2	2	1	1	5
FR96/BK77	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK78	Germany	2	4	1	1	3	1	3	3	2	2
FR96/BK79	Germany	2	4	1	1	3	1	3	3	2	2
ZF-1	France	3	5	3	5	2	2	2	1	3	12
FIZZ	Switzerland	5	1	1	1	2	2	2	1	1	5
J1	Japan	5	2	6	5	2	2	2	1	1	35
J4	Japan	5	2	5	4	1	2	1	1	3	23
J5	Japan	8	2	5	4	1	2	2	1	3	32
J6	Japan	5	2	6	5	2	2	2	1	1	35
J7	Japan	5	2	6	5	2	2	2	1	1	35
J8	Japan	5	2	6	5	2	2	2	1	1	35
P1	Philippines	5	2	6	5	2	2	2	2	1	38
P2	Philippines	5	2	6	5	2	2	2	2	1	38
P4	Philippines	5	2	6	5	2	2	2	2	1	38
P5	Philippines	5	2	6	5	2	2	1	2	1	37
P6	Philippines	4	2	5	4	1	2	1	2	3	36
P7	Philippines	3	2	5	5	2	2	2	1	1	10
P8	Philippines	3	2	6	5	2	2	2	1	1	21
T1	Thailand	5	2	6	5	2	2	2	1	3	39
T3	Thailand	8	2	5	4	1	2	2	1	3	32
T5	Thailand	6	2	5	4	1	2	2	1	3	6
T6	Thailand	4	2	5	4	1	2	2	1	3	33
T7	Thailand	5	2	6	5	2	2	2	2	1	38
T8	Thailand	5	1	6	5	2	2	2	2	1	20

[a] The description of intergenic spacers S1 to S9 and the primers used for their amplification and sequencing are given in Table 2.
[b] USA, United States.

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
ftsK-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-

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1    CAATCTTTTTAGAAG------------------------------------------------------------    (106)
2    CAATCTTTTTAGAAGCAATCTTTTTAGAAG---------------------------------------------    (5)
3    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG------------------------------    (11)
4    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG---------------    (3)
5    CAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAGCAATCTTTTTAGAAG    (1)
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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,[c] 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,[e] 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

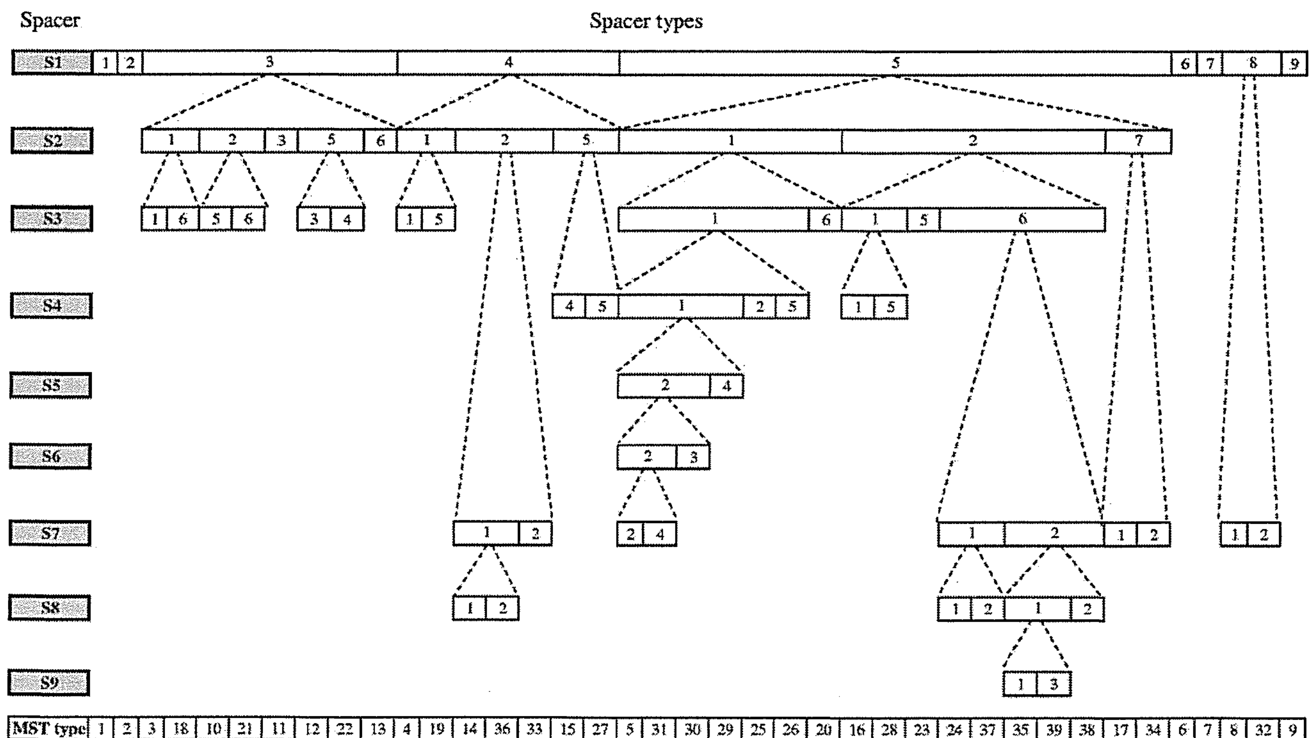
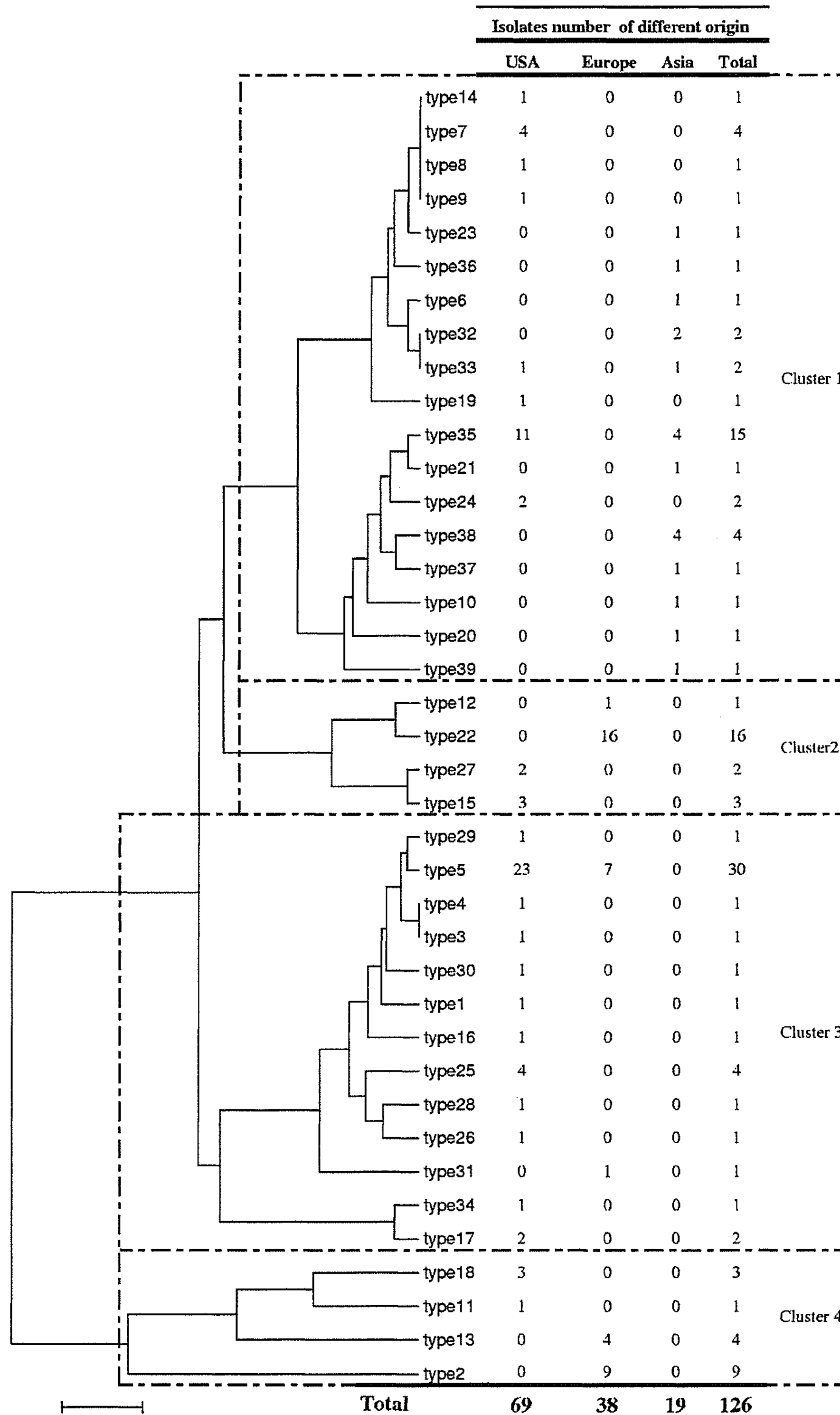


FIG. 2. Guidelines for selection of spacers for MST genotyping of *B. henselae* isolates.



	Isolates number of different origin				
	USA	Europe	Asia	Total	
type14	1	0	0	1	Cluster 1
type7	4	0	0	4	
type8	1	0	0	1	
type9	1	0	0	1	
type23	0	0	1	1	
type36	0	0	1	1	
type6	0	0	1	1	
type32	0	0	2	2	
type33	1	0	1	2	
type19	1	0	0	1	
type35	11	0	4	15	
type21	0	0	1	1	
type24	2	0	0	2	
type38	0	0	4	4	
type37	0	0	1	1	
type10	0	0	1	1	
type20	0	0	1	1	
type39	0	0	1	1	
type12	0	1	0	1	Cluster2
type22	0	16	0	16	
type27	2	0	0	2	
type15	3	0	0	3	
type29	1	0	0	1	Cluster 3
type5	23	7	0	30	
type4	1	0	0	1	
type3	1	0	0	1	
type30	1	0	0	1	
type1	1	0	0	1	
type16	1	0	0	1	
type25	4	0	0	4	
type28	1	0	0	1	
type26	1	0	0	1	
type31	0	1	0	1	
type34	1	0	0	1	
type17	2	0	0	2	
type18	3	0	0	3	Cluster 4
type11	1	0	0	1	
type13	0	4	0	4	
type2	0	9	0	9	
Total	**69**	**38**	**19**	**126**	

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.

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Short communication

Characterization of Th1 activation by *Bartonella henselae* stimulation in BALB/c mice: Inhibitory activities of interleukin-10 for the production of interferon-γ in spleen cells

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Abstract

This study was conducted to analyze cytokine production mechanisms in mice after *Bartonella henselae* stimulation. BALB/c mice were inoculated intraperitoneally with 3×10^6 colony forming units of *B. henselae* (Houston-1 strain) twice at 10-day interval. Spleen cells were harvested from the mice and stimulated with the organisms. Following the stimulation, interferon-gamma (IFN-γ) and interleukin-4 (IL-4), IL-10, IL-12 and tumor necrosis factor-alpha (TNF-α) were measured in the culture supernatants of the spleen cells by ELISA. The spleen cells specifically secreted IFN-γ, but not IL-4, indicating that T helper 1 (Th1) cells were activated following *B. henselae* stimulation. In addition, IL-10 and TNF-α productions were also detected in the culture supernatants of spleen cells. Neutralization of IL-10 in the culture supernatants significantly enhanced the production of IFN-γ from the spleen cells stimulated with *B. henselae*. These results indicate that *B. henselae* predominantly stimulated Th1 cells and resulted in secreting IFN-γ, however the production was partially inhibited by IL-10, which was produced simultaneously.
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Keywords: *Bartonella henselae*; IL-10; IFN-γ; Mice; TNF-α

1. Introduction

Although high levels of bacteremia with *Bartonella henselae* were found in cats for several months to a few years, most infected cats remain asymptomatic (Kordick et al., 1999). Immune responses against *B. henselae* infection in cats have mainly been studied from the standpoints of humoral immunity (Freeland et al., 1999). Analysis of the immune responses, especially cell-mediated immunity (CMI) is indispensable to understand the mechanisms by which the organisms are eliminated from infected animals, since

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B. henselae is an intracellular pathogen (Kordick and Breitschwerdt, 1995). However, little is known about cytokine responses against *B. henselae* in cats as well as in humans (Kabeya et al., 2006; Papadopoulos et al., 2001). The cat naturally *B. henselae*-infected were shown to promote IL4, but not IFN-γ mRNA expression when the emergence of relapsing bacteremia, suggesting that the selective induction of Th2 immune responses may contribute to establishing the persistent infection of *B. henselae* in naturally infected cats (Kabeya et al., 2006).

It has been reported that despite the fact that mice are not the natural reservoir of *B. henselae*, we used a mouse model to analyze the cellular immune response against *B. henselae*, because it has been well known the immune system of such laboratory animals (Arvand et al., 2001; Kabeya et al., 2003; Karem et al., 1999). Previous investigations have been performed to examine the immune responses against *B. henselae* stimulation in BALB/c or C57BL/6 mice (Arvand et al., 2001; Karem et al., 1999). *B. henselae* elicited cell-mediated immune responses mainly $CD4^+$ helper T cells 1 (Th1) in immunocompetent mice. Karem et al. (1999) also reported that *B. henselae*-primed BALB/c mice induced delayed-type hypersensitivity and the secretion of interferon-gamma (IFN-γ) mediated by $CD4^+$ Th1. However, the mechanism by which $CD4^+$ Th1 response in *B. henselae*-primed mice is induced remains unclear. The aim of this study was to better understand how a Th1 response is specifically induced in experimentally *B. henselae*-inoculated mice through analysis of cytokine expression profiles.

2. Materials and methods

2.1. Bacterial strains

B. henselae strain Houston-1 (ATCC49882) was used in this study. The strain was cultured on heart infusion agar (HIA) plates (DIFCO, USA) containing 5% defibrinated rabbit blood at 35 °C in an atmosphere of 5% CO_2 for 1 week. The bacterial cells were re-suspended in PBS and the concentration was adjusted to OD of 1.0 at 600 nm. Ten-fold serial dilutions were made with PBS and 100 μl of each diluted suspension were plated on two HIA plates containing 5% defibrinated rabbit blood to determine the colony-forming unit (CFU) of the inoculums.

2.2. Experimental animals

Female BALB/c mice were purchased from Nihon CLEA Corp. and used for the experiment at the age of 5 weeks. Three animals were caged together and kept under specific-pathogen-free conditions throughout the study. Animal care was carried out in accordance with the guidelines for the care and use of laboratory animals by College of Bioresource Sciences, Nihon University.

2.3. Inoculation of mice with B. henselae

Three of BALB/c mice were used for each experimental group. The mice were inoculated intraperitoneally twice at 10-day interval with 1 ml of the bacterial suspension containing 3.0×10^6 CFU of *B. henselae* Houston-1 suspended in PBS.

2.4. Induction of cytokine production from mice spleen cells

Spleen was harvested from three mice per group and suspended in complete RPMI medium consist of RPMI1640 (Invitrogen, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum, 200 U/ml penicillin, and 200 μg/ml streptomycin at a concentration of 2×10^6 cells/well of 24-well microplate. The cells were stimulated with *B. henselae* Houston-1 (2×10^6 to 2×10^8 CFU/well) with or without anti-mouse interleukin-10 (IL-10) rat monoclonal antibody (ENDOGEN, MA, USA) or IgG_1 rat isotype control (R and D systems Inc., MN, USA) at a concentration of 40 μg/ml for 24–120 h at 37 °C. Following cultivation, the concentrations of secreted cytokines (IFN-γ, IL-4, IL-10, IL-12 and tumor necrosis factor α; TNF-α) in culture-supernatants were measured by using commercial ELISA kits (Quantikine, R and D systems Inc.).

2.5. Statistical analysis

Differences between the amounts of cytokine in culture conditions (stimulation with versus without stimulant, or stimulation with live versus inactivated organisms) were determined by Student's *t*-test. *P*-values of <0.05 were regarded as significant.

3. Results

3.1. Analysis of cytokine production from spleen cells of B. henselae primed mice following in vitro stimulation

Cytokine production in the culture-supernatant of spleen cells of *B. henselae* Houston-1 primed BALB/c mice was assessed following stimulations with or without antigens at a ratio of cells to bacteria was 1:10 for 24–120 h (Fig. 1). A marked production of IFN-γ (1002.2 pg/ml) was observed following the antigen stimulation for 48 h and reached a plateau over 3000 pg/ml 72 h after the stimulation, while the production was kept at low levels (9.6–10.0 pg/ml) without *B. henselae* stimulation. By contrast, IL-4 productions showed low levels from 12.3 to 31.8 pg/ml in the culture with stimulation and from 11.1 to 13.7 pg/ml in the culture without stimulation throughout the experimental period.

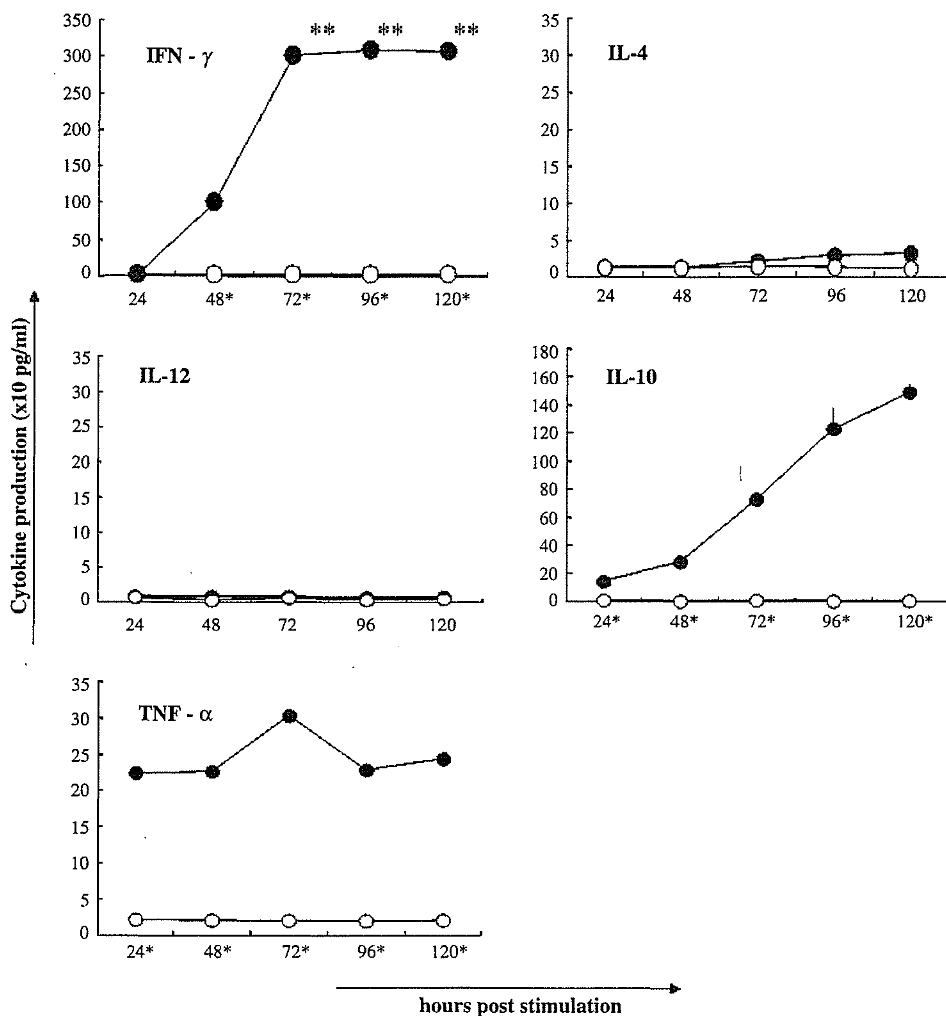

Fig. 1. Cytokine secretion in culture-supernatants of spleen cells from *B. henselae* primed mice. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with (closed circle) or without (open circle) the organism. Ratio of cells to bacteria was 1:10 from 24 to 120 h. Following cultivation, culture-supernatants were collected and the concentrations of cytokines, such as IFN-γ, IL-4, IL-12, IL-10 and TNF-α were quantitated, using ELISA. (*) $P < 0.01$; (**) production was over 3000 pg/ml.

In order to analyze the mechanisms of specific production of IFN-γ from the spleen cells of *B. henselae* primed mice, we assessed the productions of IL-12 and IL-10 which were representative cytokines of inducing and suppressing Th1 responses, respectively (Fig. 1). The spleen cells did not produce IL-12 both with and without *in vitro* antigen stimulations (5.5–8.3 pg/ml). However, production of IL-10 and TNF-α was specifically increased in the culture supernatants of antigen stimulated spleen cells. The amounts of TNF-α production were small (223.4–302.9 pg/ml), however, the cells secreted TNF-α as early as 24 h after stimulation. Production of IL-10 gradually increased from 141.6 to 1487.0 pg/ml following stimulation, although they remained at low levels from 2.7 to 7.3 pg/ml without stimulation.

3.2. Cytokine production from the primed mice spleen cells by stimulating with live or heat-inactivated B. henselae Houston-1

To examine factors involved in the induction of IFN-γ, IL-10 and TNF-α from *B. henselae* primed mice spleen cells, heat-inactivated organisms were used for *in vitro* stimulation and assessed the cytokine production (Fig. 2). The spleen cells were stimulated

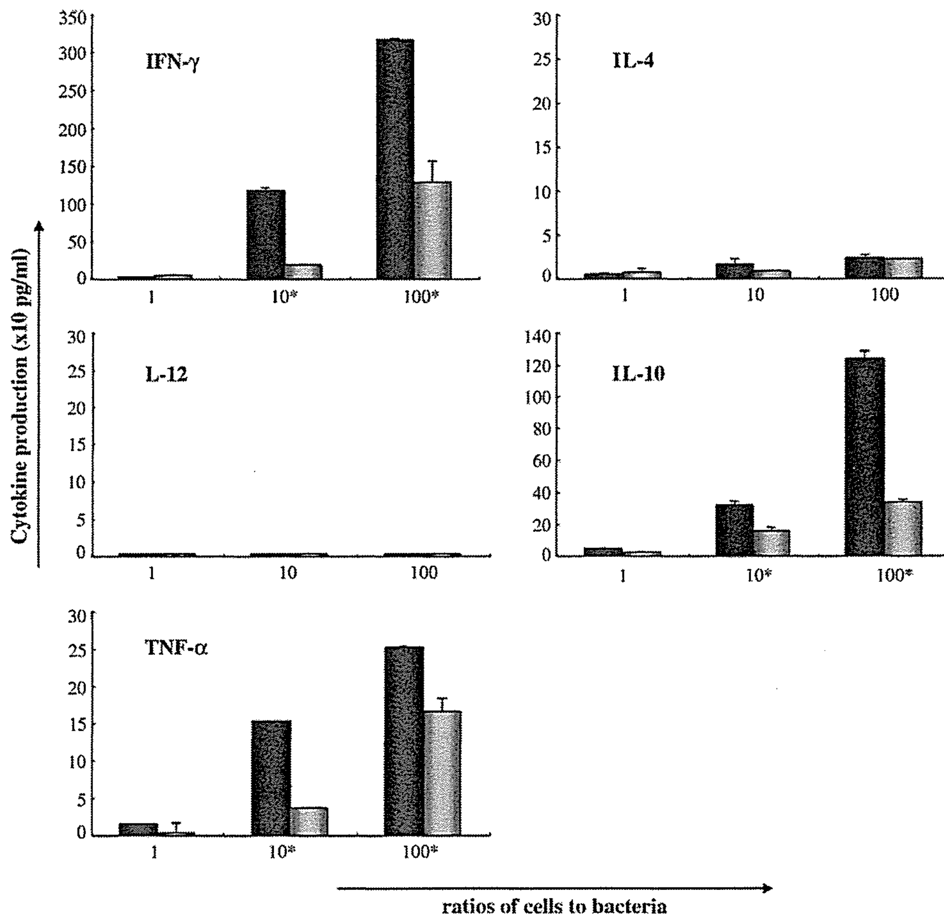

Fig. 2. Cytokine secretion by *in vitro* stimulation with live or heat-inactivated *B. henselae* in culture-supernatants of spleen cells from *B. henselae* primed mice. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with live (closed bar) or heat-inactivated (gray bar) *B. henselae* Houston-1. Ratios of cells to bacteria were from 1:1 to 1:100 for 120 h. Following cultivation, culture-supernatants were collected and cytokines, including IFN-γ, IL-4, IL-12, IL-10 and TNF-α were quantitated, using ELISA. (*) $P < 0.05$.

with live or heat-inactivated *B. henselae* Houston-1 at different concentrations (ratios of cells to bacteria from 1:1 to 1:100) for 120 h and the amount of the secreted cytokine was calculated by ELISA. The cells produced IFN-γ, IL-10 and TNF-α by stimulating with both live and heat-inactivated organisms in an antigen dose-dependent manner. However, significantly higher levels of production were observed in the cells stimulated with live organisms than the cells stimulated with heat-inactivated organisms ($P < 0.05$). At a ratio of spleen cells to bacteria equal to 1:100, the cells produced IFN-γ, IL-10 and TNF-α at concentrations of 3165.6, 1234.8 and 252.5 pg/ml, respectively when stimulated with live organisms. In comparison, stimulations with heat-inactivated organisms induced production of lower amounts of cytokines (1267.7, 334.3 and 165.2 pg/ml, respectively). Little IL-4 and IL-12 were detected in any cases of stimulation (IL-4: 5.4–21.8 pg/ml, IL-12: 2.4–2.9 pg/ml).

3.3. The effects of IL-10 on the IFN-γ production from B. henselae Houston-1—primed mice spleen cells

To clarify the effects of IL-10 on the IFN-γ production from *B. henselae*-stimulated mice spleen cells, neutralizing antibodies to mouse IL-10 were added to the cultures and the amount of IFN-γ was assessed (Fig. 3). Addition of the neutralizing antibody dramatically enhanced the IFN-γ production (66345.8 pg/ml) compared with cells without the neutralizing antibodies (34227.4 pg/ml), and with cells in which the IgG1 rat isotype control antibody was added (28588.4 pg/ml).

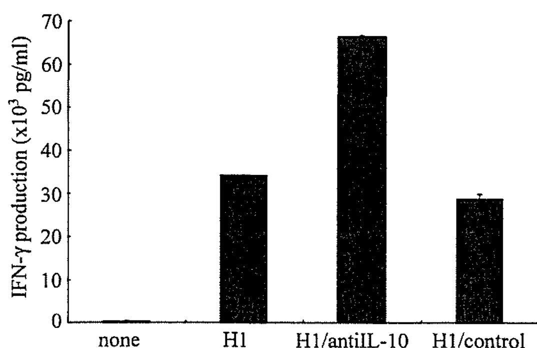

Fig. 3. Effects of neutralization of IL-10 and antigen-specificities on the production of IFN-γ. BALB/c mice were intraperitoneally infected with *B. henselae* Houston-1 twice at 10-day intervals. Spleen cells from three mice per group were harvested 5 days after the last inoculation and were cultured with or without *B. henselae* Houston-1 (H1) at ratio of cells to bacteria was 1:10. When starting the cultivation with *strain* Houston-1, the other wells were cultured in the presence of anti mice IL-10 neutralization (H1/anti IL-10) or isotype control antibodies (H1/control). Following cultivation, culture-supernatants were collected and IFN-γ was quantitated, using ELISA.

4. Discussion

In this study, we demonstrated a specific IFN-γ production from *B. henselae* (Houston-1) -primed mice spleen cells following *in vitro* stimulation with the bacteria. Karem et al. (1999) showed that spleen cells from *B. henselae* primed BALB/c mice secreted large amounts of IFN-γ after stimulation with the same organisms. Spleen cells from unprimed BALB/c mice also produced IFN-γ after *in vitro* stimulation with *B. henselae* although the amount was smaller than those of the primed mice. These results indicate that the production was induced by antigen-specific lymphocytes as well as nonspecific cell-mediated immune responses, including activated macrophages.

Resto-Ruiz et al. (2003) also reported the *B. henselae*-specific IFN-γ secretion from spleen cells of primed A/J mice. They found that *in vitro* stimulation with *B. henselae* also resulted in the significant production of IL-12 from spleen cells of the mice. IL-12 is known to induce Th1-related immune responses, including inductions of IFN-γ by NK and T cells, and resulted in stimulating cytotoxic T lymphocytes and NK cells (Trinchieri, 1998). In contrast, we did not detect any significant production of IL-12 from the spleen cells. This might be caused by the differences of the mice strains used in these experiments. The other possible reason to explain the difference is the ratio of cell to bacteria for the stimulation of spleen cells. We used the ratio of cell to bacteria at 1:1 to 1:100 for *in vitro* stimulation, while Resto-Ruiz et al. applied the ratio of 1:500 (Resto-Ruiz et al., 2003).

Patients with cat-scratch disease also showed no induction of IL-12 in their serum (Papadopoulos et al., 2001). However, the levels of circulating IL-2, IL-6 and IL-10 were shown to be significantly higher in patients with CSD than in healthy individuals

(Papadopoulos et al., 2001). In addition, the production of some inflammatory cytokines such as TNF and IL-1β, and IL-6 was markedly higher in the patients infected with *B. quintana* than those of uninfected individuals. It has also been reported that patients with *B. quintana*-bacteremia showed specific increase of IL-10 production by mononuclear cells (Capo et al., 2003). Because IL-10 is known to suppress several inflammatory mediators, overproduction of IL-10 in bacteremic patients with *B. quintana* may result in establishing a persistent infection as well as other infectious diseases (McGuirk and Mills, 2002). In the present study, we also observed IL-10 secretion from spleen cells of *B. henselae*-primed BALB/c mice and the secretion increased gradually following stimulation with the organisms. Furthermore, secretion of TNF-α was observed just 24 h after *in vitro* stimulation of spleen cells with the organisms and reached a plateau at the same time. Thus, *Bartonella* species, including *B. henselae* stimulate mononuclear cells and/or lymphocytes and the cells secrete IL-10 as well as some inflammatory cytokines, such as TNF-α and IFN-γ. In a mouse model, it has been shown that nitric oxide derived from macrophages activated by IFN-γ play a significant role in eliminating *B. henselae* from infected cells (Musso et al., 2001). We showed that IL-10 induced by stimulation with *B. henselae* partially suppressed the secretion of IFN-γ following the stimulation with the organisms. These results suggest that the organisms may escape the host immune responses, such as the activation of macrophages by inhibiting the induction of IFN-γ by inducing IL-10, simultaneously. However, it has been shown that persistent infection of *B. henselae* does not last for long periods in mice, suggesting that these modulations, such as inhibition of the production of IFN-γ may not affect the infectivity of *B. henselae* in mice. Supporting this hypothesis, *Bartonella* species shows strict host specificities in mice (Kosoy et al., 2000).

Arvand et al. (2001) reported that heat-killed *B. henselae* stimulated spleen cells of the primed C57BL/6 mice and induced proliferation of $CD4^+$ T cells and IFN-γ production. In the present experiment, large amounts of cytokines, including IFN-γ, IL-10 and TNF-α were detected when the spleen cells of BALB/c mice were stimulated with live *B. henselae*, whereas heat-killed organisms induced lower amount of cytokines. These results clearly suggest that some heat-stable components of *B. henselae* as well as secreted factors from live bacteria and/or invasion into cells may be involved in the induction of the cytokines in mice. Recent studies have revealed that *B. henselae* uses type IV secretion systems for intercellular delivery of effector molecules that modify host cellular functions in favor of the pathogen (Schmid et al., 2004). It has also been proven that the type IV secretion systems of *B. henselae* mediates invasion, a NF-κB-dependent pro-inflammatory activation (Schmid et al., 2004). Thus, type IV secretion systems may be involved in the induction of cytokines in mice.

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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*.
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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 $500 \times g$ 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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Comparison of Japanese Isolates of *Coxiella burnetii* by PCR-RFLP and Sequence Analysis

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***Abstract*: The genetic variation of Japanese isolates of *Coxiella burnetii*, the agent of Q fever, was found for the first time. Forty-nine out of 72 isolates had the chronic pattern of the *isocitrate hydrogenase* gene. Sequence analysis revealed that the isolates have a specific nucleotide sequence. The putative amino acid sequence was the same as that of chronic reference strains. These results suggest the variation of *C. burnetii* isolates in Japan.**

***Key words*: *com1* gene, *Coxiella burnetii*, *icd* gene, PCR-RFLP**

Coxiella burnetii (*C. burnetii*) is the causative agent of worldwide zoonosis, Q fever. It is the only known species of genus *Coxiella* (family *Coxiellaceae*, order *Legionellales*, gammaproteobacteria) (3). Q fever has a wide range of acute and chronic manifestations. Acute Q fever develops mainly as self-limited febrile illness, pneumonia or hepatitis, and sometimes in neurological or miscellaneous manifestations (11). The manifestations of chronic Q fever are mainly endocarditis, occasionally vascular infection, osteomyelitis, hepatitis and prolonged fever (18). The reason for these variable manifestations is still under debate.

Although a comparatively high prevalence of *C. burnetii* antibody in Japanese people has been reported (1, 10, 22), Q fever patients are rarely reported in Japan. Because the chronic reference strains have the same antigenicity as the acute reference strains but they have lower pyrogenicity than acute reference strains in guinea pigs (12), Japanese isolates have been suspected to have common characteristic with chronic reference strains. However, the hypothesis that Japanese isolates have a low pathogenicity like the chronic reference strains has not been proved yet.

C. burnetii has been isolated from various sources all over the world. It has been reported that *C. burnetii* isolates could be divided by their plasmid types (21), LPS profiles (4) and chromosomal DNA restriction endonuclease fragment patterns (5). Recently, two genetic markers, isocitrate dehydrogenase (*icd*) and *Coxiella* outer membrane protein 1 (*com1*) genes, were reported to distinguish *C. burnetii* reference strains (15, 25). The *C. burnetii icd* gene encodes an immunogenic dimeric $NADP^+$-dependent isocitrate dehydrogenase. The sequence of the *C. burnetii icd* gene of 11 reference strains and 8 Japanese isolates was analyzed, and a PCR-restriction fragment length polymorphism (PCR-RFLP) was described which may differentiate isolates from acute and chronic forms of Q fever (15). The *icd* genes were divided into three groups based on their nucleotide sequences: acute group (acute reference strains), chronic+plasmid group (chronic reference strains containing plasmids) and chronicΔplasmid group (plasmidless chronic reference strains). The *C. burnetii com1* gene encodes an immunogenic 27-kDa outer membrane protein (6). The sequence of the *com1* gene of 11 reference strains and 10 Japanese isolates was analyzed, and a PCR-RFLP was described which may screen plasmidless chronic isolates (25). With the PCR primers used in this study, the detection sensitivity of *com1* gene PCR is lower than that of *icd* gene nested PCR (data not shown).

All Japanese isolates have the same sequences as the

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Abbreviations: com1, *Coxiella* outer membrane protein 1; DNA, deoxyribonucleic acid; icd, isocitrate dehydrogenase; LPS, lipopolysaccharide; NADP, nicotinamide adenine dinucleotide phosphate; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism.

Table 1. Japanese isolates of *C. burnetii* studied

Species	Originating materials	Geographical sources (Prefecture)	Number of isolates	*icd* gene pattern	
				chronic	acute
Human[a]	Serum	Shizuoka	19	19	0
	Blood	Shizuoka	2	2	0
	Swab of throat	Shizuoka	7	7	0
Cattle	Raw milk	Chiba	9	0	9
		Shizuoka	13	13	0
		Ehime	3	0	3
	Swab of vagina	Mie	4	0	4
	Aborted fetus	Mie	1	0	1
	Mammary gland	Gifu	3	0	3
Pet cat	Blood	Shizuoka	5	5	0
	Swab of vagina	Shizuoka	1	1	0
Pet dog	Blood	Shizuoka	2	2	0
Tick	Whole body	Gifu	3	0	3
Total			72	49	23

[a] With flu-like symptom.

acute reference strain, Nine Mile, in the previous reports (15, 25), suggesting that there is no chronic type of *C. burnetii* in Japan. Until now, no Japanese isolate was reported to be involved in chronic Q fever. However, this might be caused by the use of *C. burnetii* isolates which were successfully adapted in cultured cells in these reports. All Japanese isolates were first isolated using mice, and then adapted to cultured cells. However, some isolates could not be adapted to cultured cells. Since each *C. burnetii* isolate has a different infectivity to cultured cells (19, 20), the process of *in vitro* cultivation may select a certain property of *C. burnetii*. Acute reference strains are known to have higher infectivity to cultured cells than chronic reference strains (19, 20). Therefore, there is a possibility that all Japanese isolates used in the previous studies were unknowingly selected to have a common character with acute reference strains. To avoid unintentional selection, all isolates used in this study have no history of *in vitro* cultivation. To investigate genetic variation, particularly to find the chronic type of *C. burnetii*, a large number of Japanese isolates was used. The aim of this study was to find Japanese isolates that may be involved in chronic Q fever or whose pathogenicities are different from those of foreign reference isolates.

The *C. burnetii* isolates used in this study were derived from various sources in Japan, such as humans with flu-like symptoms, cattle, pet cats, pet dogs and ticks (Table 1). A total of 72 isolates were used. The isolation was done by mouse inoculation in two laboratories: the Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Gifu, Japan from 1992 to 1993, and the Department of Microbiology, Shizuoka Prefectural Institute of Public Health and Environmental Science, Shizuoka, Japan from 1994 to 2000 (7, 13, 14). The possibility of laboratory contamination was ruled out by using blind negative controls. The spleens of the mice used in the isolation were stored as isolated *C. burnetii* materials at −80 C until use. DNA was extracted from the homogenate of the mouse spleen using a DNA extraction kit, Sepagene (Sanko Junyaku Co., Tokyo). Normal uninfected mouse spleen was used for a negative control to rule out the false amplification of mouse genome DNA. *Icd* gene PCR-RFLP was performed as reported by Nguyen and Hirai (15). Briefly, an *icd* gene 370-bp fragment was amplified by nested PCR using primer pairs of *icd*1/5 and *icd*N1/N2. The PCR products were purified and digested with *Acc*II, and its RFLPs were analyzed. The 370-bp fragment that was digested into two bands (221 and 149 bp) was considered to be an acute pattern (acute group: Nine Mile, Ohio, California, Bangui and El Tayeb strains and 8 Japanese isolates). Another fragment that was not digested by *Acc*II was considered to be a chronic pattern (chronic+plasmid group: Priscilla, ME and MAN strains; chronicΔplasmid group: GQ212, SQ217 and KoQ229 strains). *Com1* gene PCR-RFLP was performed as reported by Zhang et al. (25). Briefly, the *com1* gene 1,061-bp fragment was amplified by PCR using primer pair OMP1/2. The PCR products were purified and digested with *Pst*I, and RFLPs were analyzed. The 1,061-bp fragment that was digested into two bands (740 and 321 bp) was considered to be the chronicΔplasmid pattern (GQ212, SQ217 and KoQ229 strains). A fragment that was not digested by *Pst*I was considered to be another type, i.e. acute or