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Detection of *Coxiella burnetii* by DNA Amplification Using Polymerase Chain Reaction

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The polymerase chain reaction (PCR) was used for the detection of *Coxiella burnetii*, an obligate intracellular bacterium and the etiologic agent of Q fever. A pair of primers derived from the *C. burnetii* superoxide dismutase gene served to amplify a targeted 257-bp fragment of genomic DNA. These primers were chosen on the basis of GenBank analysis, G+C ratio, and absence of secondary structure. This technique allowed the detection of as few as 10 *C. burnetii* organisms. *C. burnetii* was detected in tissue culture and in specimens from patients (heart valves). In all, 8 reference isolates and 22 new isolates of *C. burnetii* from France were successfully amplified. No amplification products were found when PCR was performed with 25 bacterial species that had been isolated in a clinical laboratory from patients with clinically similar infections. Amplification products of *C. burnetii* were confirmed by restriction enzyme digestion and dot blot hybridization. The method used here, a combination of PCR and restriction analysis, is a faster and more sensitive assay for *C. burnetii* than standard culture techniques.

Coxiella burnetii is an obligate intracellular bacterium that causes Q fever in animals and humans (11, 27, 28). In humans, the acute disease normally occurs as a febrile illness, with recovery occurring in 1 to 4 weeks. Approximately 5% of patients reported to have Q fever develop chronic disease (11). Endocarditis is the most common manifestation of chronic Q fever, but hepatitis, osteomyelitis (16), and infection of vascular prostheses (17) have also been described (18). Diagnosis is usually established by serological tests.

Current serological methods for detecting antibodies to *C. burnetii* antigens include indirect immunofluorescence, complement fixation, and enzyme-linked immunosorbent assays (ELISA) (11, 27). These serological tests are adequate for some aspects of clinical medicine but in order to evaluate cures for chronic Q fever, the isolation of organisms or detection of DNA is more useful (8). The direct detection of *C. burnetii* or its DNA is also important in veterinary pathology (milk and placenta samples).

The direct recovery of *C. burnetii* by culture in embryonated eggs, by cell culture, or by experimental infections in laboratory animals has been used (12). These techniques are time-consuming, hazardous, and expensive and require extensive laboratory support. Direct immunofluorescence of infected tissues remains the easiest method for direct detection of *C. burnetii*.

The application of the polymerase chain reaction (PCR), which uses specific oligonucleotide primers and *Taq* DNA polymerase to synthesize copious quantities of DNA from a single template (20), provides a valuable new approach in view of its sensitivity and broad applicability. We recovered 22 new isolates of *C. burnetii* from human patients suffering from chronic Q fever. Sixteen patients had endocarditis, one had an infection of a vascular prosthesis, two had a vascular aneurysm, and three patients had acute Q fever. These isolates and eight reference isolates were cultured in L929 cells.

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We report the detection and identification of all these *C. burnetii* isolates by PCR in tissue culture material and in direct patient specimens (heart valves) using oligonucleotide primers derived from the superoxide dismutase gene sequence of *C. burnetii* (5).

MATERIALS AND METHODS

Microorganisms. *C. burnetii* isolates used in this study included the following prototype strains: Priscilla Q 177 and GQ 212 (obtained from T. Hackstadt) and Nine Mile phase I (ATCC VR 615), Ohio 314 (ATCC VR 542), Henzerling (ATCC VR 145), Bangui (ATCC VR 730), California 76 (ATCC VR 614), and Dyer (ATCC VR 147) (obtained from the American Type Culture Collection).

All new *C. burnetii* isolates were isolated from patients suffering from acute or chronic Q fever by a centrifugation shell-vial technique. Samples (heart valves, blood, and an arterial prosthesis) were inoculated onto human embryonic lung fibroblast cell monolayers growing in shell vials. *C. burnetii* was detected 6 days later by direct immunofluorescence (19). The supernatant of the shell-vial culture was stored at −20°C. The 22 new *C. burnetii* isolates examined in this study were named M (for Marseille), E (for endocarditis), AN (for aneurysm), P (for prosthesis), I (for immunocompromised), and AC (for acute illness) and were numbered from 1 to 22. The designations and origins of these *C. burnetii* isolates are presented in Table 1. Other bacteria used in this study were clinical isolates obtained from the culture collection maintained by D. Raoult (Laboratoire de Microbiologie, Centre Hospitalier Universitaire de la Conception, Marseille, France). These microorganisms were *Brucella abortus*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Citrobacter freundii*, *Enterobacter aerogenes*, *Enterobacter intermedium*, *Enterococcus faecalis*, *Escherichia coli*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Mycoplasma hominis*, *Neisseria meningitidis*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Pseudomonas cepacia*, *Rickettsia conorii*, *Salmonella typhimurium*, *Serratia marcesens*,

TABLE 1. *C. burnetii* isolates tested

Isolate	Source	Origin	Disease, symptoms, or clinical status
Nine Mile	ATCC[a]	*Dermacentor andersoni*	
Priscilla Q 177	T. Hackstadt	Goat, placenta	
GQ 212	T. Hackstadt	Human	Endocarditis
Bangui	ATCC	Human	Fever and rash
California 76	ATCC	Cow, milk	
Dyer	ATCC	Human, blood	
Henzerling	ATCC	Human, blood	
Ohio 314	ATCC	Cow, milk	
ME 1		Human	Endocarditis
MEI 2		Human	Endocarditis, immunocompromised
ME 3		Human	Endocarditis
ME 4		Human	Endocarditis
ME 5		Human	Endocarditis
ME 6		Human	Endocarditis
MP 7		Human	Vascular prosthesis
ME 8		Human	Endocarditis
ME 9		Human	Endocarditis
MAN 10		Human	Aneurysm
MAC 11		Human	Acute Q fever
MAC 12		Human	Acute Q fever
ME 13		Human	Endocarditis
ME 14		Human	Endocarditis
ME 15		Human	Endocarditis
MAN 16		Human	Aneurysm
ME 17		Human	Endocarditis
ME 18		Human	Endocarditis
MEI 19		Human	Endocarditis, immunocompromised
MAC 20		Human	Acute Q fever
ME 21		Human	Endocarditis
ME 22		Human	Endocarditis

[a] ATCC, American Type Culture Collection.

Staphylococcus aureus, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Ureaplasma urealyticum*.

Growth and purification of organisms. All *C. burnetii* isolates were subcultured in murine L929 fibroblasts at 37°C in a 5% CO_2 atmosphere. Antibiotic-free Eagle minimal essential medium with 4% fetal calf serum and 1% glutamine was changed every 4 to 10 days.

The cytopathic effect of *C. burnetii* was observed in L929 cells. When at least 80% of the cells were infected, they were harvested and propagated in other flasks. Cell lysis was achieved by digestion with 0.5% trypsin for 30 min at 37°C. Rickettsiae were partially purified by density gradient centrifugation (19) (7% Renografin in sucrose-phosphate-glutamate buffer [pH 7.2] [0.720 g of L-glutamic acid, 0.490 g of KH_2PO_4, 1.236 g of K_2HPO_4, and 74.6 g of sucrose in 1,000 ml of sterile double-distilled water]). First, the cell fragments were pelleted by centrifugation with a Sorvall RC 2-B with a Sorvall rotor type SS-34 at 4°C for 15 min at 1,000 rpm. *C. burnetii* was then pelleted by centrifugation with a Sorvall RC 2-B with a Sorvall rotor type SS-34 at 4°C for 15 min at 8,000 rpm. The pellets were washed twice for 10 min each time with Rinaldini buffer (6.8 g of NaCl, 0.4 g of KCl, 0.156 g of NaH_2PO_4, 2.2 g of $NaHCO_3$, 1.0 g of glucose, and 1.0 mg of phenol red in 1,000 ml of sterile double-distilled water), examined by Gimenez staining (4), and frozen at −20°C.

During this study, a new isolate was obtained (MAC 20), and PCR was performed directly on the supernatant of the shell vial.

The other bacteria were cultivated on the appropriate media, washed twice with sterile water for 10 min each time, and frozen at −20°C.

Clinical samples. The clinical samples examined were heart valves obtained from surgical procedures on patients with chronic Q fever. The samples were initially frozen and then homogenized in 2 ml of phosphate-buffered saline (8 g of NaCl, 0.2 g of KCl, 1.15 g of Na_2HPO_4, and 0.2 g of KH_2PO_4 in 1,000 ml of sterile double-distilled water). Five valves were obtained from five different patients (ME 3, MP 7, ME 18, ME 21, and ME 22).

Titration of the inoculum. Titrations were performed for the two reference strains, Nine Mile and GQ 212. Cell cultures containing 90% infected cells were trypsinized and resuspended in culture medium (18). After thorough homogenization to free microorganisms from the phagolysosomal vacuoles, the infected-cell suspension was stored at −80°C in 0.5- or 1-ml aliquots. To establish the number of infecting units per milliliter of the infected-cell suspension, serial decimal dilutions (10^{-1} to 10^{-8}) of the suspension were inoculated onto confluent monolayers of human embryonic lung fibroblast cells in centrifugation shell vials. After removal of the medium from the shell vials, the human embryonic lung fibroblast monolayers were inoculated with 0.4-ml samples of the various dilutions of infected-cell suspension and centrifuged at 30°C for 1 h at 5,000 rpm. The supernatants were discarded, 1-ml portions of fresh medium were added, and the shell vials were incubated for 6 days at 37°C. Infected cells were demonstrated by indirect immunofluorescence with rabbit antiserum to *C. burnetii*. The number of infective *C. burnetii* bacteria per milliliter of inoculum was calculated from the highest dilution yielding at least one infected cell.

Analysis by PCR. (i) Preparation of samples for PCR analysis. All samples were prepared in two ways. The first method consisted of proteinase K/sodium dodecyl sulfate (SDS) digestion followed by three phenol-chloroform extractions and absolute-ethanol precipitation (21). The purified DNA was then washed with 70% ethanol, repelleted, lyophilized, and suspended in small volumes of 10 mM Tris-HCl (pH 8) and 1 mM EDTA (21). The second method was simply boiling 100-μl samples of the bacterial suspension for 10 min.

(ii) Selection of sequences for primers. A pair of 20- and 19-residue oligonucleotide primers (primer C.B.-1 [5′-ACT CAA CGC ACT GGA ACC GC-3′] and primer C.B.-2 [5′-TAG CTG AAG CCA ATT CGC C-3′]) were synthesized (Eurogentec, Seraing, Belgium) according to the published DNA sequence of the gene encoding the superoxide dismutase enzyme of *C. burnetii* (5). The length of the rickettsial genome targeted for amplification was predicted to be 257 bp. Sensitivity was evaluated by using 10-fold dilutions of an initial inoculum of 10^6 infecting units of the two reference strains, Nine Mile and GQ 212. The specificities of these two primers were tested in PCR with purified DNA from 25 other bacterial species and uninfected L929 cells.

(iii) DNA amplification. PCR was performed on 10 μl of each prepared sample in a total volume of 100 μl (1, 2). The final reaction mixture contained a 1 μM concentration (each) of primers C.B.-1 and C.B.-2, 200 μM (each) dATP, dCTP, dGTP, and dTTP, 50 mM of KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM $MgCl_2$, 0.01% gelatin, and 2.5 U of *Thermus aquaticus* enzyme (*Taq* DNA polymerase; Promega, Madison, Wis.) overlaid with 100 μl of mineral oil. Samples were

subjected to 30 cycles of amplification in a DNA thermal cycler (LEP Scientific, Andover, Hampshire, England). An amplification cycle consisted of denaturation for 20 s at 95°C, primer annealing to the template at 50°C for 1 min, and primer extension at 72°C for 2 min.

(iv) Detection of the amplification product. Samples (10 μl) were removed from the reaction mixture, examined by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed under UV illumination (21). The room that was used for preparation of samples for PCR was used for only this purpose. Clinical specimens were stored in a different room in which no aspect of sample preparation or analysis was done. Clinical specimens and frozen cultures of *C. burnetii* were stored in separate boxes. All specimens tested were stored and treated in the same manner.

Restriction endonuclease digestion. To ensure that the amplified products of the expected size represented the target sequence, the products were digested with restriction enzymes known to cut within the target sequence (21). The 257-bp amplification products were digested with *Alu*I and *Taq*I. The known sequence of the superoxide dismutase gene of *C. burnetii* has two *Alu*I and three *Taq*I sites in the amplified region. We performed restriction analysis on nine new isolates from humans and three reference strains, Nine Mile phase II (acute Q fever), GQ 212, and Priscilla Q 177 (chronic Q fever). Samples (30 μl each) of the amplified target sequences were digested with *Alu*I and *Taq*I (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. The restriction products were examined by electrophoresis on 20% polyacrylamide gels stained with ethidium bromide and viewed under UV illumination.

Dot blot analysis. To test reactivity of the amplification product with purified chromosomal DNA, the 257-bp fragment from the *C. burnetii* Nine Mile strain was chosen as the probe. Ten microliters of each amplification product, 90 μl of sterile water, and 100 μl of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) were added to each well. Samples were boiled for 10 min, chilled on ice, and applied to a nylon filter (Hybond-N; Amersham) with a dot blot microfiltration apparatus (Bio-Rad) according to the instructions of the manufacturer. The nylon membrane was washed in a denaturation solution (1.5 M NaCl, 0.5 M NaOH) and then in a neutralization solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 7.2], 0.001 M EDTA) and fixed by UV cross-linking (7).

Hybridization. Probe DNA was labelled by using the random primed DNA labelling kit (Boehringer) according to the directions of the manufacturer.

The hybridization reaction buffer contained 5× SSC, 5× Denhardt's solution, 0.5× SDS, and 10 μg of salmon sperm DNA per ml. After hybridization for 12 h at 65°C, 30-min washes were done in SSC at 65°C as follows: two washes in 2× SSC, one wash in 1× SSC, and two washes in 0.1× SSC.

RESULTS

Detection of *C. burnetii* in tissue culture by PCR. The ability of a pair of synthetic oligonucleotide primers to amplify segments of the *C. burnetii* superoxide dismutase gene was tested on tissue culture specimens of various *C. burnetii* isolates (in all, 22 isolates from French patients and 8 reference strains). The primer pair C.B.-1 and C.B.-2 amplified the predicted products (Fig. 1). No product was generated by using uninfected control L929 cells as the template (Fig. 1). In 2% agarose gels, all isolates produced bands of the same intensity containing the 257-bp fragment. As for the two techniques of preparing samples for PCR analysis, extraction of purified *C. burnetii* DNA was not necessary, and studies could be performed with simple boiling. The isolate obtained during our study (MAC 20) needed no subculture on L929 cells. In fact, amplification was possible by using directly the supernatant of the shell vial.

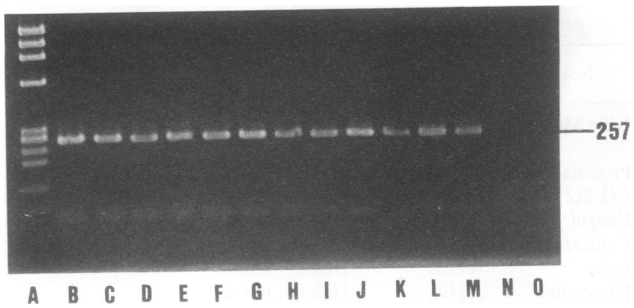

FIG. 1. 257-bp amplification products from 12 *C. burnetii* isolates. An agarose gel electrophoretogram of amplified DNA after 30 cycles of amplification and ethidium bromide staining is shown. Lane A, molecular size markers (ϕX174 cleaved with *Hae*III); lanes B to J, nine human *C. burnetii* isolates (ME 5, ME 1, ME 8, MAC 12, ME 4, MEI 2, MAC 11, ME 3, and ME 6, respectively); lane K, reference strain Nine Mile phase I; lane L, reference strain Priscilla Q 117; lane M, reference strain GQ 212; lane N, uninfected L929 cells; and lane O, reagent control.

Specificity of primers for *C. burnetii*. Cleavage of the amplification product of 257 bp at the *Alu*I sites produced three fragments of 186, 68, and 3 bp (Fig. 2). Cleavage at the *Taq*I sites generates four fragments of 118, 57, 43, and 39 bp (Fig. 3). These digestions produced the expected fragments, and the restriction patterns were identical for all 12 isolates of *C. burnetii*. All 12 amplification products were recognized in dot blot hybridization when the radiolabelled 257-bp fragment of *C. burnetii* Nine Mile was used as the probe (data not shown). We also tested the ability of our pair of primers to amplify extracellular and intracellular bacterial pathogens causing infections in humans. No amplification product was detected by using the DNAs of the 25 bacterial species listed in Materials and Methods as the template.

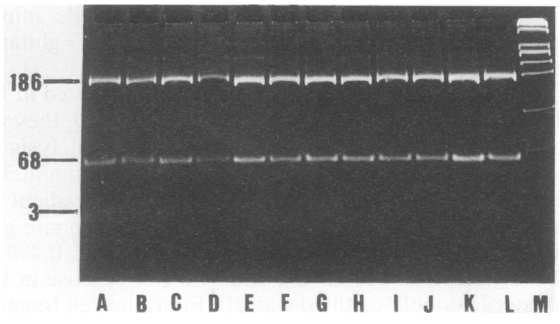

FIG. 2. Restriction endonuclease profile analysis of the 257-bp amplification products of 12 *C. burnetii* isolates. The amplification products were digested with *Alu*I, electrophoresed on polyacrylamide gels, and stained with ethidium bromide. Lanes A to I, nine human *C. burnetii* isolates (ME 5, ME 1, ME 8, MAC 12, ME 4, MEI 2, MAC 11, ME 3, and ME 6, respectively); lane J, reference strain Nine Mile phase I; lane K, reference strain Priscilla Q 117; lane L, reference strain GQ 212; and lane M, molecular size markers (ϕX174 cleaved with *Hae*III). The values to the left of the gel are sizes (in base pairs).

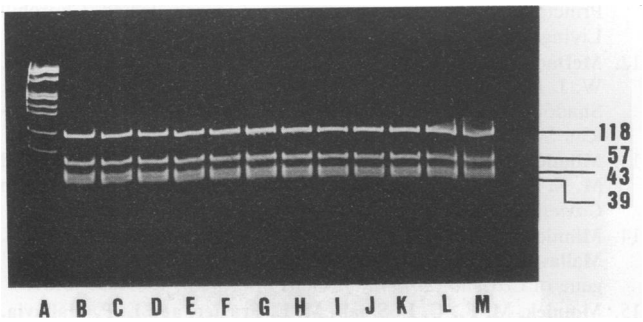

FIG. 3. Restriction endonuclease profile analysis of the 257-bp amplification products of 12 *C. burnetii* isolates. The amplification products were digested with *Taq*I, electrophoresed on polyacrylamide gels, and stained with ethidium bromide. Lane A, molecular size markers (ϕX174 cleaved with *Hae*III); lanes B to J, nine human *C. burnetii* isolates (ME 5, ME 1, ME 8, MAC 12, ME 4, MEI 2, MAC 11, ME 3, and ME 6, respectively); lane K, reference strain Nine Mile phase I; lane L, reference strain Priscilla Q 117; and lane M, reference strain GQ 212.

Sensitivity of primers for *C. burnetii*. Two reference strains, Nine Mile phase II and GQ 212, were tested in various dilutions to determine detection limits of amplification products. As shown in Fig. 4, our pair of primers detected as few as 10 infecting units per ml.

Detection of *C. burnetii* in clinical specimens. Heart valve specimens from five patients suffering chronic Q fever were tested. All specimens yielded specific PCR products that were detected by agarose gel electrophoresis (data not shown).

The concentrations of $MgCl_2$, primer oligonucleotides, and *Taq* were the same as in the procedure employed for amplification of *C. burnetii* DNA from tissue culture. Only the cycle number was modified, because 40 cycles were necessary to obtain a clearly visible band in the agarose gel.

DISCUSSION

We report here the first pair of primer sequences which allow the detection of *C. burnetii* by PCR. Gene amplification by PCR has been used extensively for detection of viral and bacterial pathogens. *C. burnetii* is a fastidious intracellular bacterium. Isolation requires several days (and sometimes weeks) and is time-consuming, difficult, and hazardous. The different strains of *C. burnetii* show heterogeneity in their growth conditions (25), adding to the complexity of culture.

When culture facilities for these intracellular bacteria are not available, diagnosis is accomplished by serological procedures. ELISA and microimmunofluorescence tests are the standard diagnostic procedures (11, 12). Since antibodies often persist for years after the illness, discrimination between current and past infections may require demonstration of a significant increase in specific antibody titer in successive serum samples. As a result, these retrospective analyses are of no value in treating patients.

A technique using radiolabelled DNA probes for detection and identification of *C. burnetii* strains has been reported previously (3, 10). That method uses specific hybridization of labelled DNA probes to nucleic acid in clinical samples. By performing Southern blotting in conjunction with PCR, these assays detect as few as two to nine *C. burnetii* organisms. Thus, hybridization with radiolabelled probes increases sensitivity, but these techniques are available only at specialized research laboratories.

In fact, no primer sequence for *C. burnetii* has been published. Detection of *C. burnetii* in tissue culture and in specimens from patients by DNA amplification expands the spectrum of pathogens amenable to PCR diagnosis. Few data on the molecular biology of *C. burnetii* are available; only five genes have been sequenced (3, 5, 6, 14, 15), and little is known of the molecular aspects of pathogenesis (9, 13, 22–24, 26). We used the nucleotide sequences of the *C. burnetii* superoxide dismutase gene for primer selection. These primers allowed generation of PCR fragments for various isolates causing acute or chronic Q fever. Thus, these primers allowed for fragment identification by size, restriction enzyme analysis, and dot blotting. We found the number of cycles to be an important variable in performing PCR on clinical specimens. Tissue culture specimens were easily amplified after 30 cycles of amplification, whereas patients' specimens or samples from early tissue culture for isolation (supernatant of shell vials) required 40 cycles of amplification to be clearly visible by ethidium bromide staining. Low numbers of *C. burnetii* genomes in some clinical samples may account for this finding. A titration test showed that our PCR assay detects as few as 10 infecting units. Host cell DNA has no effect on sequence-specific amplification, and there was no amplification of the DNA of microorganisms that cause clinical symptoms similar to those of Q fever or that are commonly found in a laboratory for clinical microbiology. Our studies revealed that DNA extraction and purification from *C. burnetii* were not necessary in the PCR assay. In fact, DNA extracted from a given quantity of *C. burnetii* produced a PCR band with the same intensity as the band produced by the same quantity of organisms which had been boiled for 10 min prior to PCR assay and used without further processing. Consequently, the PCR results can be obtained in a timely fashion, generally in less than 6 h.

PCR is useful for detection of *C. burnetii* in early shell-vial cultures for diagnosis of both acute and chronic infections (19) and also for detection of bacteria in clinical specimens (heart valves). This is very important because prompt antibiotic treatment may lead to a better prognosis for individuals suffering from Q fever. Because PCR needs no viable

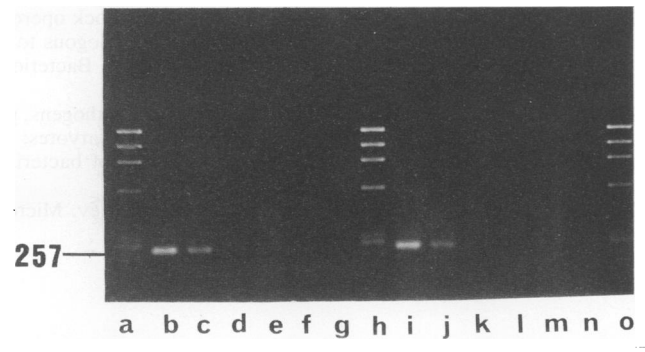

FIG. 4. Detection limits of *C. burnetii* organisms by PCR followed by agarose gel electrophoresis. Serial decimal dilutions (10^{-4} to 10^{-8}) of an initial inoculum of 10^{-6} infecting units (reference strains Nine Mile and GQ 212) were amplified as described in the text. Lanes a, h, and o, molecular size markers (ϕX174 cleaved with *Hae*III); lanes g and n, reagent control; lanes b to f, Nine Mile in dilutions from 10^{-4} to 10^{-8}, respectively; lanes i to m, GQ 212 in dilutions of 10^{-4} to 10^{-8}, respectively.

microorganisms, it is better for retrospective diagnosis on stored samples (frozen tissues and frozen cell cultures) than standard culture techniques. The PCR also has utility in management of chronic infections due to *C. burnetii*. In fact, in patients with chronic Q fever and undergoing an antibiotic regimen, the possible persistence of microorganisms after several months of treatment needs to be documented and would guide further therapeutic treatments.

Moreover, for treated patients, the antibiotic activity in clinical specimens can inhibit the multiplication of *C. burnetii* in cell culture or embryonated eggs, although the bacteria can be easily detected by gene amplification. In veterinary pathology, PCR could also be a simple and rapid detection assay for *C. burnetii* (milk, urine, and placenta samples).

We believe that our pair of primers would not only be very efficient for the detection and identification of *C. burnetii* by PCR in other research laboratories but also could serve as a valuable tool for diagnosis in clinical microbiology laboratories.

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