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Retrospective Survey of Chronic Q Fever in Japan by Using PCR To Detect *Coxiella burnetii* DNA in Paraffin-Embedded Clinical Samples

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We used PCR to detect *Coxiella burnetii* DNA in paraffin-embedded tissues obtained from patients with chronic endocarditis in which the etiological agent had been unknown. On the basis of the published nucleotide sequence of the *C. burnetii htpB* gene, primers were chosen to produce an amplified fragment of 285 bp. A total of 60 samples from 56 patients were tested for the presence of *C. burnetii* DNA. Five samples from four patients were found to be positive. All of the amplified DNA fragments possessed a *Tth*HB8I restriction site, as predicted from the published sequence of *C. burnetii*. In one of the four positive patients, rickettsia-like particles were found in sections of tissue stained by Gimenez's method. This is the first report of chronic Q fever in Japan.

Coxiella burnetii, a unique rickettsial agent, causes a wide spectrum of human diseases. The most common is a self-limited febrile illness often accompanied by pneumonia (acute Q fever), and less common ones are endocarditis, osteomyelitis, and granulomatous hepatitis (chronic Q fever) (9, 14). Cases of acute Q fever are often misdiagnosed as influenza or the common cold, and many patients with various manifestations present with fever of unknown origin. Thus, Q fever should be part of the differential diagnosis of acute flu-like illness, pneumonia, endocarditis, and chronic bone and liver diseases. *C. burnetii* has been found to be widely distributed in nature, and Q fever is now recognized as being present in most countries of the world (1). However, the naturally occurring disease has not been reported in Japan, except for seven cases in laboratory workers who had been engaged in studies on *C. burnetii* (7, 18), so it has been thought that Q fever does not exist in Japan.

Recently, we isolated a *C. burnetii* strain from a patient with acute Q fever in Japan (11). It was unclear, however, whether the isolate, named TK-1, is an imported or indigenous strain. Then we made a serological study of *C. burnetii* infection in Japan and found that about 30% of dairy cattle and 2 of 10 veterinarians possessed the antibody against *C. burnetii* (21). After that, Htwe et al. (5, 6) reported seroepidemiological data suggesting that *C. burnetii* had spread widely among wild animals, domestic animals, and humans in Japan.

For further investigation of the actual status of infection with *C. burnetii* in humans in Japan, we made a retrospective study. In this report we describe the PCR assay (12) that we used to detect *C. burnetii* DNA in preserved paraffin-embedded tissue samples obtained from patients with culture-negative endocarditis. Through this investigation, we showed that human cases of chronic *C. burnetii* infection exist in Japan.

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MATERIALS AND METHODS

Organisms and growth conditions. *C. burnetii* TK-1, a clinical isolate from a patient with acute Q fever, was inoculated intraperitoneally into BALB/c mice treated with cyclophosphamide as described previously (11). A suspension of infected spleen cells harvested on the 10th day after inoculation was used to test the sensitivity and specificity of the PCR assay. The rest of the infected spleen tissues were fixed with 10% (vol/vol) formalin in saline and were embedded in paraffin wax by the standard histological technique. The sections of these samples were used as a positive control for the PCR assay. The Nine Mile strain of *C. burnetii* phase II, supplied by K. Hirai, Gifu University, was also used after it was cultured in yolk sacs of embryonated chicken eggs. The following microorganisms were used to test the specificity of the PCR assay: *Rickettsia tsutsugamushi* Gilliam, *Rickettsia sibirica* 246, *Rickettsia japonica* Katayama, *Escherichia coli* B, *Mycobacterium bovis* BCG Tokyo strain, and *Legionella pneumophila* Philadelphia I. All of the rickettsial strains were cultivated in L929 cells at 35°C for 6 days in a 5% CO_2 atmosphere. The other bacterial strains were cultivated on the appropriate medium. The rickettsia-infected L929 cells and the other bacterial cells were washed twice with phosphate-buffered saline (PBS) and were suspended in PBS.

Primers and expected product of PCR. The oligonucleotide primers designated CB-1 (5′-GACGCCGGTGACATTAAAAAC-3′) and CB-2 (5′-CTCAA CTTCCACTGAATC-3′) were synthesized on the basis of the published nucleotide sequence data for the *C. burnetii htpB* gene, which encodes a 62-kDa immunoreactive protein (19). The expected product of amplification of the target sequence with these primers was 285 bp in length. This 285-bp sequence contained a single *Tth*HB8I restriction site and was predicted to be digested to 209- and 76-bp fragments by the enzyme.

Specimen processing. Suspensions of *C. burnetii*-infected mouse spleen cells or chicken egg yolk sacs were centrifuged and were resuspended in 50 μl of lysis buffer consisting of 50 mM Tris hydrochloride, 1 mM EDTA, 0.45% Tween 20, 0.45% Nonidet P-40, and 100 μg of proteinase K per ml. The samples were incubated at 55°C for 60 min, and then the proteinase was inactivated at 95°C for 10 min and centrifuged at 12,000 × *g* for 3 min. The supernatants were used to test the sensitivity and specificity of the PCR assay. The suspensions of rickettsia-infected L929 cells and the other bacterial cells were also processed as described above.

Two sections (5 μm thick) from each paraffin-embedded sample were immersed in xylene to remove the paraffin wax and were washed with ethyl alcohol (15). After being dried, the samples were immersed in lysis buffer and were processed as described above, and the final supernatants were used for PCR assay.

Clinical samples. Paraffin-embedded tissues obtained from patients clinically and pathologically diagnosed as having chronic endocarditis of which the etiological agent had been unknown were subjected to PCR assay. The paraffin-embedded endocardial tissue and liver tissue samples had been prepared from 1988 through 1992. They had been obtained from 55 patients who were operated on for chronic heart disease and an autopsy at the Kagoshima University Hospital.

PCR amplification. Amplification was performed in an automatic thermal controller (PC-700; Astec Ltd., Fukuoka, Japan) with a GeneAmp reagents kit (Perkin-Elmer Cetus, Norwalk, Conn.). A total volume of 100 μl of reaction

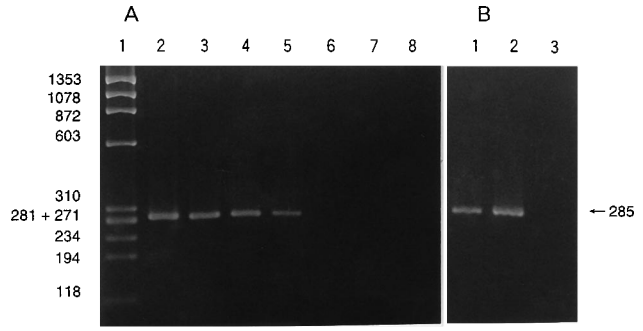

FIG. 1. Detection of *C. burnetii* DNA by PCR. (A) Serially diluted spleen cells of a BALB/c mouse infected with *C. burnetii* TK-1. Lane 1, molecular mass standards (ϕ X174 DNA digested with *Hae*III); lanes 2 to 6, serial 10-fold dilutions of *C. burnetii* TK-1-infected spleen cells; lane 7, spleen cells of uninfected BALB/c mouse (10^6 cells); lane 8, no DNA. (B) Lane 1, unfixed spleen tissue of a BALB/c mouse infected with *C. burnetii* TK-1; lane 2, formalin-fixed and paraffin-embedded spleen tissue of a BALB/c mouse infected with *C. burnetii* TK-1; lane 3, negative control (TE buffer). The numbers on the left and right are in base pairs.

mixture contained 10 mM Tris hydrochloride (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.2 mM (each) deoxynucleotide, 0.5 μM (each) oligonucleotide primer, 2.5 U of *Taq* polymerase, and 10 μl of sample DNA. Prior to the addition of sample DNA, the reaction mixture was overlaid with 50 μl of mineral oil to prevent evaporation. The amplification cycle consisted of an initial denaturation of target DNA at 94°C for 2 min; this was followed by denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72°C for 2 min. The final cycle included extension for 8 min at 72°C to ensure full extension of the product. Samples were amplified through 40 consecutive cycles. The PCR-amplified products were analyzed by agarose gel electrophoresis. A 10-μl sample of PCR-amplified products was electrophoresed on a 4% gel (NuSieve 3:1 agarose; FMC Corp., Rockland, Maine) in Tris-acetate-EDTA (TE) buffer. The gel was stained with ethidium bromide, and the bands were visualized under UV light.

Restriction endonuclease digestion. Samples (10 μl) of amplification products obtained by PCR were subjected to *Tth*HB8I restriction endonuclease (Takara Shuzo Co., Ltd., Kyoto, Japan) digestion for 1 h at 65°C in 20-μl volumes as recommended by the manufacturer. The digested samples were analyzed by agarose gel electrophoresis as described above.

RESULTS

Sensitivity of the PCR assay. Amplification of the DNA templates released from the mouse spleen cells infected with *C. burnetii* by using CB-1 and CB-2 primers produced the expected 285-bp fragment (Fig. 1A). The sensitivity of the PCR assay was investigated with serial dilutions of the *C. burnetii*-infected mouse spleen cells. The smallest detectable numbers of *C. burnetii* organisms, as determined by direct counting in Gimenez-stained samples under a microscope, ranged from 5 to 50.

Specificity of the PCR assay. Amplification carried out with DNA from the Nine Mile strain of *C. burnetii* also yielded the predicted 285-bp fragment (Fig. 2B, lane 2). Amplification performed with *L. pneumophila*, which is known to have a sequence highly homologous to the *htpB* gene of *C. burnetii* (13, 19), yielded nothing but about a 400-bp band (Fig. 2A, lane 5). Other bacteria with sequences homologous to the sequence of this gene, including *M. bovis* and *E. coli*, did not yield the 285-bp fragment (Fig. 2A, lanes 3 and 4). *R. tsutsugamushi*, *R. sibirica*, and *R. japonica* yielded no DNA band (Fig. 2B, lanes 3 to 5, respectively). To detect unknown bacteria having target sequences the same as those of our primers, amplification was performed with human feces containing numerous kinds of bacteria. No amplified fragment was observed in the feces sample examined (Fig. 2A, lane 6).

Furthermore, we searched the fragments with sequences

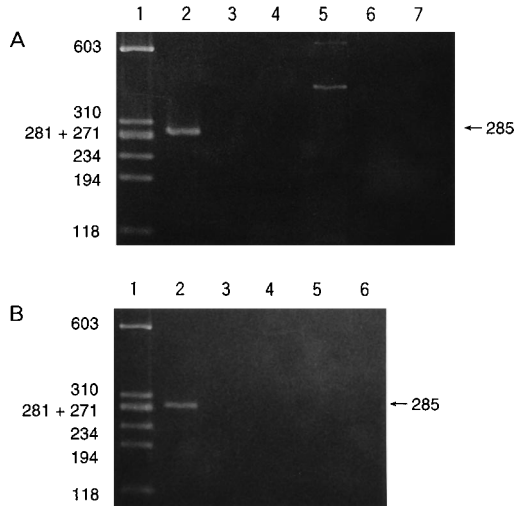

FIG. 2. Specificity of PCR for detection of *C. burnetii*. Amplified PCR products were analyzed by electrophoresis. (A) Lane 1, molecular mass standards; lane 2, *C. burnetii* TK-1; lane 3, *M. bovis* BCG Tokyo; lane 4, *E. coli* B; lane 5, *L. pneumophila* Philadelphia I; lane 6, human feces; lane 7, spleen cells of a BALB/c mouse. (B) Lane 1, molecular mass standards; lane 2, *C. burnetii* Nine Mile phase II; lane 3, *R. tsutsugamushi* Gilliam; lane 4, *R. sibirica* 246; lane 5, *R. japonica* Katayama; lane 6, negative control (TE buffer). The numbers on the left and right are in base pairs.

that were the same as those of our primers in a DNA sequence database including data from GenBank and the European Molecular Biology Laboratory (EMBL) with DNA sequence analysis software (DNASIS-Mac; Hitachi Software Engineering Co. Ltd., Yokohama, Japan). Sequences that matched completely the sequences of both primers in a single gene were found only in the heat shock operon of *C. burnetii*. Several sequences matched the sequence of one of our primers, with more than 85% homology. These sequences were found in seven species of viruses, bacteria, protozoa, and fungi (four species for CB-1 and three species for CB-2), but a single species of these microbes did not possess sequences that matched those of both primers.

Detection of *C. burnetii* DNA in paraffin-embedded clinical samples. Paraffin-embedded endocardial tissue and liver tissue samples taken from patients with culture-negative endocarditis were stored for 1 to 4 years. A total of 60 samples obtained from 56 patients were tested for the presence of *C. burnetii* DNA. Five samples from four patients yielded the expected

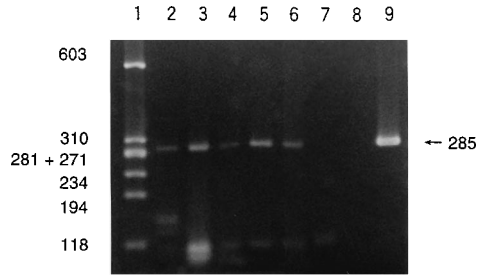

FIG. 3. Detection of *C. burnetii* DNA from paraffin-embedded tissue samples from four patients with endocarditis. Lane 1, molecular mass standards; lanes 2 to 5, paraffin-embedded tissues of endocarditis lesions from patients 1 to 4, respectively; lanes 6 and 7, paraffin-embedded liver tissue from patient 4; lane 8, negative control (TE buffer); lane 9, positive control (spleen cells of a BALB/c mouse infected with *C. burnetii* TK-1). The numbers on the left and right are in base pairs.

TABLE 1. Summary of the PCR-positive patients

Patient no.	Age (yr)	Sex	Clinical diagnosis
1	35	Female	Infectious endocarditis
2	56	Male	Infectious endocarditis
3	51	Male	Aortic insufficiency
4	40	Male	Hepatic cell carcinoma[a]

[a] Endocarditis was found by pathological examination at autopsy.

285-bp product (Fig. 3). The clinical diagnoses for the patients are given in Table 1.

The amplified products were confirmed by restriction endonuclease digestion to have originated from *C. burnetii*. Digestion of the 285-bp products with *Tth*HB8I yielded 209- and 76-bp fragments (Fig. 4).

Light microscopic observation. Light microscopy revealed rickettsia-like particles packed in the cytoplasms of the cells in sections of an endocarditis lesion obtained from one of the PCR-positive patients (Fig. 5).

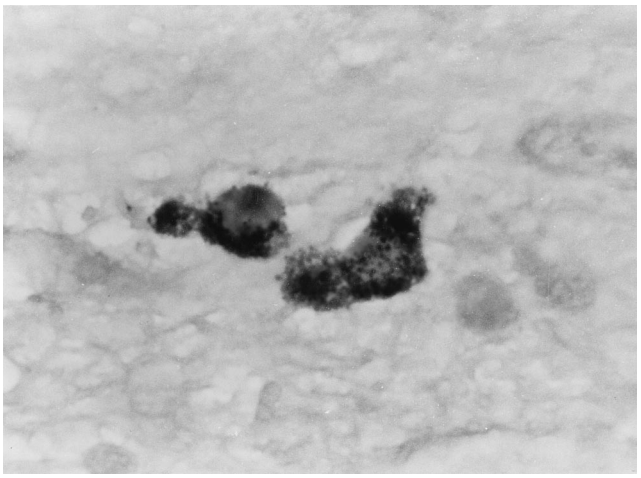

FIG. 5. Photomicrograph of an endocarditis lesion from patient 4. Two cells containing numerous rickettsia-like particles are seen. Gimenez staining was used. Magnification, ×1,800.

DISCUSSION

The PCR method is a rapid, sensitive, and highly specific diagnostic test for various microbial infections. It detects DNA sequences in a very small amount of sample or even in formalin-fixed, paraffin-embedded tissues (15). This method is useful for the detection of microorganisms which are difficult or hazardous to cultivate in the laboratory. From this viewpoint, PCR is the most suitable method for the laboratory diagnosis of *C. burnetii* infection since isolation of this organism in the standard laboratory setting is hazardous. Application of the PCR method for the detection of *C. burnetii* has been reported by some investigators (2, 3, 8, 16, 17, 20). We also developed a PCR method by using original primers and used it to detect *C. burnetii* in paraffin-embedded samples. Use of the PCR for DNA amplification requires knowledge of specific DNA sequences in the region of DNA to be amplified, which should be specific for the microorganisms to be detected. Five regions of DNA sequences have been reported for *C. burnetii*, viz., the *htpA* gene and the *htpB* gene (19), *Eco*RI fragment H of plasmid QpHI (3), the *cbeE′* gene of plasmid QpRS (10), and the superoxide dismutase gene (4). The *htpB* gene, which encodes a 62-kDa immunoreactive protein (19), is a well-conserved sequence and is thought to be common to various strains of *C. burnetii*. Therefore, the sequence is thought to be suitable as the target for detecting *C. burnetii* in clinical samples. However, this sequence is highly homologous to that of *Mycobacterium* species, *L. pneumophila*, and *E. coli* (13, 19). We constructed the primers in the *htpB* sequence, selecting the part unique to *C. burnetii*, and we obtained sensitivity and specificity adequate for detecting *C. burnetii* DNA in infected tissues with our primers.

Then we examined the state of chronic infection with *C. burnetii* in Japan by using the PCR system. We found four cases of endocarditis for which *C. burnetii* was thought to be the causative agent. Serological tests should be instituted to confirm the diagnosis, but sera were not available from these patients. This is the first report of chronic endocarditis caused by *C. burnetii* in Japan. Three of the four patients showed the typical course of infectious endocarditis; however, one patient (patient 4), whose disease had been diagnosed as hepatic cell carcinoma, showed no signs of endocarditis before autopsy. The endocarditis lesion was very small and was found by chance at autopsy. This proves the existence of inapparent chronic infection with *C. burnetii.*

A recent seroepidemiological study by Htwe et al. (6), together with our previous study (21), suggests that *C. burnetii* is widely distributed in Japan, and the prevalence of antibodies to *C. burnetii* is increasing. Our present study suggests that chronic infection with this organism also may be a more common disease than had previously been recognized in Japan. Therefore, serological investigations for *C. burnetii* should be performed for patients with a fever with an unknown cause or culture-negative endocarditis. In addition, electron microscopy can be useful in diagnosing these conditions when suitable material is obtained at the time of surgery.

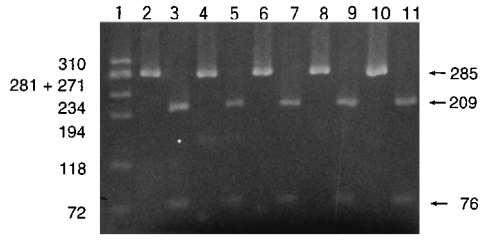

FIG. 4. Electrophoretic analysis of the PCR products obtained from four patients before and after digestion with *Tth*HBI. Lane 1, molecular mass standards; lanes 2 and 3, *C. burnetii* TK-1 before and after digestion with *Tth*HBI, respectively; lanes 4 to 11, clinical samples from patients 1 to 4 before and after digestion with *Tth*HBI in the following pairs of lanes, respectively: lanes 4 and 5, patient 1; lanes 6 and 7, patient 2; lanes 8 and 9, patient 3; lanes 10 and 11, patient 4. The numbers on the left and right are in base pairs.

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REFERENCES

1. **Baca, O. G., and D. Paretsky.** 1983. Q fever and *Coxiella burnetii*: a model for host-parasite interactions. Microbiol. Rev. **47:**127–149.
2. **Frazier, M. E., R. A. Heinzen, L. P. Mallavia, and O. G. Baca.** 1992. DNA probes for detecting *Coxiella burnetii* strains. Acta Virol. **35:**83–89.
3. **Frazier, M. E., L. P. Mallavia, J. E. Samuel, and O. G. Baca.** 1990. DNA probes for the identification of *Coxiella burnetii* strains. Ann. N. Y. Acad. Sci. **590:**445–458.

4. **Heinzen, R. A., M. E. Frazier, and L. P. Mallavia.** 1990. Nucleotide sequence of *Coxiella burnetii* superoxide dismutase. Nucleic Acids Res. **18:**6437.
5. **Htwe, K. K., K. Amano, Y. Sugiyama, K. Yagami, N. Minamoto, A. Hashimoto, T. Yamaguchi, H. Fukushi, and K. Hirai.** 1992. Seroepidemiology of *Coxiella burnetii* in domestic and companion animals in Japan. Vet. Res. **131:**490.
6. **Htwe, K. K., T. Yoshida, S. Hayashi, T. Miyake, K. Amano, C. Morita, T. Yamaguchi, H. Fukushi, and K. Hirai.** 1993. Prevalence of antibodies to *Coxiella burnetii* in Japan. J. Clin. Microbiol. **31:**722–723.
7. **Kitaoka, M.** 1954. Q fever. J. Public Health **15:**2–10. (In Japanese.)
8. **Mallavia, L. P., L. L. Whiting, M. F. Minnick, R. Heinzen, D. Reschke, M. Foreman, O. G. Baca, and M. E. Frazier.** 1990. Strategy for detection and differentiation of *Coxiella burnetii* strains using the polymerase chain reaction. Ann. N. Y. Acad. Sci. **590:**572–581.
9. **Marrie, T. J.** 1990. Acute Q fever, p. 125–160. *In* T. J. Marrie (ed.), Q fever, vol. I. The disease. CRC Press, Inc., Boca Raton, Fla.
10. **Minnick, M. F., R. A. Heinzen, M. E. Frazier, and L. P. Mallavia.** 1990. Characterization and expression of the *cbb*E′ gene of *Coxiella burnetii*. J. Gen. Microbiol. **136:**1099–1107.
11. **Oda, H., and K. Yoshiie.** 1989. Isolation of a *Coxiella burnetii* strain that has low virulence for mice from a patient with acute Q fever. Microbiol. Immunol. **33:**969–973.
12. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
13. **Sampson, J. S., S. P. O'Connor, B. P. Holloway, B. B. Plikaytis, G. M. Carlone, and L. W. Mayer.** 1990. Nucleotide sequence of *htp*B, the *Legionella pneumophila* gene encoding the 58-kilodalton (kDa) common antigen, formerly designated the 60-kDa common antigen. Infect. Immun. **58:**3154–3157.
14. **Sawyer, L. A., D. B. Fishbein, and J. E. McDade.** 1987. Q fever: current concepts. Rev. Infect. Dis. **9:**935–946.
15. **Shibata, D., N. Arnheim, and W. J. Martin.** 1988. Detection of human papilloma virus in paraffin-embedded tissue using the polymerase chain reaction. J. Exp. Med. **167:**225–230.
16. **Stein, A., and D. Raoult.** 1992. Detection of *Coxiella burnetii* by DNA amplification using polymerase chain reaction. J. Clin. Microbiol. **30:**2462–2466.
17. **Stein, A., and D. Raoult.** 1992. A simple method for amplification of DNA from paraffin-embedded tissues. Nucleic Acids Res. **20:**5237–5238.
18. **Tasaka, S., and K. Tameda.** 1952. A case of Q fever. J. Jpn. Soc. Intern. Med. **41:**74–81. (In Japanese.)
19. **Vodkin, M. H., and J. C. Williams.** 1988. A heat shock operon in *Coxiella burnetii* produces a major antigen homologous to a protein in both mycobacteria and *Escherichia coli*. J. Bacteriol. **170:**1227–1234.
20. **Williams, H., D. Thiele, and H. Krauss.** 1993. Plasmid based differentiation and detection of *Coxiella burnetii* in clinical samples. Eur. J. Epidemiol. **9:**411–418.
21. **Yoshiie, K., H. Oda, N. Nagano, and S. Matayoshi.** 1991. Serological evidence that the Q fever agent (*Coxiella burnetii*) has spread widely among dairy cattle of Japan. Microbiol. Immunol. **35:**577–581.