Identification of Rickettsiae Isolated in Japan as *Coxiella burnetii* by 16S rRNA Sequencing

TOSHIYUKI MASUZAWA,^{1*} KATSUJI SAWAKI,¹ HIROMI NAGAOKA,² MASATO AKIYAMA,²
KATSUYA HIRAI,³ AND YASUTAKE YANAGIHARA¹

Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422,¹ Department of Microbiology, Shizuoka Institute of Environment and Hygiene, Shizuoka 420,² and Department of Veterinary Microbiology, Faculty of Agriculture, Gifu University, Gifu 501-11,³ Japan

The 16S rRNA genes of Japanese *Coxiella* isolates obtained from various sources and geographical areas were directly sequenced by dideoxynucleotide chain termination methods in which *Taq* DNA polymerase was used. The levels of sequence similarity among Japanese, European, and American isolates were more than 99%, and the Japanese isolates were identified as *Coxiella burnetii*. *C. burnetii* strains isolated worldwide, including Japan, were found to be very similar.

Q fever is a zoonotic disease that is caused by the rickettsial organism *Coxiella burnetii* (1, 2) and has influenza-like symptoms, such as fever, chills, myalgia, headache, pneumonia, and bronchopneumonia. Although human Q fever was not recognized in Japan before the agent was isolated from patients, the disease is present in many countries (4). Oda and Yoshiie (6) isolated *C. burnetii* from an acute Q fever patient who returned from Canada in 1989, but could not determine whether this was an imported case or an indigenous case. Recently, To et al. (8) reported that *C. burnetii* had been isolated from raw milk and uterus swab samples originating from dairy cattle with reproductive disorders, aborted bovine fetus samples, mammary gland samples originating from healthy dairy cattle, and tick samples originating from pastures. Furthermore, Nagaoka et al. (5) isolated *Coxiella* strains from children that had Q fever and influenza-like symptoms. These isolates were identified based on electron microscopic observations, protein and lipopolysaccharide profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, PCR amplification data, and immunological reactivity with *C. burnetii*-specific antiserum (5, 8, 9), but were not analyzed genetically. The phylogenetic diversity of six species belonging to the family *Rickettsiaceae*, including *C. burnetii*, has been noted, and *C. burnetii* is a member of the γ subdivision and is specifically related to the genus *Legionella* (10). Stein et al. (7) showed that all of the *C. burnetii* strains which they studied are highly related (>99%) on the basis of their 16S rRNA sequences, although they had different geographic origins and phenotypic characteristics, and that the genus *Coxiella* contains only one species, *C. burnetii*.

In this study, we genetically analyzed Japanese isolates obtained from various sources by determining their 16S rRNA sequences and compared these isolates to other established strains. Table 1 shows the sources of the *C. burnetii* isolates used, which were obtained from various sources and different geographical areas (5, 8, 9). These isolates were propagated not only in the yolk sacs of embryonated chicken eggs but also in BGM cell cultures as described previously (8). Template DNA was extracted from a culture of each isolate infecting BGM cells by using an InstaGene DNA purification matrix (Bio-Rad, Hercules, Calif.) as recommended by the manufacturer. Approximately 1,450 bases of the 16S rRNA gene were amplified with forward primer QR-F0 (5'-ATTGAAGAGTT

TGATTCTGG-3') and reverse primer QR-R0 (5'-CGGCCTCCGAAGGTTAG-3'), which were designed by using the previously published 16S rRNA gene sequence of *C. burnetii* Q177 (GenBank accession no. M21291) (10). Each PCR was set up so that the mixture contained each deoxynucleotide triphosphate at a concentration of 200 mM, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 0.01% (wt/vol) gelatin, and 1 U of *Taq* polymerase (Takara, Otsu, Japan), as well as each primer at a concentration of 0.4 mM, in a final volume of 25 μ l. Aliquots (5 μ l) of DNAs extracted from *Coxiella* isolates were amplified with 35 cycles consisting of denaturation at 94°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 2 min by using a model 2400 GeneAmp PCR System apparatus (Perkin-Elmer Cetus, Norwalk, Conn.). An amplified DNA fragment of the expected size (1,450 bases) was purified from a 2% low-melting-temperature agarose gel (Sea-Kem GTG; FMC, Rockland, Maine) by using a PCR DNA purification column (Takara suprec-01). The purified DNA was kept at -20°C for sequencing.

The amplicons were directly sequenced by the dideoxynucleotide chain termination method (11) with a dye terminator-*Taq*-cycle sequencing kit by using a model 373A DNA sequencer (Applied Biosystems, Inc., Foster City, Calif.). The sequencing primers were chosen by using the sequence of human isolate 607; the sequencing primers used were primers QR-F0, SF319-338 (5'-TGAGACACGGCCAGACTCC-3'), SF588-607 (5'-TTTAAGTCGGATGTGAAAGC-3'), SF863-882 (5'-GTTAAGTTCTCCGCCTGGGG-3'), SF1147-1166 (5'-GGAGACTGCCGGTGATAAAC-3'), QR-R0, SR1189-1170 (5'-GACATCATCCCCACCTTCCT-3'), SR829-810 (5'-ACAGCTAGTTCTCATCGTTG-3'), SR564-545 (5'-CCCAGTGATTCCGATTAACG-3'), and SR257-238 (5'-ACCCACCAACAAGCTA

TABLE 1. *C. burnetii* isolates used

Isolate	Source	Origin
Bangui VR730	ATCC ^a	Human, blood
Ohio	ATCC	Cow, milk
GQ212	L. P. Mallavia	Human, heart valve
1M	Chiba, Japan	Cow, milk
27M	Shizuoka, Japan	Cow, milk
50F	Mie, Japan	Cow, aborted fetus
58T	Gifu, Japan	<i>Ixodes</i> spp.
607	Shizuoka, Japan	Human, blood

* Corresponding author. Phone: 81-54-264-5710. Fax: 81-54-264-5715. E-mail: masuzawa@ys7.u-shizuoka-ken.ac.jp.

^a ATCC, American Type Culture Collection.

TABLE 2. Levels of sequence similarity between 16S rRNAs

Isolate	% Sequence similarity								
	Q177	Bangui VR730	Ohio	GQ212	1M	27M	50F	58T	607
Q177	100	99.5	99.1	99.5	99.4	99.4	99.4	99.4	99.5
Bangui VR730		100	99.5	99.9	99.7	99.8	99.7	99.9	99.9
Ohio			100	99.5	99.4	99.5	99.4	99.5	99.5
GQ212				100	99.9	99.9	99.9	99.9	99.9
1M					100	99.7	99.8	99.9	99.8
27M						100	99.9	99.7	99.7
50F							100	99.8	99.8
58T								100	99.9
607									100

ATC-3'). (The numbers in the designations of the primers indicate the locations of the primers in the 16S rRNA sequence of isolate 607.) The sequences were aligned by using Genetic-Mac Automatic Connection of Sequences software (Software Development Co., Tokyo, Japan) and Genetic-Mac Genetic Information Processing software.

All of the isolates tested produced a 1,451-bp amplicon with primers QR-F0 and QR-R0. The 16S rRNA sequence of isolate 607, which was obtained from a patient who had influenza-like symptoms, was also determined by using primers QR-F0 and QR-R0. The sequencing primers used were designed on the basis of the sequence determined, and the entire sequence of the amplicon was determined by repeating the primer walking procedure. The ten sequencing primers were also used to sequence three *C. burnetii* strains (Bangui VR730, Ohio, and GQ212) from Europe and North America and five isolates (1M, 27M, 50F, 58T, and 607) from Japan. The sequences obtained in this study were aligned with the 16S rRNA sequence of *C. burnetii* Q177 (accession no. M21291). All of the strains, including the Japanese isolates obtained from various sources and geographical areas, exhibited high levels of similarity (more than 99%) (Table 2). The levels of sequence similarity among the test strains and Japanese isolates ranged from 99.1 to 99.9%. Interestingly, we did not find strains (including the five Japanese isolates) that had identical 16S rRNA sequences. The base substitutions observed are shown in Table 3. We found 17 base substitution points in the sequences of the strains used in this study. The previously published 16S rRNA sequence of strain Q177 differed at several nucleotide positions from the sequences of strains Bangui VR730, Ohio, GQ212, 1M, 27M, 50F, 58T, and 607 determined in this study. Only 12 nucleotide differences were found in the latter eight sequences, whereas 17 different nucleotides were found when the strain Q177 sequence was included. This result may imply that strain Q177 is unique.

This study revealed that the levels of sequence similarity among Japanese isolates and European and American isolates are more than 99%, and the Japanese isolates were identified as *C. burnetii*. *C. burnetii* strains isolated worldwide, including Japan, were found to be very similar, as previously reported (3, 7), and the genus *Coxiella* contains only one species, *C. burnetii*.

TABLE 3. Nucleotide differences in the 16S rRNA sequences of *C. burnetii* isolates

Isolate	Nucleotide at position:															
	74	87	97	98	177	197	216	309	549	592	638	697	779	843	859	1025
Q177	A	—	G	A	T	G	C	G	G	T	G	G	A	C	G	C
Bangui VR730	G	C	—	—	C	G	C	G	G	T	G	G	A	A	G	C
Ohio	G	C	—	—	C	A	T	G	G	T	C	C	G	A	A	—
GQ212	G	—	—	—	C	G	C	G	G	T	G	G	A	A	G	—
1M	G	—	—	—	C	G	C	G	T	C	G	G	A	A	G	—
27M	G	—	—	—	C	G	C	G	G	T	G	G	A	A	—	—
50F	G	—	—	—	C	G	C	A	G	T	G	G	A	A	—	—
58T	G	C	—	—	C	G	C	G	T	T	G	G	A	A	G	—
607	G	C	—	—	C	G	C	G	G	T	G	G	A	A	G	C

^a —, gap in the sequence.

Nucleotide sequence accession numbers. The accession numbers of the nucleotide sequences determined in this study are as follows: strain Ohio, D89791; strain 607, D89792; strain 58T, D89795; strain 50F, D89796; strain GQ212, D89797; strain Bangui VR730, D89798; strain 1M, D89799; and strain 27M, D89800.

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