# EVALUATION OF PCR AND NESTED PCR ASSAYS CURRENTLY USED FOR DETECTION OF *COXIELLA BURNETII* IN JAPAN

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**Abstract.** Detection of *Coxiella burnetii*, the etiologic agent of Q fever, is important for diagnosis of Q fever. PCR-based methods have been widely used for the detection mostly because isolation of *C. burnetii* is time-consuming. Recent reports showed that PCR-positive rates of Q fever infection widely differed. We have evaluated the PCR and nested PCR assays currently used in Japan. The nested PCR assay detected as few as 6 microorganisms and was 10 times more sensitive than the regular PCR assay. The nested PCR assay did not show any non-specific bands with 12 other bacteria, whereas the PCR assay showed some extra bands for 5 of the 12 bacteria. These results suggest that the nested PCR is more sensitive and specific than the PCR in the detection of *C. burnetii*. However, nested PCR generally has a risk of cross-contamination during preparation of the 2nd PCR. Using blood specimens serially collected from an acute Q fever patient, the PCR and the nested PCR assays gave very similar results, suggesting that sensitivity of the PCR assay is at an achieved level of the detection for clinical specimens although the nested PCR assay is more sensitive. It is recommended that both the PCR and nested PCR assays should be performed for the detection of *C. burnetii* to obtain reliable results.

## INTRODUCTION

Q fever is an important zoonosis in many areas of the world (Marrie, 1990). *Coxiella burnetii*, the etiologic agent of Q fever, is an intracellular bacterium similar to rickettsia, but it can survive in a dry and harsh environment (Williams and Thompson, 1991). Prevalent sources of human infection are domestic and companion animals: cows, goats, ewes and cats (Dupuis *et al*, 1987; Rauch *et al*, 1987; Embil *et al*, 1990; Lyytikainen *et al*, 1998; Hatchette *et al*, 2001). *C. burnetii* is contained in urine, stool, and milk, and is most heavily in amniotic fluid and placenta of the infected animals. Humans are usually infected by inhalation of aerosol and dust containing *C. burnetii* in a contaminated environment.

Detection of *C. burnetii* DNA by PCR-based methods has been used for diagnosis of Q fever and investigation of infectious sources. PCR assay designed for the superoxide dismutase gene (Stein and Raoult, 1992) and nested PCR assay designed for the 29kDa outer membrane gene (Zhang *et al*, 1998) have been mainly used. The positive rates of *C. burnetii* infection determined by PCR-based methods in atypical pneumonia and healthy populations differed among reports. Interestingly the positive rates were higher in the reports from Japan than in those from other countries (To *et al*, 1996; Musso and Raoult, 1997; Kato *et al*, 1998). A more resent report has suggested that the PCR-based methods using blood samples are prone to false positive results (Maurin and Raoult, 1999). In the present study, we evaluated the sensitivity and specificity of the PCR and the nested PCR assays and examined some clinical specimens by using the two methods.

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## MATERIALS AND METHODS

### PCR assay

A pair of primers [CB1 (5′-ACT CAA CGC ACT GGA ACC GC-3′) and CB2 (5′-TAG CTG AAG CCA ATT CGC C-3′)] designed for the *C. burnetii* superoxide dismutase gene was used according to the previous report (Stein and Raoult, 1992). A total of 30 μl of reaction mixture contained 3 μl of DNA sample, 30 pmol of each

primer, 3 μl of 10xPCR buffer, 3 μl of 10xdNTP mixture and 0.75 U of AmpliTaq Gold (Applied Biosystems, Foster City, California, USA). After heating at 95ºC for 10 minutes, the annealing temperature was changed from 60ºC to 50ºC by 1ºC per cycle during the initial 10 cycles. This step called 'touch down' was an additional innovation to the original protocols of amplification. After the touch down step, this was followed by 35 cycles of amplification of denaturation at 95ºC for 30 seconds, annealing at 50ºC for 20 seconds and a final extension at 72ºC for 1 minute, and sequence extension at 72ºC for 5 minutes. The amplification was performed in a thermalcycler, PC-806 (ASTEC, Fukuoka, Japan). The products of PCR were subjected to electrophoresis on 1.5% agarose gels and visualized by ethidium bromide staining.

**Nested PCR assay**

Two pairs of primers [OMP1 (5′-AGT AGA AGC ATC CCA AGC ATT G-3′) and OMP2 (5′-TGC CTG CTA GCT GTA ACG ATT G-3′) for 1st PCR, and OMP3 (5′-GAA GCG CAA CAA GAA GAA CAC-3′) and OMP4 (5′-TTG GAA GTT ATC ACG CAG TTG-3′) for 2nd PCR] designed from the *C. burnetii* 29 kDa outer membrane protein gene were used according to the previous report (Zhang *et al*, 1998). A total of 30 μl of mixture contained 3 μl of DNA samples (1 μl of 1st PCR reaction mixture in 2nd PCR), 30 pmol of each primer, 3 μl of 10 x PCR Buffer, 3 μl of 10 x dNTP and 0.75 U of Takara EX Taq (Takara Shuzo, Shiga, Japan). PCR was performed under conditions shown in Table 1. In the 1st PCR, after heating at 94ºC for 3 minutes, the annealing temperature was changed from 63ºC to 53ºC by 1ºC per cycle during the initial 10 cycles, a 'touch down' step. After the touch down step, this was followed by 25 cycles of amplification consisting of denaturation at 94ºC for 30 seconds, annealing at 53ºC for 30 seconds and extension at 72ºC for 1 minute, and a final sequence extension at 72ºC for 5 minutes. In the 2nd PCR, after heating at 94ºC for 3 minutes, 35 cycles of amplification consisting of denaturation at 94ºC for 30 seconds, annealing at 53ºC for 30 seconds and extension at 72ºC for 1 minute, and a final sequence extension at 72ºC for 5 minutes. Electrophoresis and visualization of PCR products were performed as described in the section of PCR assay.

**Growth and purification of *C. burnetii***

*C. burnetii* Nine Mile phase II was propagated in Vero cells in MEM containing 3% fetal calf serum at 37ºC and 5% $CO_2$. After the cytopathic effect was observed, a portion of the cells were collected and stained by indirect immunofulorescence assay (IF) using a convalescent serum from the acute Q fever patient and FITC-conjugated anti-human IgG antibody (BioSource International, Camarillo, California, USA). When at least 90% of the cells were positive for *C. burnetii*, all the cells were harvested and *C. burnetii* was purified according to the previous report (Ho *et al*, 1995). The solution of the purified microorganisms was serially diluted 10-fold and 5 μl aliquot of each dilution was placed in each well of a multiwell-glass plate. The number of *C. burnetii* in each well was counted after IF staining and the concentration of the microorganism was calculated.

Table 1
Comparison of the PCR and nested PCR in the detection of *Coxiella burnetii* from blood samples of a Q fever patient[a].

| | Primers | | 12[b] | 14 | 15 | 17 | 18 | 20 | 21 | 30 | 47 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| PCR | CB1-2 | Serum | ++ | ++ | ++ | + | - | + | - | - | - |
| | | Buffy coat | NT | NT | NT | NT | NT | NT | ++ | - | - |
| Nested PCR | OMP1-2 and 3-4 | Serum | ++ | ++ | ++ | + | + | + | - | - | - |
| | | Buffy coat | NT | NT | NT | NT | NT | NT | ++ | - | - |

[a]These results were obtained in the separated tests. Each symbol shows as follows; ++: both results were positive, +: one of the results was positive, -: both results were negative, NT: Not tested. [b]Days after onset of the disease

**Sensitivity of PCR and nested PCR assays**

Solutions of purified *C. burnetii* were prepared ranging from $1x10^6$ to 1 microorganisms/100 μl. DNA was extracted by QIAamp tissue kit (Qiagen, Tokyo, Japan), according to the instruction manual. Two hundred μl of the solution at each microorganism concentration were used, and the extracted DNA was dissolved in 100 μl of distilled water. Three μl of the DNA solution were used in the PCR and nested PCR assays. Results are shown as number of microorganisms in one PCR-tube (microorganisms/PCR-tube) and in 200 μl of sample (microorganisms/sample).

**Specificity of PCR and nested PCR assays**

DNA samples of *C. burnetii* and 12 other bacteria were used in the PCR and the nested PCR assays in order to evaluate specificity. Bacterial DNA were kindly provided by Dr A Wada, Department of Bacteriology 1, National Institute of Infectious Diseases, Japan. The bacteria used in the experiments were *Chlamydia pneumoniae, Hemophilus influenzae, Legionella pneumophila, Mycobacterium avium, M. gondonae, M. intracellulare, M. kansasii, M. tuberculosis, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus pneumoniae,* and *S. pyogenes*.

**DNA extraction from clinical samples**

DNA was extracted from serum and buffy coat sample of an acute Q fever patient by PUREGENE BLOOD KIT (Gentra systems, Minneapolis, Minnesota, USA), according to the instruction manual.

## RESULTS

**Sensitivity of the PCR and nested PCR assays**

Sensitivity was compared between the PCR and the nested PCR assays in the detection of *C. burnetii* DNA. The PCR assay with the CB1 and 2 primers detected 60 microorganisms/PCR-tube, equivalent to $2x10^3$ microorganisms/sample, whereas the nested PCR assay with two pairs of primers, OMP1 and 2, and OMP3 and 4, detected 6 microorganisms/PCR-tube equivalent to $2x10^2$ microorganisms/sample (data not shown), indicating that the nested PCR assay was 10 times more sensitive than the PCR assay.

**Specificity of the PCR and the nested PCR assays**

Twelve other bacteria as well as *C. burnetii* were examined by the PCR and the nested PCR assays. In the PCR assay with CB1 and 2 primers, some non-specific bands were observed with *Staphylococcus aureus, Legionella pneumophila, Hemophilus influenzae, Streptococcus pneumoniae* and *Chlamydia pneumoniae* (Fig 1). All the bands were determined to be non-specific, because the sizes were different from the specific band of *C.burnetti*. On the other hand, in the nested PCR assay with two pairs of primers, OMP1 and 2, and OMP3 and 4, no band was observed with any of the 12 bacteria, except for one band with the expected size of *C. burnetii*.

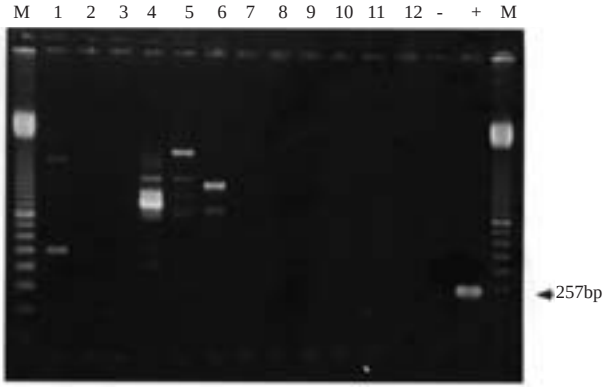


Fig 1–PCR assay with *Coxiella burnetii* and twelve other bacteria. PCR assay was performed with primers of CB1 and CB2 as described in Material and Methods. Lanes; 1, *Staphylococcus aureus*; 2,*Streptococcus pyogenes*; 3, *Pseudomonas aeruginosa*; 4, *Legionella pneumophila*; 5, *Haemophilus influenzae*; 6, *Streptococcus pneumoniae*; 7, *Mycobacterium tuberculosis*; 8, *Mycobacterium intracellulare*; 9, *Mycobacterium avium*; 10, *Mycobacterium kansasii*; 11, *Mycobacterium gondonae*; 12, *Chlamydia pneumoniae*; -, negative control; +, *Coxiella burnetii*; M, 100bp DNA ladder marker. 1.5% agarose gel was used.

**Detection of *C. burnetii* DNA from clinical specimens**

Serum and buffy coat samples serially collected from one Q fever patient were tested for *C. burnetii* DNA by the PCR and the nested PCR assays (Table 1). In the nested PCR assay, serum samples collected on 12, 14, and 15 days after onset of the disease were positive, whereas those on day 17 and 18 were positive in one of separate tests. Buffy coat sample collected on day 20 after onset was positive, although serum sample on the

same day was negative. In the PCR assay, similar results were obtained except that serum sample on day 18 after onset was negative. Non-specific bands were not observed in any of the tests.

## DISCUSSION

In this study, we evaluated the PCR and the nested PCR assays that were previously reported and widely used in Japan. The nested PCR assay detected as few as 6 microorganisms/PCR-tube equivalent to $2x10^2$ microorganisms/sample. The nested PCR assay was 10 times more sensitive than the PCR assay, and did not show any non-specific bands with 12 other bacteria, whereas some extra bands were observed with several bacteria in the PCR assay. These results suggest that the nested PCR assay is more sensitive and specific for detection of *C. burnetii*.

When serum and buffy coat samples serially collected from one Q fever patient were tested, *C. burneti* DNA was detected at very similar sensitivity by either technique. This result suggests that sensitivity of PCR assay is at an achieved level of the *C. burnetii*-detection from clinical specimens. As nested PCR assay has a risk of cross-contamination during preparation of the 2nd PCR, it is recommended that the PCR assay should be performed along with the nested PCR assay to obtain more reliable results.

In our experiments using human serum samples, *C. burnetii*-DNA was detected in 50 μl of serum sample collected at acute phase. Interestingly, *C. burnetii* DNA was detected in the buffy coat sample but not in the serum sample on day 20 after onset of the illness, suggesting that detection of *C. burnetii* is more sensitive in the buffy coat than in serum. Although no non-specific band was detected in the present study, PCR-based methods sometimes amplified non-specific bands from human genome that contained in clinical specimens. A previous report has shown that false positive results are sometimes observed in PCR using blood samples (Maurin and Raoult, 1999 ). It is recommended that subsequent DNA-sequencing assay should be performed using positive case of PCR or/and nested PCR products to get the most reliable results.

In conclusion, it is recommended that both the PCR and the nested PCR assays should be performed for the detection of *C. burnetii* to get reliable results. In future study, further validation should be done using more clinical samples.

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