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

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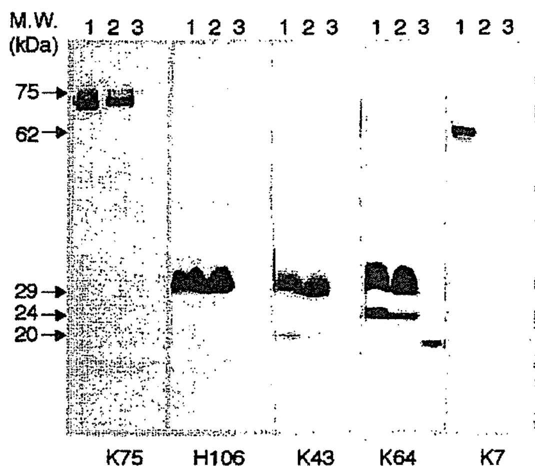

Fig. 1. Reactions of the MAb to whole cell lysate, OM and proteinase K-digested antigen of Nine Mile strain phase II cells in Western blots (lanes 1 to 3, respectively). Based on the reaction patterns, the MAbs were divided into 5 groups (IV to VIII). Reactions of the representative MAb of groups IV 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.

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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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Identification and Characterization of an Immunodominant 28-Kilodalton *Coxiella burnetii* Outer Membrane Protein Specific to Isolates Associated with Acute Disease

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Coxiella burnetii **causes acute Q fever in humans and occasional chronic infections that typically manifest as endocarditis or hepatitis. Isolates associated with acute disease were found to be distinct from a group of chronic disease isolates by a variety of biochemical parameters and in a guinea pig fever model of acute disease, suggesting a difference in virulence potential. We compared antigenic polypeptides among *C. burnetii* isolates and found an immunodominant 28-kDa protein in acute group isolates but not in chronic group isolates (T. Ho, A. Hotta, G. Q. Zhang, S. V. Nguyen, M. Ogawa, T. Yamaguchi, H. Fukushi, and K. Hirai, Microbiol. Immunol. 42:81-85, 1998). In order to clone the *adaA* gene, the N-terminal amino acid sequence of *adaA* was determined and a 59-bp fragment was amplified from Nine Mile phase I DNA by PCR. The putative gene fragment was used to screen a lambda ZAP II genomic DNA library, and an open reading frame expressing a 28-kDa immunoreactive protein was identified. Sequence analysis predicted a gene encoding an ~28-kDa mature protein with a typical signal sequence. The *adaA* (acute disease antigen A) gene was detected in acute group *C. burnetii* isolates but not identified in chronic group isolates by PCR and Southern blotting. A typical signal peptide was predicted in *adaA*, and specific antibody to *adaA* reacted with the purified membrane fraction of acute group isolates by Western blotting, suggesting that *adaA* is exposed on the outer surface of *C. burnetii*. *adaA* was overexpressed in pET23a as a fusion protein in *Escherichia coli* to develop anti-recombinant *adaA* (anti-*radaA*) specific antibody, which recognized a ~28-kDa band in acute group isolates but not in chronic group isolates. In addition, immunoblotting indicates that *radaA* reacted with sera derived from animals infected with acute group isolates but did not react with sera from animals infected with chronic group isolates. These results support the idea that an *adaA* gene-targeted PCR assay and an *radaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever.**

Coxiella burnetii is an obligate intracellular bacterium that causes acute and chronic forms of Q fever in humans. Acute Q fever is an influenza-like illness that usually is self-limiting and effectively treated by antibiotics (11). In contrast, chronic Q fever is a severe, sometimes fatal disease, and patients have responded poorly to various antibiotics (8, 20). Endocarditis is the most common chronic manifestation, while vascular infection, bone infection, and chronic hepatitis are also reported (21). Infection in most animals is mainly subclinical, but abortion and infertility are common manifestations in ruminants (2). Domestic animals, especially cattle, sheep, and goats, are important reservoirs of the agent responsible for infection of humans (7, 11).

C. burnetii has been isolated from various sources including milk, ticks, and humans with acute and chronic Q fever worldwide (2, 7, 8, 10). Previous studies have demonstrated that *C. burnetii* isolates originating from milk, ticks, and humans with acute Q fever differ in plasmid type (22), lipopolysaccharide profiles (3), and chromosomal DNA restriction endonuclease fragment patterns (5) from many isolates originating from chronic Q fever. The differences at the phenotypic and molecular levels between acute and chronic disease-associated isolates suggested that there may be a virulence potential characteristic of each group of isolates. Samuel et al. first reported that *C. burnetii* isolates associated with acute Q fever contained the QpH1 plasmid, while isolates associated with chronic Q fever possessed the QpRS plasmid or the plasmid sequences were integrated into the chromosome (22, 23). More recent studies of several *C. burnetii* isolates from Europe detected either the QpH1 plasmid-specific sequences (25, 26) or plasmid type QpDV (27) in both acute and chronic disease-associated isolates, suggesting that there was no specific gene(s) on plasmids responsible for a specific virulence phenotype. These data supported the notion that chronic disease could result from isolates associated with acute disease and might result from unique patient factors associated with immune status (25–27). However, no chronic disease-associated organisms have been isolated from acute Q fever patients. Therefore, it is quite possible that there are bacterial genetic factors responsible for acute disease. This hypothesis was supported in a

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TABLE 1. Original source, pathogenic characteristics, genetic group, and plasmid type of *C. burnetii* strains

Group[a]	Plasmid type[b]	Isolate[c]	Phase	Original source	Disease or type	Passage[d]
I	QpH1	Nine Mile RSA493	I	Montana, tick, 1935		307GP/1TC/1EP
		Turkey RSA333	II	Turkey, human blood, 1948	Acute	31EP
		African RSA334 (>[e])	I	Central Africa, human blood, 1949	Acute, Congolese red fever	3HP/4EP
		Giroud RSA431 (>[e])	I	Central Africa, human blood, 1949	Acute, Congolese red fever	2GP/2EP
		El Tayeb RSA342	I	Egypt, tick, 1967		4GP/2EP
		Panama RSA335	I	Panama, chiggers, 1961		4EP
		California 33 RSA329	I	California, cow's milk, 1947	Persistent	6EP
		Ohio 314 RSA270	I	Ohio, cow's milk, 1956	Persistent	4EP
II		Henzerling RSA331	II	Italy, human blood, 1945	Acute	36EP
IV	QpRS	Priscilla	I	Montana, goat cotyledon, 1980	Abortion	GP/2EP
		KQ154	I	Oregon, human heart valve, 1976	Endocarditis	HV/2EP
V	NP	GQ212	I	Nova Scotia, Canada, human heart valve, 1981	Endocarditis	HV/2EP
		SQ217	I	Montana, human liver biopsy sample, 1981	Hepatitis	BX/2EP
		KoQ229	I	Nova Scotia, Canada, human heart valve, 1982	Endocarditis	HV/2EP
VI	QpDG	Dugway 7E22-57	I	Utah, rodents, 1958		3EP
—[f]	QpDV	MAN	I	French, human blood	Aortic aneurysm	?
—[f]		ME	I	French, human heart valve	Endocarditis	?

[a] As defined by restriction enzyme banding patterns (5).
[b] Plasmids were described elsewhere (22, 27).
[c] Provided by Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont. Reference strains were determined by complement block titration (M. G. Peacock, Rocky Mountain Laboratories).
[d] Numbers indicate passage number; GP, guinea pig passage; TC, tissue culture; EP, egg passage; HP, hamster passage; HV, heart valve; BX, liver biopsy sample; ?, passage prior to receipt in authors' laboratory not known.
[e] >, passage history variants.
[f] —, MAN and ME were not classified (27).

study by Moos and Hackstadt (17) comparing virulence of a prototype isolate from each group in guinea pigs. The acute disease group prototype isolate (Nine Mile phase I RSA493) caused infection and fever when delivered intraperitoneally with less than 10 organisms, while the chronic disease group prototype isolate (Priscilla Q177) required at least 10^5 organisms to cause fever.

Our previous study identified a 28-kDa protein (P28) that was immunodominant in isolates originating from milk, ticks, and humans with acute Q fever but not immunogenic in isolates originating from chronic Q fever (6). This finding suggested that *adaA* could be associated with a pathogenic factor of acute Q fever. *adaA* may also have value as a marker to distinguish isolate groups. In order to clone and characterize the *adaA*-encoding gene, the N-terminal amino acid sequence of the protein was determined by protein sequencing. A 59-bp gene fragment was amplified from Nine Mile phase I DNA by PCR with one primer pair designed based on the N-terminal amino acid sequence and was used as a probe to screen a genomic library by Southern hybridization. The gene encoding P28 was cloned and sequenced. Outer membrane localization and antigenicity of *adaA* indicated that *adaA* may be a virulence factor related to acute Q fever, and the *adaA* gene may be a useful genetic marker for differentiation of isolates of *C. burnetii*.

MATERIALS AND METHODS

Bacterial strains, phage, and growth conditions. Seventeen *C. burnetii* isolates from various clinical and geographical sources were used in this study. The original source, pathogenic characteristics, and genetic properties of these strains are summarized in Table 1. All the isolates were propagated in BGM or L929 cell cultures and purified as described elsewhere (7, 22). The bacteriophage lambda ZAP II (Stratagene, La Jolla, Calif.) was used as the vector for construction of the *C. burnetii* expression genomic DNA library. *Escherichia coli* XL-Blue MRF' (Stratagene) was cultured in Luria broth (LB) with 12.5 μg of tetracycline/ml and used as the host strain for recombinant plasmids and bacteriophage lambda ZAP II.

Preparation of *C. burnetii* OMPs. The outer membrane proteins (OMPs) of *C. burnetii* were extracted from purified *C. burnetii* Nine Mile based on the method described by Ohashi et al. (19). Briefly, purified organisms were suspended in 10 mM sodium phosphate buffer, pH 7.4, containing 1% Sarkosyl (Sigma, St. Louis, Mo.) and 50 μg each of DNase I and RNase A and incubated at 37°C for 30 min. EDTA at a final concentration of 15 mM was added to stop the nuclease reaction. The insoluble precipitates were obtained by centrifugation at 10,000 × *g* for 1 h, washed twice with 0.1% Sarkosyl–phosphate-buffered saline, and then resuspended in STE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (Sigma).

Analysis of the N-terminal amino acid sequences of *adaA*. The OMPs of *C. burnetii* Nine Mile were separated by reversed discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride membrane as described elsewhere (19). The presence of *adaA* in the purified membrane fraction of *C. burnetii* Nine Mile was confirmed by immunoblotting as described previously (6). The portion of the polyvinylidene difluoride membrane containing *adaA* was excised and analyzed with the HP G1005A protein sequencing system (Takara Shuzo Co., Kyoto, Japan).

Preparation of DNA probe specific to the P28-encoding gene. The N-terminal amino acid sequence of *adaA* was determined as ENRPILNTINYQQQVEKWV TTDSADVMVSVN. Based on the N-terminal amino acid sequence, a pair of primers, P28a (5'-ATHAAYTAYCARCARCARGT-3') and P28b (5'-AGCAT NACRTCNGC-3'), were designed and used to amplify a 59-bp fragment of the putative *adaA* gene. The expected 59-bp product was amplified from *C. burnetii* Nine Mile DNA by PCR with these primers. The nucleotide sequence of the 59-bp fragment was determined by the dideoxy nucleotide chain-termination method as described previously (29). Sequence analysis of the 59-bp fragment indicates that the deduced amino acid sequence is identical to the chemically determined N-terminal amino acid sequence of P28, suggesting that the 59-bp fragment is specific DNA of the *adaA* gene. Based on the determined nucleotide sequence, specific primers P28a1 (5'-ATTAATTATCAACAGCAGGTTG-3') and P28b1 (5'-AGCATTACATCGGCAGAATCC-3') were designed and used to amplify the 59-bp specific fragment of the *adaA* gene from *C. burnetii* Nine Mile DNA. The amplified 59-bp fragment was labeled by the random primer

extension method with the digoxigenin DNA labeling kit (Roche Diagnostics K. K., Tokyo, Japan) and used as a DNA probe to screen the genomic DNA library of *C. burnetii* by Southern hybridization.

Construction and screening of genomic DNA library. A lambda ZAP II genomic DNA library was constructed as described by Macellaro et al. (9) and screened by Southern hybridization with the *adaA* gene-specific probe. Briefly, the genomic library was plated on *E. coli* XL-Blue MRF′ to yield about 500 plaques per plate. Plates were incubated at 37°C until plaques were 1 mm in diameter. Plaques were transferred onto a nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.) and were hybridized with the *adaA* gene-specific probe according to the protocol provided by the manufacturer (Roche Diagnostics K. K.). The positive plaques were detected by using the digoxigenin luminescent detection kit (Roche Diagnostics K. K.). In vivo excision of the pBluescript vector along with the inserted DNA of each positive clone was performed according to the protocol of the supplier of the lambda ZAP II cloning system.

Immunoblot analysis of *adaA* expression in *E. coli*. *E. coli* containing the recombinant plasmid was cultured in LB supplemented with 4 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C overnight, and then cells were pelleted by centrifugation. The cell pellet was analyzed by SDS-PAGE and immunoblotting with rabbit anti-Nine Mile serum as described previously (30).

DNA sequence analysis. Plasmid DNAs from positive clones that expressed immunoreactive protein were isolated and purified by using the FlexyPrep kit (Amersham Pharmacia Biotech). The nucleotide sequence was partially determined by the dideoxy nucleotide chain-termination method with the Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit and SEQ4x4 personal sequencer system (Amersham Pharmacia Biotech). A BLAST search against the complete genomic sequence of Nine Mile phase I (24) was achieved to identify the complete nucleotide sequence of the cloned gene. The nucleotide sequence and the deduced amino acid sequence were analyzed by the GENETYX analyzing system (Software Development Co., Ltd., Tokyo, Japan).

Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR. A pair of primers, P28F and P28R, was designed based on the *adaA* gene sequence and used to amplify a 269-bp fragment (ranging from positions 369 to 637 in the open reading frame [ORF] region of the *adaA* gene) from DNAs of 17 isolates from various clinical and geographical sources. The sequences of the primers are as follows: P28F, 5′-AATAGATTCGCTCTCTCAAGCCG-3′, and P28R, 5′-TCACCGCTGTTTTTTCAGACG-3′. PCR was performed with 2.5 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, Calif.) in 50 μl of reaction mixture containing 20 ng of genomic DNA, 0.2 μM (each) primer, and 200 μM (each) deoxynucleotide triphosphates in 10 mM Tris-HCl (pH 8.3)–50 mM KCl–2.5 mM $MgCl_2$. The reactant was subjected to 35 cycles of 30 s at 94°C, 30 s at 53°C, and 1 min at 72°C in a DNA thermal cycler (PTC-0200 DNA Engine; MJ Research, Inc., Waltham, Mass.).

Southern blotting. Restriction enzyme-digested DNAs from various clinical phenotypic isolates including two acute prototypic isolates (Nine Mile and Henzerling) and five chronic prototypic isolates (Priscilla, Q217, Q229, MAN, and ME) were tested by Southern blotting with the *adaA* gene-specific probe.

Expression and purification of the *adaA* fusion protein. The 602-bp DNA fragment of the *adaA* gene was amplified from *C. burnetii* Nine Mile DNA by PCR with primers P28EF-P28ER, which were designed from the *adaA* gene sequence and included 602 bp of the ORF region without the signal peptide-encoding sequence. Primer P28EF (5′-TTCGCTGCCACCGGATCCTTC-3′) is the 5′ end of the *adaA* gene with an additional BamHI restriction site, and primer P28ER (5′-ATCAACTCGAGGTTTCTTCG-3′) is complementary to the 3′ end of the gene with a XhoI restriction site in the sequence. The amplified *adaA* gene fragment was digested with BamHI and XhoI, ligated to expression vector pET23a, and then transformed into *E. coli* BL21(DE3)LysS competent cells. Expression of T7-tagged (N-terminal) and His-tagged (C-terminal) recombinant *adaA* (*radaA*) was induced by 4 mM IPTG. *radaA* was purified by using a ProBond resin column (Invitrogen) under denaturing conditions.

Antiserum preparation and immunoblot analysis of *adaA* among various strains of *C. burnetii*. The anti-*adaA* specific antibody was produced by immunization of BALB/c mice with purified *radaA*. Briefly, BALB/c mice (6 weeks old) were immunized with purified recombinant fusion protein in adjuvant (Titermax) three times at 14-day intervals. At each immunization, mice were subcutaneously injected with 50 μl of antigen (containing 20 μg of *radaA*) mixture with 50 μl of Titermax. After the third immunization, serum was collected and stored at −20°C.

The expression of *adaA* in various strains of *C. burnetii* was confirmed by immunoblotting with anti-*adaA* specific serum. SDS-PAGE and immunoblotting were performed as described elsewhere (31).

Reactivity of purified *radaA* with infection-derived sera. The reactivity of *radaA* with sera from guinea pigs infected with various strains of *C. burnetii* was analyzed by immunoblotting. Guinea pig serum was collected at 4 weeks postaerosol infection with 10^6 organisms of the Nine Mile phase I, Ohio, Q217, or Q229 strain and stored at −80°C until use. *C. burnetii* Nine Mile whole-cell lysate and purified rCom1, which is a protein common to all isolates tested (29, 30), were used as a control to confirm the presence of the antibodies to *C. burnetii* antigens in infection-derived sera. SDS-PAGE and immunoblotting were performed as described previously (31).

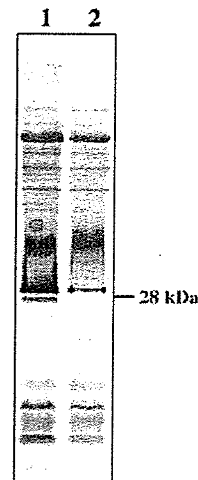

FIG. 1. Immunoblot analysis of purified OMPs of Nine Mile and Q217 strains with rabbit anti-Nine Mile hyperimmune serum. Lane 1, Nine Mile; lane 2, Q217.

RESULTS

Cloning the *adaA* gene. Immunoblotting identified an immunoreactive band at 28 kDa in the purified membrane fraction of *C. burnetii* Nine Mile but did not detect reactivity in the Q217 strain (Fig. 1). The result confirmed that the 28-kDa protein corresponds to the *adaA* previously noted (6). To identify the *adaA* gene, we determined the N-terminal 31 amino acids of a 28-kDa protein from *C. burnetii* Nine Mile. Based on the amino acid sequence, we designed several primer pairs and successfully amplified a 59-bp fragment from *C. burnetii* Nine Mile DNA by PCR. The 59-bp fragment was used as a DNA probe to screen a genomic library of *C. burnetii* Nine Mile DNA. Approximately 10,000 plaques were screened by Southern hybridization with the *adaA* gene-specific probe. Forty positive plaques were purified and compared for expression of immunoreactive proteins. Coomassie brilliant blue (CBB) staining on an SDS-polyacrylamide gel identified one clone, designated p110, expressed as an ~24-kDa protein. Immunoblotting indicates that the protein expressed by clone p110 reacted with rabbit anti-Nine Mile serum (data not shown), suggesting that the clone p110-expressed recombinant protein is specific for *C. burnetii*.

Sequence analysis. To determine the nucleotide sequence of the ORF encoding *adaA*, the purified recombinant plasmid from clone p110 was sequenced. The *adaA* gene-specific primers P28a1 and P28b1 were used in a sequence reaction to directly determine the nucleotide sequence of the *adaA* gene. The sequence of the p110 cloned insert was BLAST searched against the complete genome sequence of *C. burnetii* Nine Mile RSA493, allowing confirmation of the nucleotide sequence of

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1    cgctcttcagttacccaaagccgatcgtctttatccgcggatatttcctgttggtgggctaatagcttgc   70

71   ttatttcttgccttttgggtgccccctgatgatttggtcaattggcataggcctgattgctgttggacttg  140

141  cttggaaatattttatggctcgtttatggatatgagcaattttgaataggtggtgtatttatcagcaat   210
                                              -35

211  gttatgctaatgtaaggactgctgttctagcgcaatagaaaggaggaggtcacATGAAAAAACTAACCGT   280
     -10                                       RBS         M  K  K  L  T  V

281  AACTTTCTTAACTTTTATTAGTATTTTCTTCGCTGCCACCGCAGCCTTCGCTGAAAATCGCCCCATTTTA   350
       T  F  L  T  F  I  S  I  F  F  A  A  T  A  A  F  A  E  N  R  P  I  L
                 Signal peptide

351  AATACGATTAATTATCAACAGCAAGTTGAAAAATGGGTAACGACGGATTCTGCCGATGTGATGGTTTCCG   420
     N  T  I  N  Y  Q  Q  Q  V  E  K  W  V  T  T  D  S  A  D  V  M  V  S  V
          Chemically determined N-terminal 31 amino acids of the P28

421  TTAACGTTACAACAAAAGAAAAAAAGTTTGATGCACTGCAACACCAAGTAATGAAAAAATTGGAAGAATT   490
      N  V  T  T  K  E  K  K  F  D  A  L  Q  H  Q  V  M  K  K  L  E  E  L

491  AAGTGACGGACGTCAATGGCATATCGATTCTTTTTCAATGTCACAGGATCAGTCGGGATTGGAAGTTTTA   560
       S  D  G  R  Q  W  H  I  D  S  F  S  M  S  Q  D  Q  S  G  L  E  V  L

561  AGCTGGGAAGTCCGCTCTCGAATGCCTTTGGCCTTGGTGAACAGTCTGCGTCAAAAAATAGATTCGCTCT   630
     S  W  E  V  R  S  R  M  P  L  A  L  V  N  S  L  R  Q  K  I  D  S  L  S

631  CTCAAGCCGGACAACAATATAAAATTCAGAACGTTGATTTTGAACCTAGTTTAGTAGAAAAAGAAAAAGC   700
      Q  A  G  Q  Q  Y  K  I  Q  N  V  D  F  E  P  S  L  V  E  K  E  K  A

701  TTTCGCTGAGCTGCGCCAGCGAGTTTACGATCAAGTTAAAGTAGAGTTAGATAATTTGAATAAATCGTTT   770
      F  A  E  L  R  Q  R  V  Y  D  Q  V  K  V  E  L  D  N  L  N  K  S  F

771  CCGAATGGTCATTATTTTTTACATAGCATCGATTTTGTCTCTCCACCGCTCTACGCCGCTAACCAAAAAG   840
     P  N  G  H  Y  F  L  H  S  I  D  F  V  S  P  P  L  Y  A  A  N  Q  K  E

841  AATTAACGCTAATGCGATCTGCCCCGTCTGAAAAAACAGCGGTGACTTTGGGAAGAAACCTAATGTTGAT   910
       L  T  L  M  R  S  A  P  S  E  K  T  A  V  T  L  G  R  N  L  M  L  I

911  TGCGAATGTAAAAGTGGCGACTTTTTTAAATAAATAGgcaaataacgctagaaaagttcatactattttt   980
      A  N  V  K  V  A  T  F  L  N  K  *

981  ttacaaaacttttctaattttataaagaatagaaaaagggctttgtcattgaagcattcaatccatctg   1050

1051 atttaaaaactattttacattccaagcacgccaatatttattatttagaacattgtcgtatccttgttaa  1120

1121 tggcggtcgagtagagtatgtcactcaggaaggtagccagtcgttgtattggaatatccctattgcaaat  1190

1191 accacaacaatttattggggacgggaacttcggttactcaagcagccat  1240
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FIG. 2. Nucleotide sequence of the *adaA* gene. The deduced amino acid sequence is shown beneath the nucleotide sequence of the ORF. A putative promoter (positions −35 and −10) is underlined (thin line). The predicted ribosome-binding site (RBS) is underlined (thick line). The 20-amino-acid sequence at the N-terminal end of *adaA* is predicted to be a signal peptide. The chemically determined N-terminal 31-amino-acid sequence was identical to the N-terminal end of the deduced amino acid sequence of *adaA*.

the cloned gene (24). The nucleotide sequence (1,240 bp) from the cloned gene is shown in Fig. 2, which includes the flanking regions and the deduced amino acid sequence of P28. The *adaA* gene has a predicted ORF consisting of 684 bp, starting with an ATG codon at position 264 and ending with a TAG codon at position 947. The ORF is preceded by a putative ribosome-binding site, GGAGG, from 7 bp upstream of the ATG start codon. A predicted promoter sequence, TTGAA T-21 nt-TGTTAT, was found 36 bp upstream from the putative ribosome-binding site. The G+C content of the ORF coding region was 40%, which is similar to the value of *C. burnetii* total genomic G+C content (43%). The predicted mature P28 protein consists of 227 deduced amino acid residues and has a calculated molecular mass of 25,950 Da and a theoretical isoelectric point of 8.60. A 20-amino-acid signal peptide was also predicted for the N-terminal sequence of *adaA*. We also confirmed that the chemically determined N-terminal 31 amino acids of *adaA* were identical to the amino acid sequence of the mature protein deduced from the nucleotide sequence of the cloned *adaA* gene,.which is the region immediately adjacent to a predicted signal peptide (Fig. 2). In addition, a BLAST search of GenBank with the deduced amino acid sequence of the *adaA* gene indicated that the ORF encoding *adaA* was identical to CBU0952, which was predicted by The Institute for Genomic Research gene annotation (24). These results suggest that the ORF identified in the p110 cloned gene sequence is a gene unique to *C. burnetii* and encodes *adaA*.

Detection of the *adaA* gene in various isolates of *C. burnetii*. PCR and Southern blotting were used to test and confirm

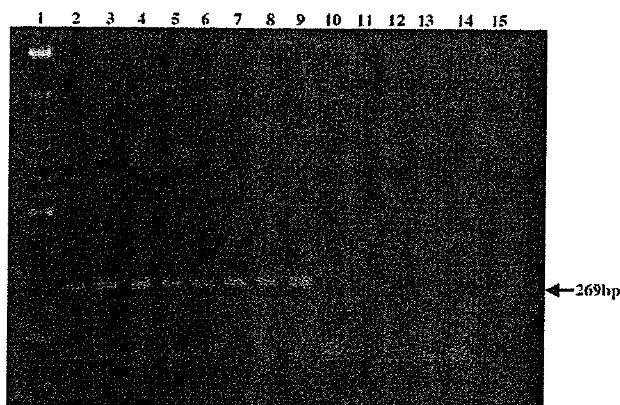

FIG. 3. Detection of the *adaA* gene from various isolates of *C. burnetii* by PCR with primers P28F-P28R. Shown is an ethidium bromide-stained agarose gel electrophoretogram of PCR-amplified products. Lane 1, molecular size markers (100-bp DNA ladder); lanes 2 to 9, isolates originating from ticks, milk, and humans with acute Q fever (Nine Mile, Ohio, California, El Tayeb, Africa, Panama, Turkey, and Giround, respectively); lanes 10 to 14, isolates originating from a goat and humans with chronic Q fever (Priscilla, KQ154, KoQ229, SQ217, and GQ212, respectively); lane 15, Dugway isolate.

whether the *adaA* gene is unique for a subgroup of isolates. The PCR result indicated that the *adaA* gene-specific fragment was amplified from isolates originating from humans with acute Q fever, ticks, cattle, and rodents, but PCR did not amplify any product from isolates from goats or humans with chronic Q fever (Fig. 3). Southern blotting also indicated that the *adaA* gene-specific probe hybridized with one band with SalI-digested DNAs of Nine Mile and Henzerling strains associated with acute Q fever but did not hybridize with any band with SalI-digested DNAs of Priscilla, Q217, Q229, MAN, and ME strains, which have been linked to chronic Q fever (data not shown). These results suggest that the *adaA* gene is specific for *C. burnetii* isolates originating from humans with acute Q fever, ticks, cattle, and rodents.

Expression of *adaA* in *E. coli* and various isolates of *C. burnetii*. The partial *adaA* protein of the Nine Mile strain was overexpressed as a fusion protein in pET23a. An IPTG-inducible fusion protein with a molecular mass of 28 kDa was detected in the *adaA* gene recombinant pET23a-transformed *E. coli* culture by CBB staining of the SDS-polyacrylamide gel and immunoblotting with a His-tagged specific monoclonal antibody (Fig. 4A and B, lanes 2 and 3). The expressed fusion protein was not detected in the negative control of pET23a-transformed *E. coli* culture (Fig. 4A and B, lanes 1). SDS-PAGE and immunoblotting also indicated that *radaA* was successfully purified from the *adaA* gene recombinant pET23a-transformed *E. coli* culture by using ProBond resin column (Fig. 4A and B, lanes 4). To confirm that P28 is expressed by acute disease isolates but not carried by chronic disease isolates, anti-*radaA* specific antibody was produced and used in immunoblotting with antigens of various strains of *C. burnetii*. Immunoblotting indicated that a ~28-kDa reaction band was detected from acute-disease-associated isolates Nine Mile and Henzerling but not observed in chronic-disease-associated isolates Priscilla and Q217 (Fig. 4C). This result confirmed that our cloned *adaA* gene encodes *adaA* and that *adaA* is expressed by acute-disease-associated isolates but not carried by chronic-disease-associated isolates.

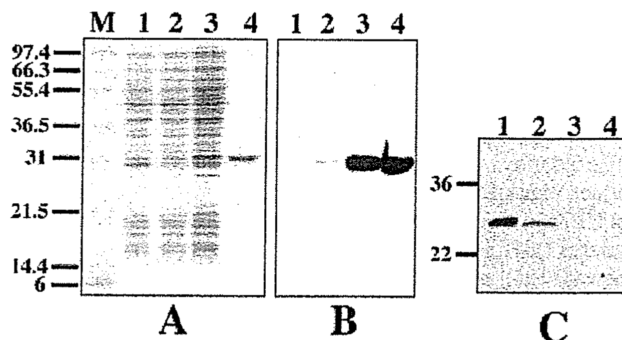

FIG. 4. Expression of the *adaA* gene in the pET23a expression vector system. Expression of the T7-tagged (N-terminal) and His-tagged (C-terminal) fusion protein was monitored by CBB staining of uninduced and induced cultures by SDS-PAGE and immunoblotting with His-tag-specific monoclonal antibody. (A) CBB staining profile of the expressed and purified fusion *adaA* protein. Lane M, molecular size markers; lane 1, pET23a-transformed *E. coli* (negative control); lanes 2 and 3, *adaA* gene recombinant pET23a-transformed *E. coli* uninduced and induced cultures, respectively; lane 4, purified fusion *radaA*. (B) Immunoblot analysis of expressed and purified recombinant proteins with His-tag-specific monoclonal antibody. The samples shown in panel B are the same as in panel A. (C) Immunoblot analysis of *adaA* in various strains of *C. burnetii* with *adaA*-specific antiserum. Lanes 1 to 4, whole-cell lysate of Nine Mile, Henzerling, Priscilla, and Q217 strains, respectively. A ~28-kDa reaction band was observed in acute disease isolates including the Nine Mile and Henzerling strains but not detected in Priscilla and Q217. Numbers at left are molecular masses in kilodaltons.

Reactivity of purified *radaA* with sera derived from infected animals. Figure 5 shows the immunoblots of the whole-cell antigen, rCom1, and *radaA* with sera derived from a guinea pig

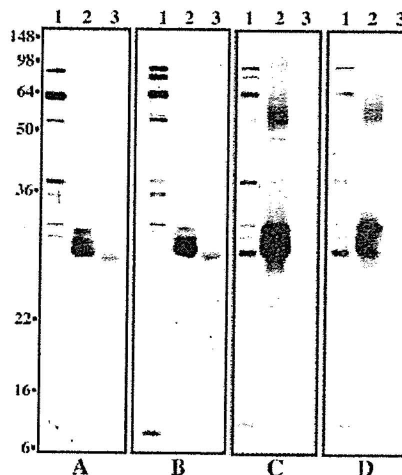

FIG. 5. Immunoblots of whole-cell antigen, rCom1, and *radaA* with sera derived from guinea pigs 4 weeks post-aerosol infection with 10^6 organisms of either the acute or the chronic prototypic isolate of *C. burnetii*. Sera (diluted 1:500) came from guinea pigs infected with Nine Mile (A), Ohio (B), Q217 (C), and Q229 (D). The samples shown in panels A to D are the same. Lanes 1, Nine Mile whole-cell antigen; lanes 2, purified rCom1 protein; lanes 3, purified recombinant *adaA* protein. Numbers at left are molecular masses in kilodaltons.

4 weeks post-aerosol infection with 10^6 organisms of the acute or chronic prototypic strain of *C. burnetii*. All sera from guinea pigs aerosol infected with 10^6 organisms of various strains strongly reacted with Nine Mile whole-cell antigen at a wide range of molecular weights and with rCom1 at similar levels (Fig. 5, lanes 1 and 2). However, r*adaA* reacted with sera from guinea pigs infected with isolates Nine Mile and Ohio (Fig. 5A and B, lanes 3) but not with sera from those infected with isolates Q217 and Q229 (Fig. 5C and D, lanes 3). These results indicate that anti-*adaA* specific antibody was present in sera derived from animals infected with the acute prototypic isolate but absent in sera from animals infected with the chronic prototypic isolate.

DISCUSSION

Cloning and characterization of *adaA* demonstrated that this protein is specific for acute-Q-fever-related isolates but deleted in chronic-disease-associated isolates despite geographical source, suggesting that *adaA* may be a virulence factor involved in the pathogenesis of acute Q fever in humans.

The predicted *adaA* mature protein consists of 227 amino acids and has a predicted molecular mass of 25,950 Da. This is very close to the molecular size of native *adaA* expressed in *C. burnetii* but about 2 kDa larger than the expression product of the *adaA* gene in *E. coli* (data not shown). The 25-amino-acid signal peptide is predicted in the N-terminal sequence of *adaA*, which is probably cleaved from the mature protein when the *adaA* gene is expressed in *E. coli*. The chemically determined N-terminal and internal peptide (data not show) amino acid sequences of *adaA* were identical to the deduced amino acid sequence of the cloned *adaA* gene, confirming that the identified ORF encodes *adaA*. The cloned *adaA* gene recombinant pUC19 expressed r*adaA* in *E. coli* DH5α cells without induction by IPTG (data not shown). A potential promoter sequence, TTGAAT-21 nt-TGTTAT, was identified in the *adaA* gene sequence, suggesting that the *adaA* gene was expressed in *E. coli* by using the endogenous promoter. A BLAST search of GenBank with either the nucleotide sequence or the deduced amino acid sequence for the *adaA* gene did not identify significant DNA or amino acid homologies, suggesting that *adaA* is unique to *C. burnetii*.

OMPs of gram-negative bacteria are employed in several important roles in the host-parasite interaction and relate to both pathogenesis and protective immunity. Due to the difficulties in cultivation and purification of *C. burnetii*, only a limited group of OMP-encoding genes have been characterized (4, 16, 28). Candidates for OMPs include the QpH1 plasmid-specific gene *cbhE'* for a 42-kDa surface protein (15) and the QpRS plasmid-specific gene *cbbE'* for a 55-kDa surface protein (12–14), which have been speculated to be virulence related and associated with acute or chronic Q fever in humans. However, recent investigations of several European isolates suggested that there were no specific genes on plasmids responsible for acute or chronic Q fever (25–27) and supported the notion that host factors may play a key role in the development of chronic Q fever. It remains unknown whether there are specific genes on the chromosome responsible for acute or chronic Q fever. Isolates from acute disease are distinct from chronic-disease-associated isolates at the molecular level (3, 5, 22) and in a guinea pig fever model of acute disease (17; K. Russell, unpublished data), suggesting different virulence potentials for groups of isolates of *C. burnetii*. In this study, we identified a novel ~28-kDa membrane-associated protein and demonstrated that *adaA* is expressed in acute group isolates but not carried by chronic group isolates, suggesting that *adaA* may be a virulence factor related to acute Q fever. Immunoblotting with purified r*adaA* antigen recognized anti-*adaA* specific antibody from sera derived from animals infected with acute group isolates but not from sera from animals infected with chronic group isolates, suggesting that *adaA* is an important antigen in acute disease. Since there has been no suitable animal model developed to represent the manifestation of chronic Q fever and because there is a lack of genetic tools for *C. burnetii*, it is not possible to directly test whether a specific gene is related to acute or chronic disease. Recently, SCID mice have been used as a model highly sensitive to lethal challenge by an acute-disease-associated isolate of *C. burnetii* (1), and preliminary comparison in this model shows dramatic differences in disease from isolates which do not carry *adaA* (M. Andoh, unpublished data). Further studies to test whether the *adaA* gene can be delivered on a stable plasmid to a *adaA*-negative isolate may allow its role in virulence to be determined.

Since prompt antibiotic therapy could lead to a better prognosis for individual patients with chronic Q fever, developing a diagnostic method for rapid differential diagnosis of acute and chronic Q fever could be very important for control of chronic disease. Recently, based on point mutations unique to isolate groups, *com1* and *icd* genes have been used as genetic markers to distinguish acute and chronic isolates (18, 29). However, comparison of nucleotide sequences of *com1* and *icd* genes among isolates indicates that they are highly conserved between acute and chronic isolates, except for these few point mutations (18, 29). The finding that the *adaA* protein and the *adaA* gene are unique to acute group isolates can be used for development of r*adaA* antigen-based serodiagnostic methods and/or an *adaA* gene-targeted PCR assay for differential diagnosis of acute and chronic Q fever in clinical samples. We have designed primers based on the nucleotide sequence of the *adaA* gene and used them to amplify products from DNA of various strains of *C. burnetii*. Amplicon products were amplified from DNA templates of isolates originating from humans with acute Q fever, ticks, cattle, and rodents but not from isolates originating from humans with chronic Q fever, suggesting that PCR for the *adaA* gene can be used for differentiation of acute- and chronic-disease-associated isolates. In addition, immunoblotting indicated that r*adaA* reacted with sera derived from animals infected with acute group isolates but was not recognized by sera derived from animals infected with chronic group isolates, suggesting that an r*adaA* antigen-based serodiagnostic test may be useful for differential diagnosis of acute and chronic Q fever in human sera. Further studies will evaluate the usefulness of an *adaA* gene-targeted PCR assay and an r*adaA* antigen-based enzyme-linked immunosorbent assay for differential diagnosis of acute and chronic Q fever in clinical samples from acute and chronic Q fever patients.

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Sensitive and Specific Detection of *Yersinia pseudotuberculosis* by Loop-Mediated Isothermal Amplification

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We developed a loop-mediated isothermal amplification method able to detect *Yersinia pseudotuberculosis* strains in 30 min by using six primers designed by targeting the *inv* gene. This method is more sensitive than PCR and might be a useful tool for detecting and identifying *Y. pseudotuberculosis*.

Yersinia pseudotuberculosis is known to be an important causal agent of zoonosis. *Y. pseudotuberculosis* infection in humans causes several diseases, such as enteritis, mesenteric lymphadenitis, reactive arthritis, erythema nodosum, and septicemia (1, 14, 15). This bacterium has been isolated from many animals, including monkeys, dogs, pigs, rodents, rabbits, deer, and birds, and is sometimes fatal to them (1, 3, 4).

Of several molecular genetic methods, PCR is the most widely used for specific amplification of a target gene, and it has also been reported to be able to detect pathogenic *Yersinia* species from foods and environmental samples (7, 16, 17, 19). Recently, a novel nucleic acid amplification method, named loop-mediated isothermal amplification (LAMP), that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions has been developed (2, 9, 12). This method simply consists of incubating a mixture of the target gene, four different primers, DNA polymerase with strand displacement activity, and substrates at a constant temperature between 60 and 65°C. The target gene is detected by the increase in the turbidity of the reaction mixture that coincides with the production of precipitate correlated with the amount of target DNA synthesized, i.e., the amplicons. The aim of this study was to develop a *Y. pseudotuberculosis* detection method, more sensitive and specific than PCR, based on the LAMP method, and to evaluate the performance of this method for detection of *Y. pseudotuberculosis* in clinical samples.

Thirty-one *Yersinia* species comprising 21 strains of *Y. pseudotuberculosis*, 4 strains of pathogenic *Y. enterocolitica*, and 6 strains of nonpathogenic *Yersinia* species strains, as well as 10 other gram-negative bacilli, were tested (Table 1). Template DNAs used for LAMP were prepared as follows. Bacterial cells of each strain from colonies on trypticase soy agar (TSA; BBL) were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA) to achieve a concentration of approximately 10^8 CFU/ml. In order to examine the detection limit for LAMP and PCR, a series of 10-fold dilutions of *Y. pseudotuberculosis* serovar 1b with TE buffer was made. The cells were heat treated in a boiling water bath for 10 min and were centrifuged for 10 min at 9,000 × *g*. The resulting supernatant was used as the template for LAMP and PCR. The LAMP reaction requires four oligonucleotide primers recognizing six distinct regions (F1, F2, F3, B1, B2, and B3) on the target DNA: the forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (12). FIP consists of a complementary sequence of F1 and a sense sequence of F2. BIP consists of a sense sequence of B1 and a complementary sequence of B2. LAMP primers targeting the *inv* gene of *Y. pseudotuberculosis*, the chromosomal virulence gene (8), were designed based on the gene sequence of *inv* (accession no. M17448) obtained from the DNA Data Bank of Japan by using the software program Primer Explorer V2 (Fujitsu, Tokyo, Japan). The sequences of the designed primers are shown in Table 2. Those four primers are sufficient to carry out the amplification reaction; however, the LAMP reaction can be accelerated by using additional primers termed loop primers (10), so loop primers LF and LB targeting the *inv* gene of *Y. pseudotuberculosis* were designed (Table 2). These loop primers were used in the reactions through which the amplification data were collected. However, the designed loop primers react with the restriction site of restriction enzyme BssHII (New England BioLabs, Beverly, Mass.), which was used to digest the obtained amplicons so as to confirm that the amplicons are of the target genes. Therefore, the loop primers were not used in the reactions involving BssHII. The LAMP reaction was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). A reaction mixture (25 μl) containing 1.6 μM each inner primer (FIP and BIP), 0.2 μM each outer primer (F3 and B3), 0.8 μM each loop primer (LF and LB), *Bst* DNA polymerase (0.5 μl), 2× reaction mix (12.5 μl), and template DNA (2 μl) was incubated at 63°C for 50 min and then heated at 80°C for 2 min to terminate the reaction. A DNA-omitted reaction mixture was used as a negative control. The amplification of the gene was confirmed by real-time monitoring of the increase of turbidity by using LA-200 (Teramecs, Kyoto, Japan), which sequentially measured the absorbance of the reaction mixture at 650 nm. To determine the detection

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TABLE 1. Bacterial strains subjected to LAMP and results

Species	Serotype	Strain	LAMP result[a]
Y. pseudotuberculosis	1a	3384	0.49
Y. pseudotuberculosis	1b	NYP95001	0.50
Y. pseudotuberculosis	1c	Kuratani	0.46
Y. pseudotuberculosis	2a	49	0.49
Y. pseudotuberculosis	2b	1799	0.49
Y. pseudotuberculosis	2c	274	0.49
Y. pseudotuberculosis	3	T-312	0.46
Y. pseudotuberculosis	4a	51	0.44
Y. pseudotuberculosis	4b	NYP01001	0.48
Y. pseudotuberculosis	5a	204	0.47
Y. pseudotuberculosis	5b	197	0.50
Y. pseudotuberculosis	6	#14	0.55
Y. pseudotuberculosis	7	141	0.50
Y. pseudotuberculosis	8	151	0.50
Y. pseudotuberculosis	9	R708Ly	0.49
Y. pseudotuberculosis	10	6088	0.48
Y. pseudotuberculosis	11	R80	0.43
Y. pseudotuberculosis	12	MW900-3	0.42
Y. pseudotuberculosis	13	N916	0.47
Y. pseudotuberculosis	14	CN7	0.45
Y. pseudotuberculosis	15	93422	0.47
Y. enterocolitica	O:3	8	0.00
Y. enterocolitica	O:5,27	S203	0.00
Y. enterocolitica	O:8	NY9306089	0.00
Y. enterocolitica	O:9	314-2	0.00
Y. enterocolitica	O:8,19	NY8904001	0.00
Y. aldovae		JCM 5892	0.00
Y. intermedia		JCM 7579	0.00
Y. cristensenii		JCM 7576	0.00
Y. bercovieri		NY8704001	0.00
Y. rohdei		JCM 7376	0.00
Campylobacter jejuni		ATCC33560	0.00
Campylobacter coli		JCM2529	0.00
Campylobacter lari		JCM2530	0.00
Citrobacter freundii		JCM1657	0.00
Enterobacter cloacae		JCM1232	0.00
Escherichia coli		JCM5431	0.00
Pasteurella haemolytica		NP8507001	0.00
Pseudomonas fluorescens		JCM 5963	0.00
Salmonella enterica serovar Typhimurium		NMJS1	0.00
Salmonella enterica serovar Enteritidis		NS9506003	0.00

[a] Turbidity after 30 min of incubation.

limit, 1 μl of the LAMP products was submitted to electrophoresis, and, to confirm the amplicon structure, the LAMP products were digested with restriction enzyme BssHII and submitted to electrophoresis. The electrophoresis was carried out in 2% Tris-acetic acid-EDTA (TAE) agarose gel, and staining was performed with ethidium bromide to confirm the presence of the expected DNA fragments. One kilobase of

TABLE 2. LAMP primers

Primer	Sequence (5′-3′)
F3	CTCGTCGCGTGATTTCTCC
B3	GATCTACCCCGACAGTGAGT
FIP	CCAGTTGTGGGAGTGCAGGTAACTATAAAG AGCGCCCAGCC
BIP	CACCGGTGAGCGTGTTGCTTTGTGTAATTGA TCCCGGCAGT
LF	CATTCGCGCGCAAATCC
LB	GCAACGCAACCCTTATGC

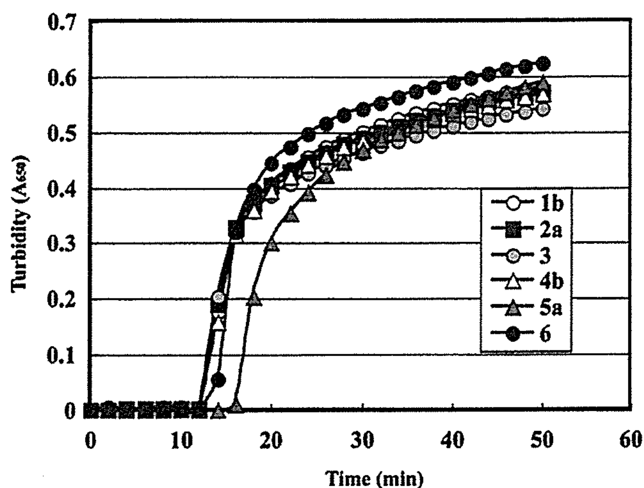

FIG. 1. Detection of the LAMP amplification signals. A total of 10^5 CFU of template DNA of *Y. pseudotuberculosis* 1b, 2a, 3, 4b, 5a, and 6 was used for the LAMP reaction.

Plus DNA ladder (Invitrogen) was used as a molecular weight standard. The PCR was carried out with the primers for the *inv* gene designed by Nakajima et al. (11). PCR was performed with a Program Temperature Control System PC-701 (ASTEC, Fukuoka, Japan) at 94°C for 1 min as an initial denaturation step and then was subjected to 30 cycles consisting of 30 s at 94°C, 1 min at 55°C, and 2 min at 70°C, followed by a single 5-min extension step at 70°C. The PCR mixture (50 μl) contained 4 μl of template DNA, 0.1 mM each of the four deoxynucleoside triphosphates, 5 μl of 10× PCR buffer (Applied Biosystems Japan Ltd., Tokyo, Japan), 0.1 μM each primer, and 0.5 U of *Taq* DNA polymerase (Promega, Madison, Wis.). Ten microliters of the PCR amplification products was subjected to electrophoresis under the same protocol of the LAMP products in a 1.5% agarose gel.

The specificity of LAMP using the newly designed primers was examined by carrying out reactions with DNAs from the *Yersinia* species and other gram-negative bacilli. The results of turbidity measurements for the LAMP reaction for 30 min at 63°C are shown in Table 1, and the representative curves are shown in Fig. 1. Turbidities derived from the LAMP reaction of *Y. pseudotuberculosis* strains began to increase after approximately 15 min of incubation, and they continued to increase as the LAMP progressed. All *Y. pseudotuberculosis* strains examined showed turbidities above 0.4 at 650 nm after 30 min of incubation. In contrast, turbidities were not observed even after 50 min of incubation when template DNA from *Y. enterocolitica*, a nonpathogenic *Yersinia* species, and other gram-negative bacilli were tested. This result proved the specificity of the developed primers. The differences among the turbidities of *Y. pseudotuberculosis* and all of the other samples became evident after 20 to 25 min of incubation. The use of loop primers shortened the reaction time for amplification by about one-half compared to that of amplification performed without loop primers (data not shown). These results showed that the LAMP method using these newly designed primers is able to detect *Y. pseudotuberculosis* specifically.

The sensitivity of LAMP and PCR for *Y. pseudotuberculosis* was determined by determining the detection limit as de-

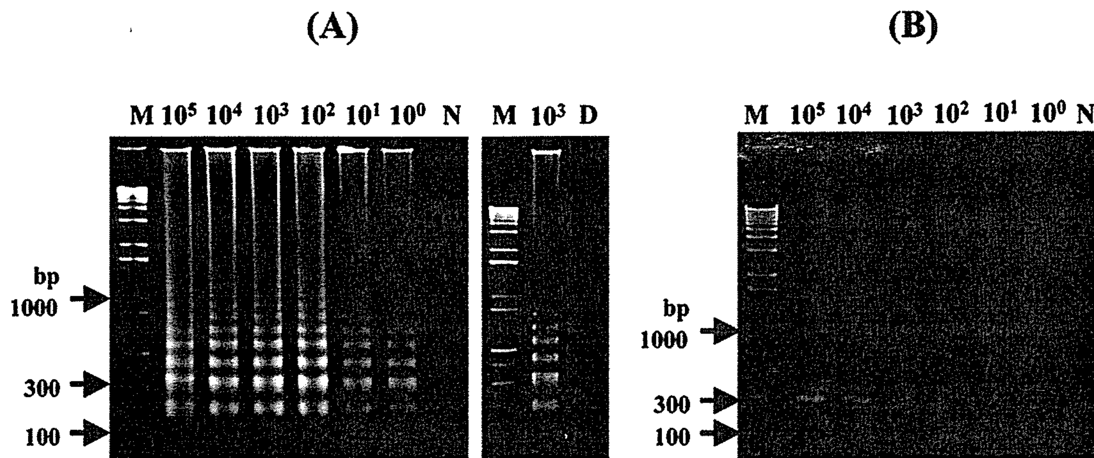

FIG. 2. Electrophoretic analysis of LAMP (A) and PCR (B) products. The numbers above each lane represent 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 CFU per reaction tube of template DNA of *Y. pseudotuberculosis* 1b. Lane D, LAMP product after digestion with BssHII; lane N, LAMP or PCR in the absence of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

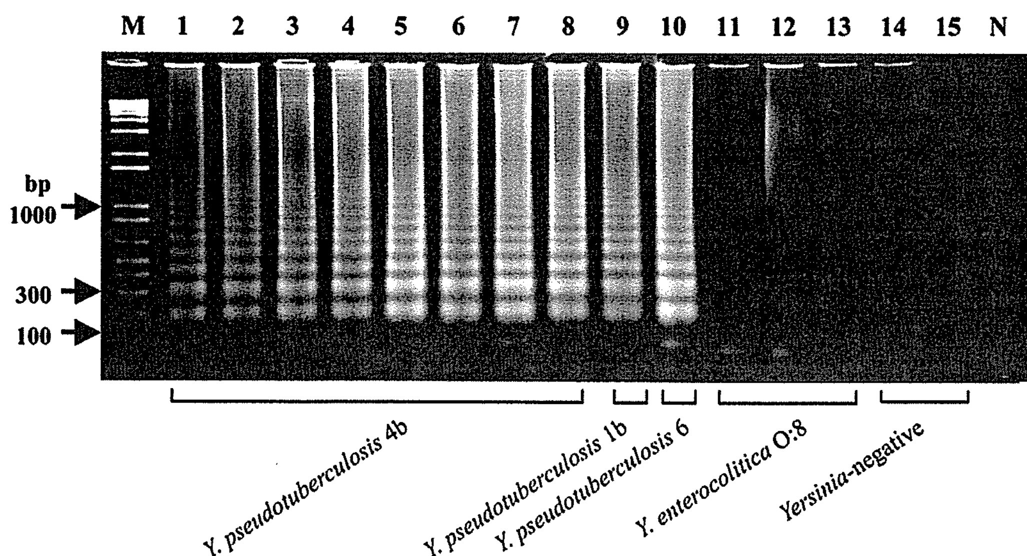

FIG. 3. LAMP detection of the *inv* gene in liver samples from *Y. pseudotuberculosis*-infected monkeys and uninfected monkeys. The samples of each lane and the number of bacteria isolated, in log CFU/gram, from each sample are the following: lanes 1 to 7, squirrel monkey, 5.1, 6.8, 6.4, 6.8, 5.1, 2.2, 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.