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Q Fever Pneumonia in Children in Japan

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The prevalence of Q fever pneumonia among children with atypical pneumonia from whom only an acute-phase serum sample was available was traced by using an indirect immunofluorescence (IF) test, nested PCR, and isolation. Twenty (34.5%) of 58 sera were found to have both polyvalent and immunoglobulin M antibodies to the phase II antigen of *Coxiella burnetii* by the IF test. Q fever pneumonia was present in 23 (39.7%) of 58 patients as determined by both the nested PCR and isolation and in 20 patients as determined by the IF test. The sensitivities for nested PCR and isolation were 100%, and that for the IF test was 87%. Our results indicate that nested PCR was faster and more sensitive than isolation and the IF test in the diagnosis of acute Q fever when a single acute-phase serum was available. These findings suggest that *C. burnetii* is an important cause of atypical pneumonia in children in Japan.

Pneumonia is an important clinical manifestation of acute Q fever (15). It is an uncommon manifestation in Australia and some parts of Russia, whereas in North America and Europe it is the major manifestation of this illness (1). Rapid diagnosis of the illness is very important, since prompt antibiotic treatment may lead to a better prognosis for individuals suffering from Q fever.

Serological methods, including indirect immunofluorescence (IF) tests and enzyme-linked immunosorbent assays, have been used for confirming a clinical diagnosis of the illness, since isolation is time-consuming, hazardous, and expensive. In patients with acute Q fever, immunoglobulin M (IgM) can be detected within the first 2 weeks after the onset of symptoms (3, 6, 9, 18). The estimation of Q fever-specific IgM by the IF test has been proven to be useful in confirming the infection when a single serum sample is taken within the second week after the onset of symptoms (9).

Recently, PCR has been used for the detection and differentiation of *Coxiella burnetii* strains (13); this seems to be the most promising direct technique because of its sensitivity and specificity.

Kitaoka reported serological evidence of *C. burnetii* infection in animals and humans in Japan in 1954 (11). Recently, Yoshiie et al. (27) showed that *C. burnetii* infection is widespread among dairy cattle. In previous studies (4, 7, 8, 23), we found a high incidence of *C. burnetii* antibodies in wild and domestic animals, patients with respiratory disorders, and healthy humans living in close contact with animals, e.g., veterinarians and meat-processing workers. Most recently, Morita et al. (16) reported the prevalence of antibody to phase II antigen of *C. burnetii* in domestic cats.

A previous publication (8) from our laboratory revealed that 9 (4.9%) and 28 (15.2%) of 184 sera of patients with respiratory disorders were positive for phase I and phase II antigens, respectively, which encouraged us to further investigate Q fever pneumonia among patients with atypical pneumonia in Japan.

This report describes the prevalence of Q fever pneumonia among children with atypical pneumonia admitted to a hospital in Gifu prefecture in Japan and evaluates the sensitivity of the IF test, nested PCR, and isolation in the diagnosis of acute Q fever from single acute-serum samples from children with atypical pneumonia.

MATERIALS AND METHODS

Sera. Fifty-eight acute serum samples collected from 1982 to 1983 from children with atypical pneumonia in Gifu prefecture were entered into the study. The 58 children were aged 2 to 10 years, with a median age of 7 years. In addition, 50 acute sera from patients suffering from acute pneumonia of viral or bacterial origin (influenza virus, parainfluenza virus, respiratory syncytial virus, *Chlamydia psittaci*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*) served as negative controls for PCR.

IF test. The IF test was performed as described previously (8) by using phase II *C. burnetii* Nine Mile strain as the antigen. Phase II *C. burnetii* Nine Mile (ATCC 615) was passaged three times in chicken embryos, purified (23), inactivated with 1% Formalin, and dialyzed against distilled water. All sera were tested at dilutions ranging from 1:16 to 1:512. Fluorescein isothiocyanate-labeled goat anti-human IgG (heavy plus light chains) (Organon Teknika N-V. Cappel Products) was used as the conjugate. Positive sera were further examined with monovalent fluorescein isothiocyanate-labeled goat anti-human IgG and IgM (Tago, Inc., Burlingame, Calif.) and IgA (Cappel Laboratories, Cochranville, Pa.) conjugates. Positive and negative controls were run with each test. Antibody titers of ≥1:32 were considered to be positive. A patient was diagnosed as having acute Q fever if his or her serum sample contained the polyvalent and IgM and/or IgG antibodies at the positive titer.

Nested PCR. (i) Microorganisms. To confirm the specificity of the PCR, 22 *C. burnetii* isolates were tested with primers for the PCR (Table 1). In addition, the following agents, which are known to cause pneumonia or found commonly in a microbiological laboratory, were also examined in this study: adenovirus (human type 3), *Bordetella bronchiseptica* GIFU 1127, *Chlamydia pneumoniae* TW183, *C. psittaci* GCP-1, *Chlamydia trachomatis* E, *Escherichia coli* C600, *Haemophilus influenzae* GIFU 3191, *Klebsiella pneumoniae* GIFU 2926, *L. pneumophila* SL94-1, *L. pneumophila* SL94-2, *M. pneumoniae*, *Rickettsia tsutsugamushi* Karp, *R. tsutsugamushi* Kato, *R. tsutsugamushi* Gilliam, and *Streptococcus pneumoniae* GIFU 8766.

(ii) Titration of inoculum. The procedures used for the titration were similar to those described previously (20), with the following minor modifications: (i) a 10% yolk sac suspension of the Nine Mile phase I strain was used for titration, and (ii) the buffalo green monkey (BGM) cell monolayers growing on wells of the 24-well plate were inoculated with 0.1-ml samples of the various dilutions of the suspension and centrifuged at 400 × *g* for 45 min at 37°C.

(iii) Analysis by PCR. To avoid false positives with PCR, samples for PCR were prepared according to guidelines described previously (12). All samples

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TABLE 1. *C. burnetii* isolates tested

Isolate	Source	Origin
Nine Mile VR 615	ATCC[a]	*Dermacentor andersoni*
Henzerling VR 145	ATCC	Human blood
Dyer VR 147	ATCC	Human blood
Bangui VR 730	ATCC	Human blood
California 76 VR 614	ATCC	Cow milk
Ohio 314 VR 542	ATCC	Cow milk
G Q 212	L. P. Mallavia	Human heart valve
Ko Q 229	L. P. Mallavia	Human heart valve
S Q 217	L. P. Mallavia	Liver biopsy
El Tayeb	L. P. Mallavia	Tick
1M[b]	Chiba, Japan	Cow milk
3M	Chiba	Cow milk
27M	Shizuoka, Japan	Cow milk
60M	Chiba	Cow milk
82M	Chiba	Cow milk
53U	Gifu, Japan	Cow udder
50F	Mie, Japan	Cow, aborted fetus
57T	Gifu	*Ixodes* spp.
58T	Gifu	*Ixodes* spp.
307	Shizuoka	Human blood
605	Shizuoka	Human blood
TK-1	Kagoshima, Japan[c]	Human blood

[a] ATCC, American Type Culture Collection.
[b] Some characteristics of 12 Japanese isolates were described elsewhere (23).
[c] Kindly provided by H. Oda.

were washed with phosphate-buffered saline (pH 7.5) three times and then prepared as follows. Sediments of serum samples were resuspended in 50 μl of 1% Nonidet P-40 and boiled for 5 min; sediments of mouse spleen samples were resuspended in 1 ml of lysis buffer (consisting of 100 mM Tris-HCl [pH 8], 10 mM EDTA, 1% sodium dodecyl sulfate, and 200 μg of proteinase K per ml) and incubated for 60 min at 55°C. DNA was then obtained by phenol-chloroform extraction and ethanol precipitation. The DNA pellet was washed with 70% ethanol, and resuspended in 50 μl of Tris-EDTA.

Oligonucleotide primers were obtained from a commercial source (Rikaken Co., Ltd., Nagoya, Japan). According to the published nucleotide sequence of the *C. burnetii htpB* gene (1,658 bp) of a 62-kDa antigenic polypeptide (24), two pairs of oligonucleotide primers, Q5 (5′-GCG GGT GAT GGT ACC ACA ACA-3′)-Q3 (5′-GGC AAT CAC CAA TAA GGG CCG-3′) and Q6 (5′-TT GCT GGA ATG AAC CCC A-3′)-Q4 (5′-TC AAG CTC CGC ACT CAT G-3′), were used to amplify 501- and 325-bp fragments, respectively.

The first amplification for nested PCR was performed in a total volume of 50 μl containing 5 μl of DNA sample, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM each dATP, dCTP, dGTP, and dTTP, 0.5 μM each primers Q5 and Q3, and 2 U of *Taq* DNA polymerase (Takara Shuzo, Co., Ltd., Shiga, Japan). The second amplification reaction mixture was processed as described above except for the DNA sample and the primers (Q4 and Q6). The mixtures were overlaid with 2 drops of mineral oil and subjected to 36 cycles of amplification in a DNA thermal cycler (Perkin-Elmer GeneAmp PCR System 9600; Takara Biomedicals, Kyoto, Japan). For the first PCR, samples were started with a 3-min denaturation step at 94°C. Each amplification cycle consisted of denaturation at 94°C for 1 min, primer annealing to the template at 56°C for 1 min, and primer extension at 72°C for 1 min. The extension time was 4 min for the final cycle. For the second PCR, amplification procedures were performed as described above except that temperature of the primer annealing step was 52°C.

Samples (10 μl) obtained after PCR or after further treatment with a restriction enzyme, *Eco*RI (Takara Shuzo, Co., Ltd.), were separated by electrophoresis (1.5% agarose gel), stained with ethidium bromide (0.5 μg/ml), visualized under UV illumination (TM-20; UVP, Inc.) at 302 nm, and photographed. The DNA samples were also dot blotted and identified by the hybridization signal obtained with a digoxigenin–11-dUTP-labeled probe (DIG DNA labeling kit; Boehringer, Mannheim, Germany).

Isolation. Specific-pathogen-free male A/J mice were 6 to 8 weeks old on entry into the studies. Preinoculation blood samples of mice were tested for the presence of *C. burnetii* antibodies and *C. burnetii* by the IF test and PCR, respectively. The mice were maintained in separate isolation units in an SR-2200 class II safety rack (Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). Proper safety precautions were taken during all procedures which call for the handling of the live bacterium. The procedure used for isolation of *C. burnetii* was similar to those described previously (23, 25), with minor modifications as follows: (i) each serum sample (1 ml) was injected intraperitoneally into two mice not treated with cyclophosphamide, and (ii) sera and spleens of mice of the second passage were collected on the 14th day after inoculation and tested for the presence of antibody to phase II antigen, antigen, and *C. burnetii* by the IF test, Gimenéz staining, and PCR. In addition, the average amount of *C. burnetii* present in the mouse spleen was evaluated as described previously (23). Mice which had an antibody titer of ≥1:32, had microorganisms and IF antigens, and/or were positive by the nested PCR were considered to have evidence of the infection.

RESULTS

The prevalence of IF antibodies to phase II antigen in 58 serum samples is listed in Table 2. Overall, the polyvalent antibody to phase II antigen was present in 20 (34.5%) of 58 sera. Of these, 20 sera were found to have IgM antibody, 7 sera had IgG antibody, and none of the sera had IgA. Twenty patients were diagnosed with Q fever pneumonia.

The specificity of the nested PCR with two pairs of primers, Q5-Q3 and Q6-Q4, was tested with 22 *C. burnetii* isolates, 15 other organisms, and uninfected control BGM cells and was further proven by subjecting 50 sera from patients without Q fever to the PCR. The paired primers amplified the predicted product (Fig. 1). No product was generated by using the sera, BGM cells, and other organisms as the template. Furthermore, the specificity of the primers for *C. burnetii* was also tested by digesting the amplified products with the restriction enzyme *Eco*RI and by using the DNA dot blotting test. The results of dot blotting and hybridization tests showed that the amplified products are specific for *C. burnetii*. A titration test showed that our PCR assay could detect as few as two infecting units; however, it is not known whether dead microorganisms were present in the suspension tested. Twenty-three of 58 patients were positive by nested PCR (Table 2). Among PCR-positive samples, two were positive with a 100-μl volume of serum but negative with a 50-μl volume of serum used for sample preparation.

The IF antibodies were found in 23 (39.7%) of 58 pairs of mouse sera, while the IF antigens, the microorganisms, and DNA of *C. burnetii* were found only in 21 (36.2%) of 58 pairs of mouse spleen specimens. The antibody titers to phase II antigen ranged from 1:64 to 1:1,024, and the scores for amounts of IF antigens and organisms were negative in some cases and positive in others.

Twenty of 58 patients were diagnosed as having acute Q fever by the IF test, while 23 were positive by both the nested PCR and isolation (Table 2). Compared with the nested PCR, the sensitivity, specificity, and positive and negative predictive values of the IF test were 87, 100, 100, and 92.1%, respectively (Table 3). Compared with the IF test, the sensitivity, specificity, and positive and negative predictive values of the nested PCR and isolation were 100%.

DISCUSSION

Our investigation has several epidemiological limitations. We did not obtain convalescent-phase serum samples or details on patients related to their exposure to potential sources of Q fever, residence (urban or rural area), and activities in the month before the onset of symptoms. With such limitations, we used three independent diagnostic methods to determine the prevalence of Q fever pneumonia among children with atypical pneumonia in Japan and also evaluated the sensitivities of these three methods in the diagnosis of acute Q fever from a single acute-phase serum sample.

Acute Q fever infection was characterized by an elevated phase II antibody titer (3, 5, 6, 9, 17, 18, 26). In addition, titers of the IF IgM antibody, which precedes the appearance of IF IgG antibody against phase II *C. burnetii*, were observed within

TABLE 2. Results of IF test, nested PCR, and isolation of *C. burnetii* from a single acute-phase serum sample from children with atypical pneumonia

Patient no.	Age (yr)	Antibody titer to phase II antigen in IF test			Nested PCR result[a]	Isolation of *C. burnetii*		
		Polyvalent	IgM	IgG		Antibody titer to phase II antigen in mouse sera	*C. burnetii* in spleens[a]	Nested PCR result[a]
1	7	512	256		+	128	+	+
2	10	512	256	64	+	512–1,024	+	+
3	6	512	256		+	64–128	+	+
4	8	512	128		+	512	+	+
5	3	512	128		+	256	+	+
6	5	512	256	256	+	256	+	+
7	10	128	128	256	+	256	+	+
8	7	64	128		+	256	+	+
9	6	64	128	32	− (+)[b]	64–512	+	+
10	2	64	64	128	+	256	+	+
11	5	64	128	128	+	128	+	+
12	8	64	128		+	512	+	+
13	6	64	128		+	256	+	+
14	2	64	128		+	64–128	−	−
15	3	64	128		− (+)[b]	256	+	+
16	2	64	128		+	256	+	+
17	5	32	64	64	+	128	−	−
18	8	32	64		+	1,024	+	+
19	6	32	64		+	128–512	+	+
20	6	32	32		+	64–128	+	+
21	5				+	256	+	+
22	8				+	512	+	+
23	2				+	512	+	+
24–58[c]					−	−	−	−

[a] +, positive; −, negative.
[b] Positive with a 100-μl volume of serum but negative with a 50-μl volume of serum used for preparation of samples for PCR.
[c] The sera of patients 24 to 58 were negative by all three methods.

the first 2 weeks of illness. In the present study, we had used phase II antigen for seroconfirmation of Q fever pneumonia.

Our IF test results for the different classes of antibodies (IgM, IgG, and IgA) to phase II antigen (87, 30.4, and 0% of 23 acute Q fever patients, respectively) were different from those obtained by Worswick and Marmion (100, 80, and 60% of 29 acute Q fever patients, respectively) (26) and Embil et al. (80 and 12.5% of 16 Q fever patients for IgM and IgA, respectively) (5). Our results for phase II IgM antibody were also different from those obtained by Hunt et al. (73.4% of 30 sera) (9) and Péter et al. (89% of 303 sera) (18). The reasons for these discrepancies are not known but could represent variation between laboratories or differences in the immune responses of adults and children.

In our study, IgM was seen earlier than IgG, which agrees with the results of Worswick and Marmion (26), Dupuis et al. (3), and Guigno et al. (6). However, in a study by Peacock et al. (17), the appearance of IgG preceded that of IgM (three of five patients with acute Q fever), and both were still present at low titers during the first 2 weeks in a study by Tissot Dupont et al. (22). The reasons mentioned above and differences in the immune responses of patients with various clinical manifestations might explain these discrepancies. Moreover, as postulated by Embil et al. (5), the heterogeneity of the immune responses among individuals who were infected from a point source of *C. burnetii* is not surprising.

The use of PCR for detection and/or identification of *C. burnetii* in clinical samples has been reported previously (13,

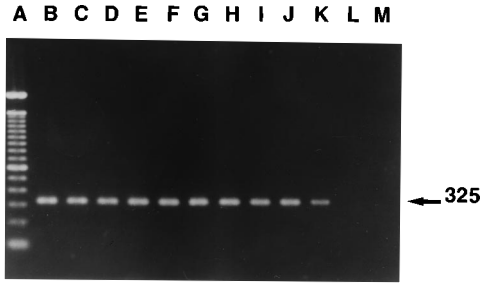

FIG. 1. Detection of 325-bp amplification products from 10 *C. burnetii* isolates. An agarose gel electrophoretogram of amplified DNA after the nested PCR and ethidium bromide staining is shown. Lane A, molecular size markers (100-bp DNA ladder); lanes B to H, seven *C. burnetii* reference strains (Nine Mile phase I, Bangui, Henzerling, Ohio, G Q212, Ko Q229, and S Q217, respectively); lanes I to K, three human *C. burnetii* isolates (307, 605, and TK-1, respectively); lane L, uninfected BGM cells; lane M, reagent control.

TABLE 3. Sensitivities of IF test, nested PCR, and isolation for diagnosis of acute Q fever with a single acute-phase serum sample from children with atypical pneumonia

Method[a]	Result[b] for the following no. of samples		
	20	3	35
IF test	+	−	−
PCR	+	+	−
Isolation	+	+	−

[a] The sensitivity, specificity, and positive and negative predictive values for nested PCR and isolation were 100%, and those for the IF test were 87, 100, 100, and 92.1%, respectively.
[b] +, positive; −, negative.

20). In this study, we used the nucleotide sequence of the *C. burnetii htpB* gene for primer selection. These primers amplified the predicted fragments of various isolates. Thus, they allowed for fragment identification by size, restriction enzyme analysis, and dot blotting and hybridization. As shown by the titration test, our primers detected as few as two infecting units. No amplification products were detected when the negative controls were used. Our experimental conditions showed that false positives with the PCR were ruled out.

The experimental conditions also showed that the infection was provided by the samples and not through cross-infection from other mice. By isolation, 23 patients were found to have Q fever pneumonia.

A good correlation was found between the IF test and the other two methods, but three samples gave discordant results. A negative IF test result with positive PCR and isolation results was found for samples from three patients. These negative results may be explained by the failure of the IF test to detect antibodies in serum samples probably taken too early in the course of the disease. The PCR had a sensitivity of 100%, which was equal to that of isolation but 13% higher than that of the IF test. The PCR was 100% specific.

This study shows that PCR is comparatively simple to perform, takes less time when a large number of samples are being tested, and also is a highly sensitive and specific assay compared with the IF test and isolation for the diagnosis of acute Q fever from a single acute-phase serum sample. This observation was also supported by the fact that *C. burnetii* was always in the acute-phase serum samples (2) but the IF antibodies were sometimes undetectable in such serum samples (9, 18).

In Japan, Q fever has long been though to occur, especially when one considers the presence of *C. burnetii* in Japan's livestock (11). The high prevalence of *C. burnetii* infection in cattle with reproductive disorders (23) and in domestic cats (16) showed that these infected animals play an important role in maintaining the infection and dispersing the pathogenic agent into the environment through their excretions, i.e., milk, colostrum, urine, and birth fluid. Thus, such excretions are considered to be potential sources of the infection in animals and humans via inhalation of infectious aerosols or airborne dust.

There have been many observations of Q fever following indirect exposure to infected animals, as reviewed by Babudieri (1) and Marrie (14). These two authors show that the inhalation of contaminated aerosols and dust is the most important route by which *C. burnetii* infects humans, and they also suggest that the age of the human host as well as the route of introduction and dose of the pathogenic agent may be important in determining whether pneumonia occurs.

The prevalence of Q fever pneumonia among patients with atypical pneumonia in Japan as determined here (23 of 58 [39.7%]) is different from those observed among patients in Canada (rural Nova Scotia; 20% of 110 patients with pneumonia) (15) and France (45.8% of 170 cases of Q fever) (21). It has been suggested that these different results may be due to differences in the virulence of the local strain and in the biology of the host.

Q fever is well known in adults but has only rarely been reported to occur in children. Acute Q fever in children has not been thought to be as rare as one would expect from the paucity of records (19). This could be explained by the fact that childhood infection may take a symptomless course and be discovered only by serological tests becoming positive (1). Q fever in children was reported by Richard et al. (18 cases) (19), Ruiz-Contreras et al. (13 cases) (19), and Jorm et al. (10).

Our results show that clinical Q fever is common in Japan, although it is not known whether subclinical Q fever occurs commonly in children. These findings could be help explain apparent immunity in adults living in close contact with animals and animal products in Japan, where clinical Q fever is seldom reported although *C. burnetii* antibodies were found to be rather common in such individuals (8).

This study suggests that *C. burnetii* is an important cause of atypical pneumonia in children in Japan. Clearly, further studies are necessary to elucidate the epidemiology of Q fever in Japan.

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