Detection of *Coxiella burnetii* in Cow's Milk by PCR–Enzyme-Linked Immunosorbent Assay Combined with a Novel Sample Preparation Method

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The use of an adequate concentration of Triton X-100 enhanced immunomagnetic separation of *Coxiella burnetii* from milk. PCR–enzyme-linked immunosorbent assay (PCR-ELISA) could detect coxiellas more sensitively than could conventional PCR. PCR-ELISA is therefore thought to be suitable for the simultaneous assay of a large number of samples. However, the number of cows from which raw milk tested positive for coxiellas by PCR-ELISA was inconsistent with that found with the antibody to coxiella by indirect immunofluorescence assay. The inconsistency is thought to be associated with the differences in the infectious route, infectious dose, or the timing of yielding the antibody and the period of duration of the antibody.

Coxiellosis in livestock are associated with the occurrence of human Q fever (5); both illnesses are caused by *Coxiella burnetii*. There is a high prevalence of the antibody to *C. burnetii* in veterinarians and meat-processing workers, who come into close contact with livestock (9). The raw milk of infected cows is an important material for detecting *C. burnetii* and diagnosing bovine coxiellosis (2, 21). PCR is a safe and useful method for detection and diagnosis; in contrast, isolation of *C. burnetii* is hazardous, difficult, and time-consuming (20).

Immunomagnetic separation (IMS) is an effective technique for rapid detection and/or isolation of several kinds of bacteria from foods, including cow's milk (10, 18, 19, 23). We previously reported that IMS improved the detection rate of coxiellas in cow's milk and that samples could be prepared quickly and easily for PCR (15). In the previous study, we used a rickettsial suspension consisting mainly of liberated, i.e., extracellular, microorganisms, as we believed that quite a large number of the coxiella existed in the extracellular environment such as in cow's raw milk in the mammary area, especially in the teat canal.

A continuous cell line, designated BGM, derived from African green monkey kidney cells has frequently been used for the propagation of coxiellas, as have chicken embryonated eggs (13, 22). We have used a culture medium of infected cells as the rickettsial antigen for indirect immunofluorescence assay (IFA) (13) and observed that the extracellular microorganisms predominated in the medium. However, further study is needed for lysis of the infected host cells and recovery of intracellular coxiellas.

Triton X-100 (TX-100) is the archetype of a group of Triton surfactants and is generally used for lysis of the cellular membrane. As for another group of Tween surfactants, it was reported that the presence of Tween 20 in the lysis buffer appeared to be beneficial when a smaller volume of the DNA preparation was used in the PCR mixture (3).

The nonradioactive PCR–enzyme-linked immunosorbent assay (PCR-ELISA) method is reported to be sensitive and/or specific for the detection of the targeted DNA (1, 8, 11, 12, 25). This method with a microtiter plate has been used for the detection of *Mycobacterium tuberculosis* (25), cytomegaloviruses (1), adenoviruses (8), and papillomaviruses (12) and used for HLA DNA typing (11). Besides the safety of nonradioisotopic detection, the method of microtiter well hybridization has advantages of speed, suitability for a large number of samples, and applicability to automation (11, 12).

In order to recover intracellular microorganisms, as well as extracellular microorganisms for preparing the PCR mixture, several concentrations of TX-100 needed for the lysis of cells infected with coxiellas were examined. Other purposes of this study were to investigate the possibility of using PCR-ELISA for the detection of coxiellas in cow's raw milk and to determine the prevalence of bovine coxiellosis in one area of Hokkaido, the northernmost island of Japan.

MATERIALS AND METHODS

Rickettsia. *C. burnetii* Nine Mile phase II (ATCC VR-616) was propagated in BGM cells at 35°C with 5% CO₂, as previously described (15). BGM cells were kindly donated by I. Kaiho, Chiba Prefectural Public Health Laboratory, Chiba, Japan. After 6 days of incubation, the culture medium containing the microorganisms, most of which were extracellular coxiellas, was collected. The number of the microorganisms was counted by IFA. The medium consisted of 8.96×10^9 rickettsiae ml⁻¹. The remaining infected cells adhering to the culture flask were trypsinized and then harvested. After two washes, the cells were suspended with 5 ml of phosphate-buffered saline (PBS) containing 50% cryopreservative (cell banker; Dia-latron, Sapporo, Japan). The number of the cells was counted by using a hemacytometer. The suspension consisted of 3.24×10^6 infected cells, which was 98.9% of the total number of cells. Both the medium and the infected-cell suspension were stored at –80°C until use.

Other microorganisms. Fourteen other bacterial species used as negative controls in this study were *Escherichia coli* (ATCC 19906), *Enterobacter aerogenes* (ATCC 13048), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 13883), *Salmonella* serovar Typhimurium (ATCC 13311), *Proteus vulgaris* (ATCC 13315), *Citrobacter freundii*, *Enterobacter cloacae* (ATCC 23355), *Staphylococcus aureus* (ATCC 25923), *Serratia marcescens* (ATCC 8100), *Bacillus subtilis* (ATCC 6633), *Listeria monocytogenes*, *Streptococcus faecalis*, and *Rickettsia japonica* YH. These bacteria, except for *R. japonica*, were cultivated in 10 ml of brain heart infusion broth. After centrifuging and washing twice, the bacteria were suspended with 500 µl of PBS and stored at –80°C until use. *R. japonica* was propagated in monolayers of Vero E-6 cells, as described elsewhere (24). After 1 week of incubation, the infected cells adhering to the flask were harvested. After washing twice, the cells were suspended with 5 ml of PBS contain-

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ing 50% cryopreservative. The infected-cell suspension was stored at -80°C until use.

Preparation of samples for PCR. The medium was serially and decimally diluted with PBS (10^{-2} to 10^{-9}). Each diluted sample was prepared to a 500- μl volume. To five of the eight serial sample groups was added 0.01, 0.02, 0.05, or 0.1% TX-100 or no TX-100 at the final concentration. Each sample was centrifuged at $15,000 \times g$ for 20 min according to the method of Corboz and Zurgilgen (4). After two washes, the pellet was resuspended in 100 μl of 1 mM EDTA in 10 mM Tris-HCl, pH 7.5 (TE).

The infected-cell suspension was serially and decimally diluted with PBS or milk (10^{-2} to 10^{-9}). The volume of each diluted sample was prepared as described above. To four of the eight serial samples diluted with PBS was added 0.01, 0.02, 0.05, or 0.1% TX-100, and the mixture was centrifuged and then resuspended with TE as described above. After addition of TX-100 to the other five groups in the same manner as described above, all samples in the five groups were subjected to the direct IMS method, as previously described (15). In brief, the immunomagnetic beads (IMB) that had been precoated with sheep antibodies against mouse immunoglobulin G (IgG) (Dynabeads M-280; 6×10^8 to 7×10^8 beads per ml; Dynal A.S., Oslo, Norway) were reacted with the mouse antiserum against *C. burnetii* at 4°C for 24 h with gentle rotation. The IMB covered with the mouse antiserum were trapped against the wall of an Eppendorf-type tube with a magnetic particle concentrator (MPC-M; Dynal A.S.), and the residual liquid was decanted. The retained IMB were washed twice with 5% bovine serum albumin in PBS (BSA-PBS). The beads were resuspended with 0.02% sodium azide in BSA-PBS at a volume equal to the original IMB suspension and stored at 4°C until use. Each sample of the infected-cell suspension diluted with PBS was mixed with a 100- μl volume of the prepared IMB suspension. During 1 h of incubation at room temperature, the mixture was gently rotated to avoid sedimentation of the beads. After trapping and washing as described above, the IMB with rosetted microorganisms were resuspended with 100 μl of TE.

Grouping and preparation of the infected-cell suspension diluted with commercial milk were performed with TX-100 and/or the IMS in the same manner as that of the infected-cell suspension diluted with PBS. After 10 min of boiling, 10 μl of all the rickettsial and other bacterial samples was subjected to amplification by PCR.

DNA amplification. PCR was performed with 10 μl of each prepared sample in a total volume of 50 μl . The region of the rickettsial genome targeted by a pair of primers for amplification was 257 bp of the gene encoding superoxide dismutase (20). The PCR mixture contained 0.8 μM primers; 200 μM (each) dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech, Tokyo, Japan); 2.0 mM MgCl_2 ; and 1.75 U of an enzyme mixture containing *Taq* and *Pwo* DNA polymerases and its PCR buffer (Expand High Fidelity; Boehringer Mannheim, Indianapolis, Ind.) overlaid with 2 drops of mineral oil (Sigma, St. Louis, Mo.). Each sample was subjected to 35 cycles of amplification in a thermal cycler (Astec, Fukuoka, Japan). The first amplification cycle consisted of denaturation for 3 min at 95°C , primer annealing to the template at 52°C for 1 min, and primer extension at 72°C for 1.5 min. In the remaining 34 cycles, each step of denaturation, annealing, and extension was performed for 1 min.

Determination of the most efficient sample preparation method for PCR. Detection and identification of the amplified products by PCR were performed as previously reported (20). To investigate the effect of TX-100 on the recovery of extracellular and intracellular coxiellas, the amplification results were compared among all groups. The most efficient recovery method according to the results of PCR was selected as the sample preparation method for PCR-ELISA. A combination of the preparation method and PCR-ELISA was applied for the detection of coxiellas in cows' raw milk obtained from a farm in Hokkaido, Japan.

Milk and serum samples from cows. Raw milk samples were collected from 62 cows. The samples were collected from one teat canal of each cow. Venous blood samples were collected from the caudal vein of 55 of the 62 cows. All of the raw milk and blood samples were collected in March 1995. The milk samples were stored at -80°C until use. One milliliter of each of the 62 milk samples was used for the detection of coxiella by PCR-ELISA. Serum obtained from the blood samples was subjected to IFA.

Conjugation of streptavidin with BSA. Ten milligrams of streptavidin (GIBCO-BRL) was dissolved in 2 ml of PBS and added to 1 ml of 10% (wt/vol) BSA fraction V (Seikagaku Corp., Tokyo, Japan). To this mixture, 300 μl of 0.5% (vol/vol) glutaraldehyde solution was added and allowed to react for 1 h at room temperature. The reaction was terminated with 1 M of ethanolamine. The streptavidin-BSA conjugates were dialyzed overnight against PBS. The conjugates were centrifuged at $100,000 \times g$ for 3 h. The pellet was resuspended with 10 ml of 0.02% sodium azide in PBS. The streptavidin-BSA solution was stored at 4°C until use.

Biotinylated capture probe. For PCR-ELISA, a 23-residue oligonucleotide probe (5'-TGT ATG GTT CCA ATG TTG TGC TG-3') was synthesized and biotinylated at the 5' end. The capture probe complemented the internal sequence in the amplification product of the gene encoding superoxide dismutase (7, 20). The sequence of the probe was selected by using the primer analysis software OLIGO, version 5.0 (National Bioscience, Plymouth, Minn.), to avoid a secondary structure and self-complementarity.

FIG. 1. Effects of different concentrations of TX-100 on the detection of extracellular coxiellas in PBS. 0.01 to 0.1%, concentrations of TX-100; no TX-100. Lanes: M, molecular size standards (base pairs); lanes 3 to 5, 10^{-3} - to 10^{-5} -diluted supernatant fluids of the infected BGM cells, respectively. The arrowhead indicates 257 bp.

PCR-ELISA. Tenfold serial dilutions of the mixture containing the supernatant and the infected-cell suspension (through 10^{-9}) were prepared by the most efficient sample preparation method, selected in accordance with the results of PCR, and the samples were subjected to PCR-ELISA. The other bacterial suspensions were used as negative controls. The PCR was performed under the same conditions as these described above, except that the final reaction mixture consisted of a PCR digoxigenin labeling mix (200 μM [each] dATP, dCTP, and dGTP; 190 μM dCTP; and 10 μM digoxigenin-labeled dUTP [Boehringer Mannheim]).

Ten microliters of the PCR product was mixed with 90 μl of hybridization solution (1) ($4 \times \text{SSC}$ [$1 \times \text{SSC}$ is 150 mM NaCl plus 15 mM sodium citrate], 20 mM EDTA, 20 mM HEPES, and 0.15% Tween 20) containing 1.3 nM biotinylated probe. The sample prepared in a total volume of 100 μl was heated at 95°C for 5 min, annealed at 60°C for 5 min, and then transferred to a 96-well microtiter plate (MTP; Dynex Technologies, Chantilly, Va.) that had been coated overnight with 100 μl of the streptavidin-BSA solution plus 200 μl of coating buffer per well, as described previously (1, 8). The solution was incubated at 37°C for 1 h on an MTP shaker. After three washes with washing solution (0.15% Tween 20 and 150 mM NaCl in 100 mM Tris-HCl, pH 7.5), each well of an MTP was filled with 200 μl of horseradish peroxidase-conjugated anti-digoxigenin-sheep IgG (Fab) (Boehringer Mannheim) diluted 1:1,000. The solution was incubated at 37°C for 30 min with shaking and then washed again. After application of 200 μl of 100- $\mu\text{g}/\text{ml}$ 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium (Sigma) to each well and incubation at 37°C for 30 min, the absorbance of the solution at 405 nm (A_{405}) was recorded by a spectrophotometer (Immuno-mini NJ-2300; Intermed, Tokyo, Japan). An A_{405} score three times higher than the maximum A_{405} score of negative control samples was regarded as positive. To evaluate the ability of PCR-ELISA to detect coxiellas, conventional PCR was performed under the same conditions as PCR-ELISA.

IFA. By using chicken embryonated eggs, the phase II IFA antigen from the Nine Mile strain was prepared as described previously (6). A 10- μl volume of the antigen suspension diluted 10-fold with PBS was spotted onto each well of a 12-well test slide. IFA was performed as previously described with slight modification (14). A 10- μl volume of a 1:40 dilution of fluorescein-conjugated goat anti-bovine IgG (Organon Teknica, Durham, N.C.) was employed for detecting the antibody. A reactivity of $\geq 1:32$ was interpreted as positive.

Statistical analysis. The χ^2 test and Student's *t* test were used to estimate associations in bivariate analysis of categorical data between two groups of cows: positive by PCR-ELISA and negative by PCR-ELISA. Geometric means of IFA titers of the two groups were compared by Student's *t* test. The χ^2 test was used to evaluate the diversity of positive rates of IFA between the two groups.

RESULTS

Effect of the detergent on extracellular coxiella in PBS. To compare the effect of each concentration of TX-100 on the detection of extracellular coxiellas, PCR was performed using samples prepared from the infectious supernatant, serially and decimally diluted with PBS. As shown in Fig. 1, none of the concentrations of TX-100 prevented detection of coxiellas. In the cases of 0.02, 0.05, and 0.1% concentrations of TX-100, coxiellas were detected with slightly higher sensitivities (Fig. 1).

Effect of the detergent on intracellular coxiellas and IMS in PBS. To compare the effect of each concentration of TX-100 on the detection of intracellular coxiellas by using IMS, PCR was performed using samples prepared from the infected-cell suspension diluted with PBS. Coxiellas were detected with higher sensitivities in the cases of 0.01 and 0.02% TX-100 treatment followed by IMS (TX-100 plus IMS) than in the case

FIG. 2. Detection of intracellular coxiellas in PBS by using different concentrations of TX-100 and IMS. 0.01 to 0.1% TX + IMS, use of the indicated concentration of TX-100 followed by IMS; TX only, use of 0.02% TX-100 (in this figure) followed by centrifugation; IMS only, IMS without TX-100. Lanes: M, molecular size standards (base pairs); lanes 2 to 6, 10^{-2} - to 10^{-6} -diluted suspensions of BGM cells harboring coxiellas, respectively. Arrowheads indicate 257 bp.

of IMS only (Fig. 2). However, in the cases of 0.05 and 0.1% TX-100 plus IMS, the sensitivity was similar to that of IMS only (Fig. 2). To evaluate the effect of the detergent on the immunoreaction between coxiellas and IMB, the results of TX-100 plus IMS were compared with those of TX-100 treatment followed by centrifugation (TX-100 only). All but 0.02% TX-100 plus IMS detected coxiellas with lower sensitivities than that of TX-100 only (Fig. 2).

Effect of the detergent on intracellular coxiellas and IMS in milk. For the detection of coxiellas in milk samples, 0.01 and 0.02% TX-100 plus IMS showed the highest sensitivity (Fig. 3). In the cases of $\geq 0.05\%$ TX-100, the sensitivity of coxiella detection decreased (Fig. 3).

The results of the detection of extra- and intracellular coxiellas showed that a combination of 0.02% TX-100 plus IMS is the most effective method. This method was used for sample preparation for PCR-ELISA.

Estimation of PCR-ELISA. The results of PCR-ELISA for negative control bacteria and the 10-fold serial dilutions consisting of the supernatant and the cells infected with coxiellas (through 10^{-9}) are summarized in Table 1. The maximum A_{405} score of the negative bacteria was 0.004. Detection by PCR-ELISA was limited to a maximum dilution of 10^{-8} , in accordance with the criterion described above (Table 1). Serial dilutions were also used for conventional PCR. Detection by PCR was limited to a maximum dilution of 10^{-7} (Table 1).

FIG. 3. Detection of intracellular coxiellas in milk by using different concentrations of TX-100 and IMS. 0.01 to 0.1% TX + IMS, use of the indicated concentration of TX-100 followed by IMS; TX only, use of 0.01% TX-100 (in this figure) followed by centrifugation. Lanes: M, molecular size standards (base pairs); lanes 2 to 5, 10^{-2} - to 10^{-5} -diluted suspensions of BGM cells harboring coxiellas, respectively. The arrowhead indicates 257 bp.

TABLE 1. Evaluation of the results of PCR-ELISA

Sample	Dilution	PCR-ELISA result	Absorbance
Rickettsia	10^{-4}	+	0.198
	10^{-5}	+	0.112
	10^{-6}	+	0.076
	10^{-7a}	+	0.049
	10^{-8}	+	0.015
	10^{-9}	—	0.008
Negative bacteria	Not done	All —	0.000–0.004

^a The limitation of coxiella detection by conventional PCR.

Detection of coxiellas in cow's raw milk by PCR-ELISA and antibody to the coxiellas by IFA. The results of the detection of coxiellas in raw milk of 62 cows by PCR-ELISA showed that 21 cows (33.9%) were positive and 41 cows (66.1%) were negative (Table 2). Of the cows positive by PCR-ELISA, 11 were also positive by IFA and 3 were not tested. In the group of cows negative by PCR-ELISA, 17 were positive by IFA and 4 were not tested.

The geometric mean of IFA titers of the cows positive by PCR-ELISA was 48.1090, and that of the cows negative by

TABLE 2. Detection of coxiellas in milk by PCR-ELISA and comparison of geometric means and positive ratios of IFA between PCR-ELISA-positive and -negative cows

Cow(s) tested	PCR-ELISA		IFA ^a			
	Result	A_{405}	Result	Titer	GM	PR (%)
1	+	1.725	+	128	48.1090 ($t = 1.4756$, NS)	64.7 ($\chi^2 = 1.8741$, NS)
2	+	0.270	+	128		
3	+	0.192	—	16		
4	+	0.134	+	64		
5	+	0.131	—	16		
6	+	0.127	+	64		
7	+	0.121	+	128		
8	+	0.109	+	64		
9	+	0.100	—	16		
10	+	0.092	—	16		
11	+	0.090	+	64		
12	+	0.087	+	128		
13	+	0.058	—	16		
14	+	0.053	+	128		
15	+	0.032	—	16		
16	+	0.031	+	64		
17	+	0.027	+	64		
18	+	0.080	NT			
19	+	0.078	NT			
20	+	0.053	NT			
21	+	0.047	NT			
22	—	0.011	—	16	33.1890	44.7
23	—	0.011	—	16		
24	—	0.008	+	128		
25	—	0.007	—	16		
26	—	0.005	—	16		
27	—	0.002	—	16		
28	—	0.002	+	128		
29–44	—	0.000	—	16		
45–55	—	0.000	+	64		
56–59	—	0.000	+	128		
60	—	0.001	NT			
61, 62	—	0.000	NT			

^a NT, not tested; GM and PR, geometric mean and positive ratio of IFA, respectively, in PCR-ELISA-positive or -negative cows; NS, not significant.

PCR-ELISA was 33.1890. The positive ratios of IFA in the groups of cows positive and negative by PCR-ELISA were 64.7 and 44.7%, respectively (Table 2). No statistically significant differences in the geometric means and positive rates of IFA were found between the two groups. A sample obtained from a mammary area infected with mastitis showed the highest A_{405} score (cow 1, Table 2). All the remaining 61 samples were collected from a normal mammary area.

DISCUSSION

The combination of 0.02% TX-100 plus IMS was found to be the most efficient method for preparation of the PCR mixture from milk. With this preparation method followed by PCR, it was possible to detect coxiella in as few as 32 infected cells ml^{-1} . The addition of 0.02% TX-100 both in PBS and milk appeared to be beneficial when a sample was prepared for PCR. By using a combination of the surfactant and IMS, the efficiency for detection of coxiellas in milk was further improved, though the influence of the milk could not be completely excluded. Even in the present study, the detection limit for the microorganisms in PBS was 10-fold higher than that in milk. This finding supports an assumption that some component of the milk interferes with the immune reaction (3, 15). Also, when preparing samples from milk, centrifugation produced hard pellets, which cannot be dissolved easily. The results of the PCR in this case were not satisfactory as was previously reported (15). Therefore, in the present study we attempted to apply IMS for the detection of coxiellas in milk.

PCR-ELISA detected coxiellas with a sensitivity 10-fold higher than that of conventional PCR. Techniques such as gel electrophoresis combined with analyses of restriction fragment length polymorphisms and/or Southern blotting are commonly used for detecting and identifying PCR products. However, these methods are time-consuming, are limited to a small number of samples, and cannot easily be automated. PCR-ELISA, on the other hand, can detect and identify PCR products more rapidly. This colorimetric method is easy and objective and requires no electrophoresis apparatus, UV illuminator, or darkroom (25). By using the appropriate standard, PCR-ELISA can be used to quantify the PCR products and can be automated for the simultaneous assay of a large number of samples.

In this study, no concentration of TX-100 interfered with the recovery of extracellular coxiellas. PCR after the addition of 0.02 to 0.1% TX-100 and centrifugation detected as few as 9.0×10^3 rickettsiae ml^{-1} . This low detection rate may be due to the fact that centrifugation detected coxiellas with a sensitivity 100-fold lower than that following IMS (15). The sensitivity of coxiella detection increased slightly by using 0.02, 0.05, and 0.1% concentrations of TX-100 (Fig. 1). The supernatant medium contained a small number of suspended cells in which coxiellas existed. Hence, it was assumed that cell lysis with TX-100 caused the recovery of the microorganism from the intracellular environment in these cases. Prior to this experiment, the effect of 0.001 to 0.1% TX-100 each on normal BGM cells suspended in PBS was observed under a microscope. The cellular membrane was quickly lysed with $\geq 0.02\%$ TX-100. However, cell lysis with 0.01% TX-100 took a longer time (data not shown). In the case of 0.01% TX-100, the cell lysis was thought to be inhibited by some component of the medium.

For the detection of intracellular coxiellas in PBS, 0.02% TX-100 plus IMS was a more efficient sample preparation method than IMS only. However, TX-100 alone was as efficient as the 0.02% TX-100 plus IMS. Both methods followed by PCR could detect coxiellas in as few as 3.2 infected cells ml^{-1} .

The sensitivities of both 0.05 and 0.1% TX-100 plus IMS were 10-fold lower than the sensitivity of 0.02% TX-100 plus IMS. This was thought to be due to the interference of TX-100 in the immune reaction between the microorganisms and IMB.

There is a considerable inconsistency between the results of coxiella detection in the raw milk of cows by PCR-ELISA and that of the serological survey by IFA. Of 38 cows positive by IFA and/or PCR-ELISA, 17 were positive by IFA but not by PCR-ELISA and 6 others were positive by PCR-ELISA but not by IFA. The period of shedding coxiellas was thought to vary according to differences in the infectious route. In a milk cow inoculated with coxiellas, it was reported that milk from the mammary area inoculated via the teat canal was infectious for 17 days, milk from the mammary area inoculated via the glandular tissue was infectious for over 200 days, and milk from the mammary area inoculated subcutaneously remained non-infectious (2). The serum of the cow which continued to shed coxiellas in its milk remained positive, while the other cow inoculated with coxiellas only by way of the teat canal yielded the antibody for over 10 days while its milk was noninfectious (2). The results for the 17 IFA-positive cows in the present study are similar to those of the above experiment, and these cows are thought to be in the convalescent stage of coxiellosis. Another reason for the inconsistency was thought to be differences in the infectious dose of coxiellas. In cows experimentally infected with coxiellas, the difference in the infectious dose influenced both the timing of the antibody yield and the duration of the antibody for coxiellosis (5). The six PCR-ELISA-positive, IFA-negative cows in the present study were presumably exposed and affected with a small quantity of the microorganism. Consequently, the immune response was thought to be delayed and the persistence of the antibodies was shortened. Alternatively, these cows were thought to be in the early stage of the infection.

The inconsistency may also be due to the biological properties of the rickettsial strain manifested among these cows. It is possible that certain *C. burnetii* strains with a low or attenuated virulence are prevalent in the area of Japan from which samples were collected. Even if such strains were transmitted to animals or humans, the response would simply be to develop antibodies. Concerning the detection of coxiellas in the *Ixodes* sp. tick, a similar phenomenon has been reported in an area of the Bavarian forest in Europe (16, 17). The results of the present study suggest that an unidentified strain has insidiously infiltrated Japan. This strain is assumed to have properties that are different from those of any other known rickettsial strain. A more rapid, easy, and efficient isolation system is needed to investigate the serological and biological properties of this strain in detail.

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