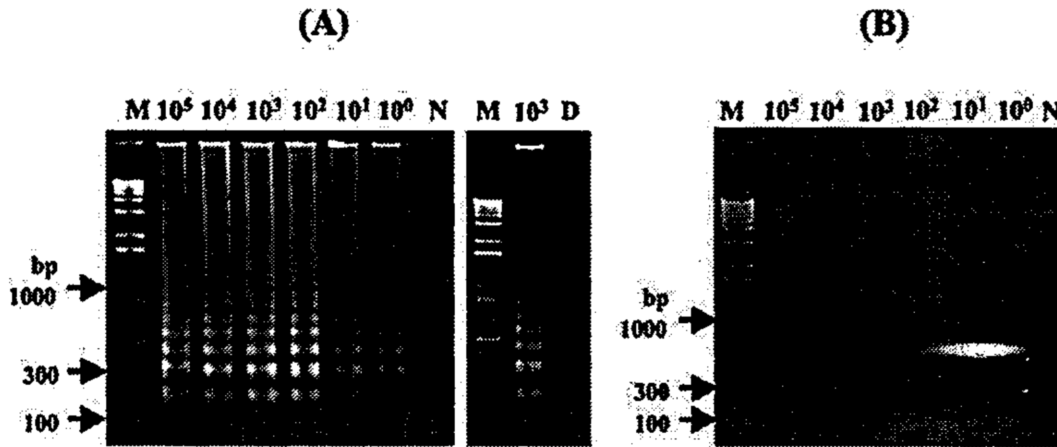

FIG. 2. Electrophoretic analysis of LAMP (A) and PCR (B) products. The numbers above each lane represent 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU per reaction tube of template DNA of *Y. pseudotuberculosis* 1b. Lane D, LAMP product after digestion with BssHII; lane N, LAMP or PCR in the absence of template DNA; lane M, 1-kb ladder DNA size marker.

scribed above. The results showed that the LAMP method is able to detect the target gene even with 10^0 CFU of bacteria present in the tube (Fig. 2). In contrast, the detection limit of PCR was 10^2 CFU. Thus, LAMP was 100 times more sensitive than PCR. The products of LAMP from *Y. pseudotuberculosis* that were submitted for confirmation by digestion with restriction endonuclease, with cleavage sites within the amplicon, showed the expected size band of 246 bp (Fig. 2).

It is known that PCR inhibitors in samples reduce the sensitivity of PCR when attempting to detect a target gene (6, 13, 17, 18). Notomi et al. (12) reported that the sensitivity of LAMP is not influenced by the copresence of nontarget DNA in samples, and Enosawa et al. (2) reported that LAMP was not inhibited by blood serum and plasma heparin, which are known to inhibit PCR. Therefore, we evaluated the performance of this method in clinical specimens. A total of 15 livers from dead monkeys were used. Of the 15 monkeys, 9 squirrel monkeys (*Saimiri sciureus*) and 1 orangutan (*Pongo pygmaeus*) died by natural *Y. pseudotuberculosis* infection, 2 squirrel monkeys and 1 dark-handed gibbon (*Hylobates agilis*) died by natural *Y. enterocolitica* O:8 infection, and 2 other squirrel monkeys died by other causes and no *Yersinia* species was isolated. Isolation of *Yersinia* from those monkeys was carried out as described previously (5). The number of bacteria in the *Y. pseudotuberculosis*-positive samples ranged from 2.2 to 6.8 log CFU/g. DNA for LAMP from liver samples was extracted by using the Wizard Genomic DNA Purification kit (Promega). The LAMP reaction was positive only for those samples from

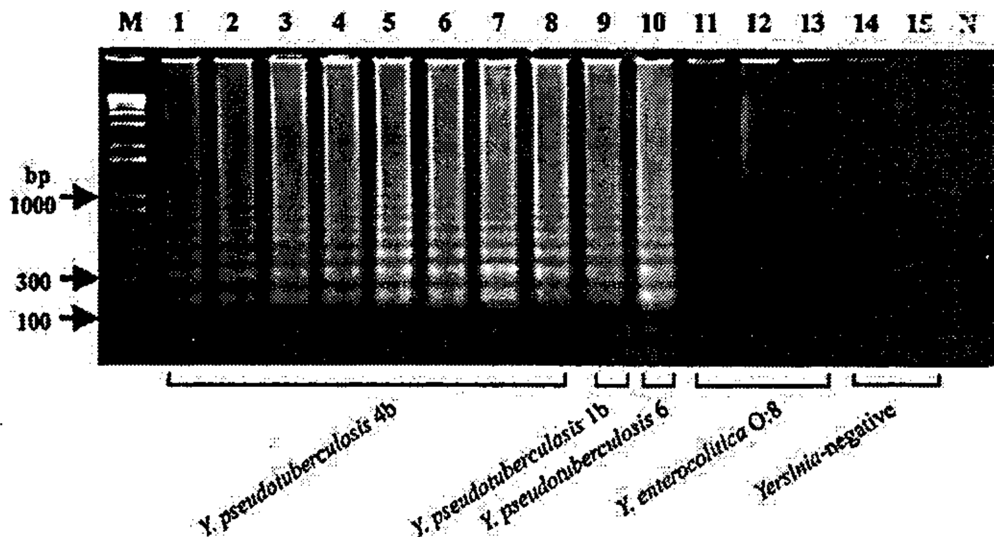

FIG. 3. LAMP detection of the *inv* gene in liver samples from *Y. pseudotuberculosis*-infected monkeys and uninfected monkeys. The samples of each lane and the number of bacteria isolated, in log CFU/gram, from each sample are the following: lanes 1 to 7, squirrel monkey, 5.1, 6.8, 6.4, 6.8, 5.1, 2.2, and 5.0, respectively; lane 8, orangutan, 5.2; lanes 9 to 12, squirrel monkey, 4.9, 6.3, 6.7, and 5.6, respectively; lane 13, dark-handed gibbon, 5.2. Lanes 14 and 15, squirrel monkeys from which no *Yersinia* species were isolated. Lane N, LAMP in the absence of template DNA. Lane M, 1-kb ladder DNA size marker.

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

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

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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

Table 2. Group-specific nucleotide sequences of the *C. burnetii* isocitrate dehydrogenase gene 370-bp fragment

Group	Position[a]				
	649	735	745	900	942
Consensus nt[a]	C	C	G	A	G
Acute[a]	—	—	—	—	—
Chronic+plasmid[b]	—	—	A	G	—
ChronicΔplasmid[c]	G	T	A	—	T
Japanese-specific	—	—	A	—	—

[a] According to the *icd* gene nucleotide sequence of Nine Mile strain (GenBank accession number AF069035).

[b] According to the *icd* gene nucleotide sequence of Priscilla strain (GenBank accession number AF146294).

[c] According to the *icd* gene nucleotide sequence of GQ212 strain (GenBank accession number AF146295).

chronic+plasmid pattern. The PCR products of *icd* gene which was considered to be a chronic pattern were cloned into pT7Blue Vector (Novagen, Inc., U.S.A.). The nucleotide sequence was determined by the dideoxynucleotide chain-termination method using the Thermo Sequenase Cy5.5 Dye Terminator Cycle sequencing kit (Amersham Pharmacia Biotech) and SEQ4x4 personal sequencer system (Amersham Pharmacia Biotech). T7 promoter and/or M13 reversal sequence primers were used. The nucleotide sequence, the deduced amino acid sequence and the secondary structure (CHOU-FASMAN analysis) were analyzed by the GENETYX analyzing system (Software Development Co., Ltd., Tokyo). Comparison with published strains and isolates was analyzed by the BLAST search system of the DNA Data Bank of Japan (DDBJ, National Institute of Genetics, Mishima, Japan). The nucleotide sequences reported in this paper have been assigned GenBank accession numbers AB128770 to AB128818.

The *icd* gene fragments of 49 out of 72 Japanese isolates were not digested by *Acc*II, and these isolates were considered to have the chronic pattern of the *icd* gene. The hosts of the isolates that had the chronic pattern of the *icd* gene were humans, cattle, pet cats and pet dogs from Shizuoka Prefecture (Table 1). The *icd* genes of the other 23 isolates were digested into two bands of the expected sizes of 221 and 149 bp, respectively, and were considered to have the acute pattern of the *icd* gene. No false positive PCR product was observed in *icd* gene PCR with the normal mouse spleen DNA.

The nucleotide sequences of 49 *icd* gene fragments which had the chronic pattern were completely identical (data not shown). No identical nucleotide sequence was found within the DNA database. This sequence does not belong to the reported group of *icd* genes (acute, chronic+plasmid and chronicΔplasmid groups) (Table 2). The isolates would be called Japanese-specific isolates. The nucleotide sequence of the *icd* gene of the Japanese-specific group differed from the sequence of the acute group by only one point mutation (99.7% homology), which resulted in a single difference in the amino acid sequences (99.2% homology): alanine to threonine in position 55. The nucleotide sequence of the *icd* gene of the Japanese-specific group differed from that of chronic+plasmid group by only one point mutation (99.7% homology), which did not affect the amino acid sequence (100% homology). The nucleotide sequence of the *icd* gene of the Japanese-specific group differed from that of chronicΔplasmid group by three point mutations (99.2% homology), which resulted in a single difference in the amino acid sequences (99.2% homology): glutamate to glutamine in position 23. The secondary structure predicted by CHOU-FASMAN analysis of the Japanese-specific group was different from the predicted structure of the acute group and identical to the predicted structures of the chronic groups (data not shown). The nucleotide sequences of the *icd* gene which had the acute pattern were not analyzed.

The *com1* gene fragment was amplified in only 24 out of 49 Japanese-specific isolates. This is because the PCR of the *com1* gene is less sensitive than the nested PCR of the *icd* gene. None of the amplified *com1* fragments were digested by *Pst*I. The *com1* gene PCR-RFLP of Japanese-specific isolates was the same as that of the acute and chronic+plasmid groups. No false positive PCR product was observed in the *com1* gene PCR with the normal mouse spleen DNA.

Japanese isolates of *C. burnetii* that had the chronic pattern of the *icd* gene were found for the first time. However, the *icd* gene sequence of these Japanese isolates was different from that of the chronic reference

strains, and there is no identical sequence in the database. The results suggest the presence of a specific *C. burnetii* isolate in Japan.

This is the first study to suggest that Japanese isolates are molecularly different from strains in other countries. Although recent seroepidemiological studies suggested a wide spread of Q fever in Japan (1, 10, 22), actual cases of Q fever have rarely been reported. The possibility that Japanese people have certain specific host factors can be ruled out by the fact that Japanese Q fever patients who were originally infected in foreign countries showed typical Q fever manifestations (16, 17). Japanese isolates of *C. burnetii* have been suspected to have a specific pathogenicity, and possibly a low pathogenicity. However, no studies have described the specificity of Japanese isolates.

Up to 1999, a limited number of Japanese isolates were studied after they were adapted for cell culture for large-scale cultivation (15, 23–25). The use of cell-adapted isolates raises the possibility that the isolates were selected to have a certain *in vitro* character of acute strains (high infectivity to cultured cells). In this study, the isolates that had no history of *in vitro* cultivation were used. PCR-RFLP of the *icd* gene revealed the presence of Japanese isolates which might be classified under the chronic group. However, the sequences of the PCR products demonstrated that they are not the same as the chronic reference *C. burnetii* isolated in other countries. Although all isolates from Shizuoka Prefecture used in this study had the chronic pattern of the *icd* gene, a geographical factor might not be correlated with the genetic variation because isolates from Shizuoka Prefecture that have the acute pattern of the *icd* gene are also reported (15). The results of the present study suggest the following two hypotheses. One hypothesis that can explain these results is that there exists a Japanese-specific *C. burnetii*. Another hypothesis is that the *icd* gene PCR-RFLP may not be a suitable marker for distinguishing the pathogenicity of *C. burnetii*. Further molecular-biological and pathophysiological studies are needed to confirm the character of the isolates that belong to the Japanese-specific group.

Much can be learned about antigenicity and pathogenicity of the Japanese isolates by using monoclonal antibodies (8, 9) and a new animal model for Q fever which has been reported previously (2). Additional studies of the antigenicity and pathogenicity of Japanese isolates can reveal not only the character of the Japanese isolates of *C. burnetii*, but also the practicality of PCR-RFLP of the *icd* gene for the typing of *C. burnetii* isolates. These studies will provide a better understanding of Q fever in Japan.

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NOTE *Bacteriology*

Use of Monoclonal Antibodies for Analyses of *Coxiella burnetii* Major Antigens

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ABSTRACT. Monoclonal antibodies (MAbs) to major antigens of *Coxiella burnetii* were produced. Some of the MAbs to a 62-kDa protein antigen, peptidoglycan protein complex and lipopolysaccharide (LPS) O-chains reacted with other bacteria whereas none of the MAbs to outer membrane proteins and LPS outer-core did. The LPS outer-core and OMPs may be useful antigens for specifically detecting antibodies to *C. burnetii*.

KEY WORDS: antigenic property, *Coxiella burnetii*, monoclonal antibody.

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Coxiella burnetii, the agent of Q fever in man and the agent of coxiellosis in other species, is most closely related to the genus *Legionella* and resides within the gamma subdivision of the *Proteobacteria* [10]. Its cell envelope is composed of an outer membrane (OM), a peptidoglycan layer and an inner membrane. The OM contains lipopolysaccharide (LPS) and OM proteins (OMPs). Mutational variation in the LPS is linked to shifts in antigenicity and virulence, termed phase variation. The virulent phase I organism has a complete length LPS whereas the avirulent phase II organism possesses a truncated LPS. This LPS variation makes OMPs accessible for antibodies [6]. LPS and OMPs play a role in the development of immunity to infection by *C. burnetii*, although their antigenic properties are poorly understood.

For analyzing antigenic properties, monoclonal antibodies (MAbs) are useful tools because they recognize a single epitope with high specificity. Recently, we produced several MAbs to *C. burnetii* LPS and analyzed the antigenic change during phase variation [7] and the antigenic difference among strains [8]. In this study, we produced MAbs to OMPs [11], 62-kDa protein antigen and peptidoglycan protein complex (PG-PC) [1]. With the MAbs, we obtained invaluable data to elucidate the antigenic properties of *C. burnetii*.

C. burnetii Nine Mile strain phases I and II cells and 18 other strains (California 76, Bangui, El Tayeb, G Q212, Henzerling, Ko Q229, MAN, ME, Ohio 314, Priscilla, S Q217, 307, 1M, 3M, 50M, 57T, 58T and 60M) of phase I cells were obtained elsewhere [15]. The MAbs were generated as described previously [7] by using BALB/c mice that had been immunized with formalin-inactivated Nine Mile strain phase II cells. Screening assay for hybridomas was performed by indirect immunofluorescence assay (IFA). The antigens used were intact purified phase II organisms and buffalo-green-monkey (BGM) cells which were infected with phase II organisms. The MAbs were characterized by their reactions to whole cell lysate, purified LPSs, purified OM and proteinase K-digested antigen of phase II cells by Western blotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). OM was partially purified from the sodium lauroyl sarcosine (Nacalai Tesque, Inc., Kyoto, Japan)-insoluble fraction of Nine Mile strain phase II cells as described by Caldwell *et al.* [4]. Proteinase K-digested antigen was prepared from the whole cell lysate by incubation with proteinase K (Takara Shuzo, Co., Ltd., Shiga, Japan) for 8 hr at 56°C as described previously [14].

The MAbs produced in this study were used for further antigenic analyses together with the MAbs to LPS, which were produced previously [7]. The MAbs of groups I (H5A, H5B, H45 and H83) and II (H21) recognize LPS O-chains and the MAbs of group III (H70, H73, H78 and H80) recognize the LPS outer-core [7]. The specificities of the MAbs were analyzed from the reactions with other bacteria in Western blots after SDS-PAGE. Nine other bacteria, *Escherichia coli, Salmonella* Typhimurium, *Klebsiella pneumoniae, Yersinia enterocolitica, Legionella pneumophila, Brucella abortus, Bacillus anthracis, Bartonella henselae* and *Chlamydia psittaci*, were propagated in the authors' laboratory at Gifu University. The reactivities of the MAbs to *C. burnetii* natural phases I and II cells and KIO_4- and trichloroacetic acid (TCA)-treated phase I cells were analyzed by IFA. KIO_4-treated phase I cells were prepared by incubation in 10 mM KIO_4 in phosphate buffered saline (PBS) at 37°C for 2 hr [13]. TCA-treated phase I cells were prepared by incubation in 10% (wt/vol) TCA in PBS at 4°C for 4 hr [5].

Nine MAbs (H106, K7, K24, K43, K57, K59, K64, K75 and K82) were produced. The MAbs K7, K24, K57 and K64 did not react with intact phase II cells in IFA although other MAbs reacted with both intact phase II and phase II-infected BGM cells. All MAbs reacted with Nine Mile strain phases I and II cells and 18 other strains similarly but did not react with the purified LPS in Western blots (data not shown). These MAbs were divided into 5 groups (IV to VIII) based on their patterns of reaction to whole cell lysate, OM and proteinase K-digested antigens. Figure 1 shows the

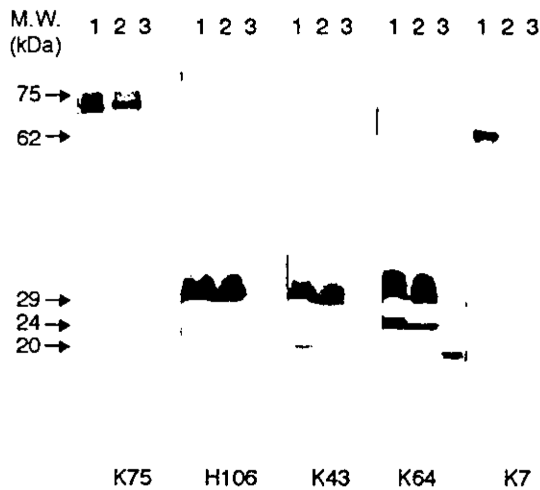

Fig. 1. Reactions of the MAb to whole cell lysate, OM and proteinase K-digested antigen of Nine Mile strain phase II cells in Western blots (lanes 1 to 3, respectively). Based on the reaction patterns, the MAbs were divided into 5 groups (IV to VIII). Reactions of the representative MAb of groups IV to VIII (K75, H106, K43, K64 and K7, respectively) are shown. The MAbs used are indicated at the bottom of each strip. Molecular weights are indicated on the left.

reactions of the representative MAbs of groups IV to VIII (K75, H106, K43, K64 and K7, respectively). The MAbs of groups IV to VI reacted with both whole cell lysate and OM similarly, whereas they did not react with proteinase K-digested antigen. The MAb of group IV (K75) reacted with 67 to 75-kDa smeared bands. The MAbs of group V reacted with 29- to 31-kDa smeared bands. The MAbs of group VI (K43) reacted with 29- to 31-kDa smeared bands and a 20-kDa band as well. The MAb of group VII (K64) reacted with 29- to 31-kDa and 24-kDa bands of whole cell lysate and OM, and an 18-kDa band of proteinase K-digested antigen. The MAbs of group VIII (K7, K24 and K57) reacted with a 62-kDa band of whole cell lysate but did not react with OM and proteinase K-digested antigen (Fig. 1).

The reactions of the MAbs produced in this and previous studies [7] were characterized. MAbs H21, H45 and K57 reacted with some of the gamma subgroup of *Proteobacteria* such as *E. coli*, *S.* Typhimurium, *K. pneumoniae*, *Y. enterocolitica* and *L. pneumophila*. MAb K64 reacted with *E. coli*, *S.* Typhimurium, *Y. enterocolitica* and *B. anthracis* as well. The other MAbs did not react with the other bacteria examined (data not shown). The reactions of the MAbs to various *C. burnetii* antigens in IFA are summarized in Table 1. The MAbs to LPS O-chains (groups I and II) reacted with natural and TCA-treated phase I cells but did not react with KIO_4 -treated phase I cells or natural phase II cells. The MAbs against the LPS outer-core (group III) reacted with KIO_4 and TCA-treated phase I cells but did not react with natural phase I or II cells. The MAbs to OMPs (groups IV, V and VI) reacted with KIO_4 and TCA-treated phase I cells and natural phase II cells but did not react with natural phase I cells. The MAbs of groups VII and VIII did not react with the antigens examined.

Our results suggest that the nine MAbs (H106, K7, K24, K43, K57, K59, K64, K75 and K82) produced in this study recognize the antigens conserved among all *C. burnetii* strains tested and both phase I and II cells. Based on their reactions to whole cell lysate, OM and proteinase K-digested antigen, the MAbs were divided into 5 groups (IV to VIII). Our results suggest that the MAbs of groups IV (K75), V (H106, K59 and K82) and VI (K43) recognize OMPs. Since the 29- to 31-kDa OMP aggregate contains 18.3-kDa PG-PC that is resistant to proteinase K as well [1, 3], the MAb of group VII (K64) may recognize PG-PC. The MAbs of group VIII (K7, K24 and K57) appear to recognize a 62-kDa protein antigen. This 62-kDa protein antigen is likely to be *C. burnetii* heat shock protein 60 (HSP60) [16] since the MAbs strongly reacted with a recombinant *C. burnetii* HSP60 in our preliminary study (data not shown). The 29.5- and 31-kDa of OMPs are considered to be porin proteins that allow passive diffusion of nutrients across the OM [3]. The PG-PC is considered to be an important antigen for resistance to environmental conditions and intracellular digestion [2]. *C. burnetii* 62-kDa antigen is reported as the primary immunodominant antigen [12]. Therefore, the MAbs obtained should be useful tools for analyzing the infection mechanisms and the developmental cycle of *C. burnetii*.

The MAbs produced in this study were characterized together with the MAbs produced in our previous study [7].

Table 1. Reactions of monoclonal antibodies to various *Coxiella burnetii* antigens in indirect immunofluorescence assay

Monoclonal antibodies			*Coxiella burnetii* antigens			
			Phase I			Phase II
Antigen recognized	Group (s)	No. of clones	Natural	KIO_4 treated	TCA treated	Natural
LPS O-chains	I	4	+	–	+	–
LPS O-chains	II	1	–	–	+	–
LPS outer-core	III	4	–	+	+	–
OMPs	IV,V,VI	6	–	+	+	+
PG-PC	VII	1	–	–	–	–
62-kDa antigen	VIII	3	–	–	–	–

+ = Reaction; – = No reaction.

The reactions of the MAbs suggest that *C. burnetii* LPS O-chains, PG-PC and 62-kDa antigen contain the conserved epitopes that are possibly related to the serological cross-reactions between *C. burnetii* and other bacteria. On the other hand, the LPS outer-core and OMPs may be *C. burnetii*-specific antigens among the bacteria tested in this study. Our results also showed the antigenic difference among KIO_4- and TCA-treated phase I and natural phase II antigens. Although both of these chemically treated phase I cells have been used as artificial phase II antigens in serological tests of Q fever [9], TCA-treated phase I cells might be more cross-reactive with the antibodies to other bacteria. These differences among the *C. burnetii* antigens may cause confusion when interpreting the serological results for clinical samples.

Diagnoses of *C. burnetii* infections are based upon serological methods, but the antigenic properties of *C. burnetii* are poorly understood and the antigen used in serological tests is not standardized in each laboratory. In this study, we analyzed the antigenic properties of *C. burnetii* LPS, OMPs, PG-PC and 62-kDa antigen by using the MAbs. Since these antigens induce humoral and/or cell-mediated immunity in experimental animals [2, 12, 17], our results should help to elucidate the immunoreactions in the serological tests. It is yet unclear whether the results of this study can be applied to reactions with clinical samples, but conceivably LPS O-chains, PG-PC and 62-kDa antigen contain the epitopes that cross-react with antibodies to other bacteria. Although additional studies are needed, we suggest that the LPS outer-core and OMPs can be useful antigens for specifically detecting antibodies to *C. burnetii.*

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In Vitro Susceptibility to Tetracycline and Fluoroquinolones of Japanese Isolates of *Coxiella burnetii*

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***Abstract*: *Coxiella burnetii* is the agent of the worldwide zoonosis, Q fever. The *in vitro* susceptibility to tetracycline and fluoroquinolones of Japanese isolates of *C. burnetii* was evaluated for the first time. The MICs against Japanese isolates were almost the same as the MICs against the foreign reference isolates. The results suggest that the common antibiotics therapy for Q fever used in other countries is also effective for Japanese Q fever patients.**

Key words: *Coxiella burnetii*, Fluoroquinolone, Tetracycline, MIC

The obligate intracellular bacterium *Coxiella burnetii* (*C. burnetii*) is the causative agent of a worldwide zoonosis, Q fever. Q fever has various acute and chronic manifestations, ranging from latent to fatal. Acute Q fever is mainly a self-limited febrile illness, pneumonia or hepatitis, and it sometimes has neurological or miscellaneous manifestations (7). Chronic Q fever mainly occurs in the form of endocarditis, occasionally and as vascular infection, osteomyelitis, hepatitis and prolonged fever (10). Antibiotic treatment of acute Q fever is satisfactory, but chronic Q fever, occasionally takes several years to respond to antibiotics (3). Tetracycline derivatives are the first choice for treating Q fever. Fluoroquinolones have been used as an alternative to tetracyclines (8).

Recently, seroepidemiological studies suggested the presence of considerable numbers of Q fever patients in Japan (1, 6, 14). However, the actual cases of Q fever are rarely reported. Japanese isolates of *C. burnetii* are suspected of differing from foreign isolates in various characteristics; e.g., they might have a lower pathogenicity than the foreign isolates.

Since Q fever patients are rare in Japan, clinical data on Japanese patients is limited. Moreover, the antibiotic susceptibility of Japanese isolates has not been studied yet. In this study, the *in vitro* susceptibility of *C. burnetii* derived from Japanese Q fever patients to tetracycline and quinolones was examined. The method in the present study was based on the shell vial assay, a common system of antibiotic susceptibility testing for *C. burnetii* (11). However, we used buffalo green monkey kidney fibroblast (BGM) cells, because of their higher sensitivity for various *C. burnetii* strains including Japanese isolates.

Four foreign reference strains and two Japanese isolates of *C. burnetii* were used (Table 1). *C. burnetii* was propagated in BGM cells grown with antibiotic-free medium as described elsewhere (5). Proper safety precautions were taken during all procedures which call for the handling of live bacteria. One tetracycline derivative and four fluoroquinolones were used. The tetracycline derivative, doxycycline (DOXY) (Sigma), used in this study was a commercial product. Three fluoroquinolones, ciprofloxacin (CPFX), levofloxacin (LVFX) and gatifloxacin (GFLX), were extracted from commercially available tablets. Moxifloxacin (MFLX) was synthesized at the Research Laboratories of Toyama Chemical Co., Ltd. (Toyama, Japan). The DOXY was solubilized in sterilized distilled water, and the fluoroquinolones were solubilized in filter-sterilized (pore size, 0.22 μm) 0.1 M NaOH. The inoculum titer was determined as reported elsewhere (11) with some modi-

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Abbreviations: BGM, buffalo green monkey; CPFX, ciprofloxacin; DOXY, doxycycline; GFLX, gatifloxacin; LVFX, levofloxacin; MFLX, moxifloxacin; MIC, minimum inhibitory concentration.

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

Isolate	Origin	Disease	Geographical source
Nine Mile[a]	Tick	No apparent Reference strain for acute Q fever	United States
Priscilla[b]	Goat (placenta)	Abortion Reference strain for chronic Q fever	United States
Ohio[a]	Cow (milk)	Unknown, persistent infection	United States
GQ212[c]	Human (heart valve)	Endocarditis	Canada
307[d]	Human (serum)	Flu-like illness	Japan (Shizuoka)
605[d]	Human (serum)	Flu-like illness	Japan (Shizuoka)

[a] Obtained from the American Type Culture Collection.
[b] Obtained from Dr. Kazar J., Institute of Virology, Bratislava, Slovakia.
[c] Obtained from Dr. Mallavia L.P., Washington State University, Pullman, Wash.
[d] Obtained from Dr. Nagaoka H., Institute of Public Health and Environment Science, Shizuoka, Japan.

fications. Briefly, monolayers of BGM cells in a 24-well cell-culture plate were inoculated with serially diluted *C. burnetii* suspensions. Six wells were used for each dilution. The cell-culture plates were centrifuged at 300×*g* for 1 hr at room temperature, the inoculum was removed and 1 ml of fresh medium was added. After 6 days of incubation (37 C, 5% CO_2), cells were fixed with methanol and stained by indirect immunofluorescence with anti-*C. burnetii* serum. The anti-*C. burnetii* serum was made in our laboratory by immunizing a rabbit with inactivated purified *C. burnetii*. The fluorescence was detected with an inverted fluorescence microscope, Axiovert 200 (Carl Zeiss Co., Ltd., Germany). The cell which had intracytoplasmic vacuole with *C. burnetii*-antigens or which contained aggregated *C. burnetii*-antigens was considered to be infected. By counting the ratio of infected to non-infected cells, the dilution that would result in 30 to 50% of the cells becoming infected was determined and used for the inoculum. Minimum inhibitory concentration (MIC) was determined as reported elsewhere (11) with some modifications. Briefly, BGM cell monolayers in a 24-well cell-culture plate were inoculated with 0.5 ml of the *C. burnetii* suspension per well, and centrifuged at 300×*g* for 1 hr at room temperature. The inoculum was removed and 1 ml of medium containing antibiotics was added. The antibiotics were tested at two-fold serial dilutions (0.008 to 16 μg/ml) and each dilution was overlaid onto 4 wells of the cells. Uninfected cells and infected cells of each strain were cultivated in medium without antibiotics as negative and positive controls respectively. Also, the cells fixed immediately after inoculation with each isolate were used as negative controls. After 6 days of incubation, cells were fixed with methanol and stained by the indirect immunofluorescence method. The activity of the antibiotics was evaluated by their capacity to inhibit *C. burnetii* growth: the absence of infected cells or the presence of isolated bacteria, meaning no growth. MIC was determined as the minimum antibiotic concentration that exhibited the growth of *C. burnetii*. In this study, we tested a single treatment effect. The test was done in duplicate.

The MICs of the antibiotics against the isolates in BGM cells are summarized in Table 2. Against the four reference isolates, the tetracycline DOXY was more effective (MIC: 0.25 to 1.0 μg/ml) than the fluoroquinolones. The fluoroquinolones LVFX (MIC: 0.5 to 1.0 μg/ml) and GFLX (MIC: 0.5 to 1.0 μg/ml) were more effective against reference isolates than MFLX (MIC: 1.0 to 2.0 μg/ml) and CPFX (2.0 to 8.0 μg/ml). Against the two Japanese isolates, DOXY (MIC: 0.25 to 1.0 μg/ml) and LVFX (MIC: 0.5 μg/ml) were most effective and CPFX was least effective (MIC: 4.0 to 8.0 μg/ml). MFLX and GFLX had approximately equal effects. No apparent difference was observed in the MICs against the reference and Japanese isolates.

The *in vitro* antibiotic susceptibility of Japanese isolates of *C. burnetii* from patients was studied for the first time. The susceptibilities of the Japanese isolates were equivalent to those of the reference strains. The present results suggest that antibiotic therapy with tetracycline derivatives or fluoroquinolones may be helpful in treating Japanese Q fever patients as in treating Q fever patients in foreign countries.

The BGM cell line was chosen for this study because it is more suitable for propagating *C. burnetii* than other cell lines (Vero, L929 mouse fibroblast, HEL and chicken embryo fibroblast cells) (To, H. 1996. Ph. D. Thesis, Gifu University). It is well suited for growing both reference strains and freshly derived isolates of *C. burnetii* and is easy to maintain (To, H. 1996. Ph. D. Thesis, Gifu University). The use of a 24-well culture plate made the assay simpler than the shell vial assay. The modified method developed in this study can help to popularize the antibiotic susceptibility test of *C. bur-*

Table 2. MICs of antibiotics against reference and Japanese isolates of *C. burnetii* in BGM cells

Drugs	MIC (μg/ml) against:					
	Reference isolates				Japanese isolates	
	Nine Mile	Priscilla	GQ212	Ohio	307	605
DOXY	0.25–0.5	0.5	0.5	0.25–1.0	0.5–1.0	0.25–0.5
MFLX	1.0–2.0	2.0	1.0	1.0–2.0	0.5–1.0	1.0
CPFX	2.0–8.0	8.0	8.0	4.0–8.0	4.0–8.0	8.0
LVFX	0.5	0.5	0.5–1.0	0.5	0.5	0.5
GFLX	0.5–1.0	0.5	0.5	1.0	1.0	1.0

netii isolates.

In vitro studies of *C. burnetii* have some difficulties. Although *C. burnetii* can grow in many kinds of cell lines (15), the infectivity of *C. burnetii* isolates to cell lines and the sensitivity of the cell lines are different from each other (9, 11, 13). Moveover, the growth feature (formation of inclusion body) of *C. burnetii* isolates in fibroblast cells differ (9, 13) and the number of infected bacteria cannot be compared in *in vitro* system. A determination of the inoculum dose of *C. burnetii* is somewhat more ambiguous than it is for other intracellular pathogens. Therefore, to test antibiotic susceptibility of an unknown strain of *C. burnetii* in a certain cell line, it is important to confirm the procedure using the reference strains of *C. burnetii* and the antibiotics reported before. Based on these points, the methods of this study were designed to be used as an *in vitro* antibiotics susceptibility test of *C. burnetii* isolates. The DOXY was more effective against reference strains in BGM cells than it was in L929 (MIC: 1.0–2.0 μg/ml) (12) and Vero cells (MIC: 1.0–2.0 μg/ml) (4). The MICs of MFLX and CPFX, against reference strains in BGM cells were almost equivalent to the MICs evaluated in L929 (MIC: 0.5–1.0 μg/ml) and Vero cells (MIC: 4.0–8.0 μg/ml). The MICs of LVFX and GFLX against the reference strains were slightly smaller than the MIC of other fluoroquinolones in BGM cells. The effective order of antibiotics used in this study against *C. burnetii* isolates was DOXY>GFLX>LVFX>MFLX>CPFX.

Evaluation of the antibiotic susceptibility of *C. burnetii* isolates seems to be too time-consuming to do in clinical settings. However, in cases of chronic Q fever, it will be important to confirm the antibiotic susceptibility of the agent since chemotherapy for chronic Q fever takes several months or years (3) and an evaluation of the success of therapy requires prolonged follow-up due to late relapses. Particularly, the heterogeneity of susceptibility of the isolates from humans implicated in endocarditis must be considered as reported (11).

Experimental evaluation of antibiotic therapy for Q fever is difficult because the drugs have different effects against *C. burnetii in vitro* and *in vivo* (16), and because a suitable animal model for chronic Q fever endocarditis is not available. However, as we described previously, a successful mouse model for Q fever endocarditis was established (2). This SCID mouse model may make it possible to evaluate the *in vivo* antibiotic susceptibility of *C. burnetii*.

In conclusion, Japanese strains of *C. burnetii* had almost the same susceptibilities to tetracycline and fluoroquinolones as foreign reference strains *in vitro*. The proposed technique is an important tool for the evaluation of *in vitro* antibiotic susceptibility of *C. burnetii*. The results of this study can help to popularize an antibiotic susceptibility test of *C. burnetii* isolates and improve the therapeutic treatments for Q fever patients in Japan.

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

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

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

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

Materials and Methods

Case history. Between December 2002 and January

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Results

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

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

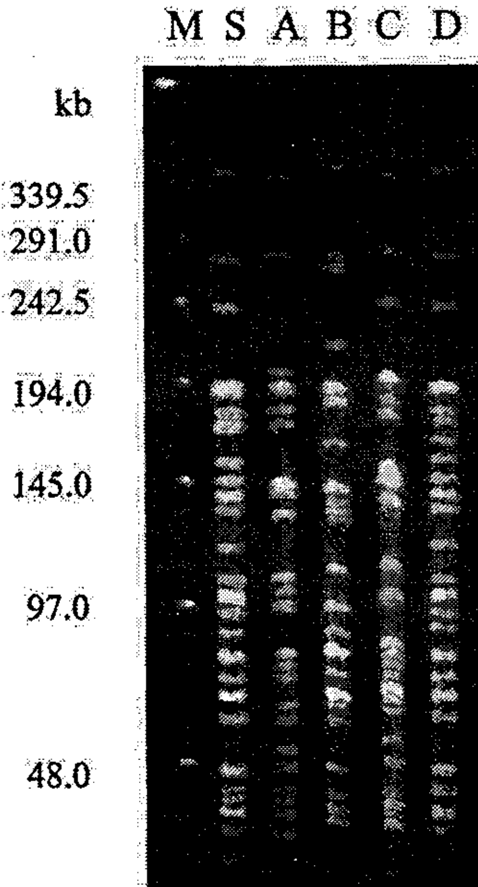

Fig. 1. Comparison of the PFGE patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes A to D). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); A, YE89023 (human patient); B, NY936005 (wild rodent); C, NY9504002 (wild rodent); D, YE9809001 (wild rodent).

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

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

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

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

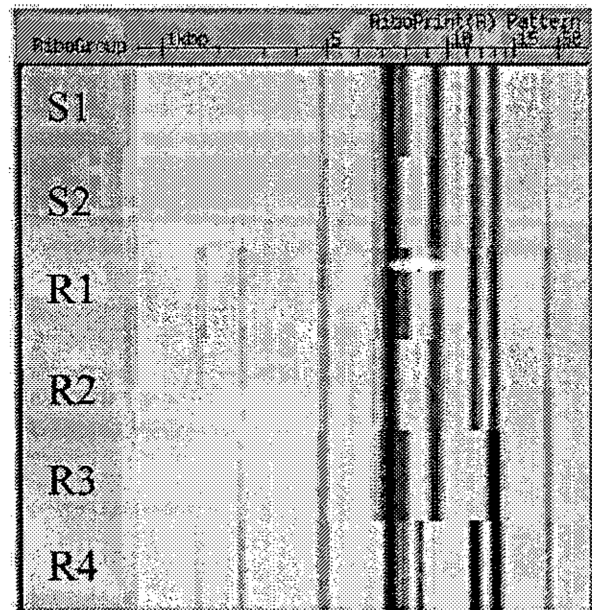

Fig. 2. Comparison of the ribotypes of representative outbreak isolates (lanes S1 and S2) and Japanese isolates (lanes R1 to R4). Lanes: S1, NY0212001 (squirrel monkey); S2, NY0304008 (black rat); R1, NY936005 (wild rodent); R2, YE9809001 (wild rodent); R3, YE87069 (human patient); R4, YE89023 (human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

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

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

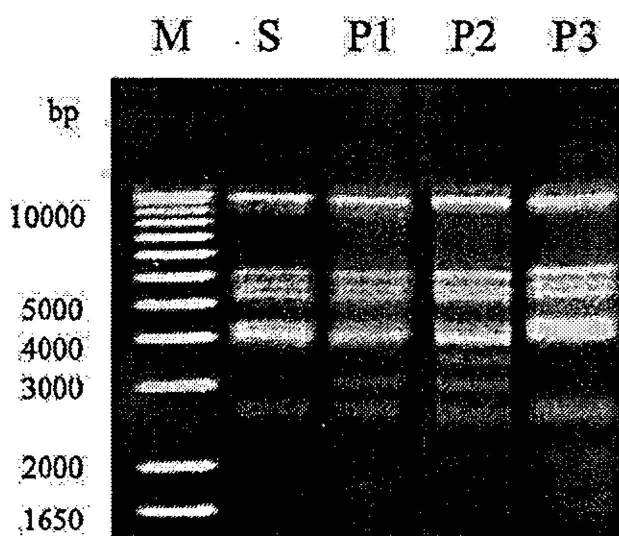

Fig. 3. Comparison of the REAP patterns of representative outbreak isolate (lane S) and Japanese isolates (lanes P1 to P3). Lanes: M, molecular weight marker; S, NY0212001 (squirrel monkey); P1, NY9306089 (wild rodent); P2, NY936005 (wild rodent); P3, YE9809001 (wild rodent).

Discussion

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

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

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

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

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

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

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

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

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

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