Evaluation of Coxiella burnetii Antibiotic Susceptibilities by Real-Time PCR Assay

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Received 22 October 2002/Returned for modification 18 January 2003/Accepted 13 February 2003

Coxiella burnetii is an obligate intracellular bacterium. The inability to cultivate this organism on axenic medium has made calculation of infectious units challenging and prevents the use of conventional antibiotic susceptibility assays. A rapid and reliable real-time PCR assay was developed to quantify C. burnetii cells from J774.16 mouse macrophage cells and was applied to antibiotic susceptibility testing of C. burnetii Nine Mile, phase I. For calculation of bacterial replication, real-time PCR performed equally as well as immunofluorescent-antibody (IFA) assay when J774.16 cells were infected with 10-fold serial dilutions of C. burnetii and was significantly (P < 0.05) more repeatable than IFA when 2-fold dilutions were used. Newly infected murine macrophage-like J774.16 cells were treated with 5 μg of chloramphenicol per ml, 4 μg of tetracycline per ml, 4 μg of rifampin per ml, 4 μg of ampicillin per ml, or 1 μg of ciprofloxacin per ml. After 6 days of treatment, tetracycline, rifampin, and ampicillin significantly (P < 0.01) inhibited the replication of C. burnetii, while chloramphenicol and ciprofloxacin did not. In general, these results are consistent with those from prior reports on the efficacy of these antibiotics against C. burnetii Nine Mile, phase I, and indicate that a real-time PCR-based assay is an appropriate alternative to the present methodology for evaluation of the antibiotic susceptibilities of C. burnetii.

Coxiella burnetii, the etiologic agent of Q fever, is an obligate intracellular bacterium. C. burnetii is widely distributed in nature and infects a variety of mammals, birds, reptiles, fish, and ticks (19). In humans, infection is usually the result of inhalation of contaminated aerosols associated with infected sheep, goats, and, to a lesser extent, cattle. The prevalence of C. burnetii infections has been difficult to determine, in part, due to the lack of surveillance. One recent retrospective study indicated that seropositivity rates in Japan were greater than 20% in at-risk individuals (7). Q fever may manifest as either an acute or chronic illness (13). Acute disease commonly presents as a self-limiting influenza-like illness accompanied by fever and severe headaches, and the prognosis is usually favorable (16). Recovery from acute infections usually occurs within 1 to 2 weeks and can be accelerated with antibiotics such as tetracyclines. However, diagnosis of acute infections often occurs too late for tetracyclines to be effective, and misdiagnoses can lead to inappropriate treatment or a lack of treatment altogether (10, 24). Chronic disease can be life threatening and most often presents as endocarditis or hepatitis (13). Even with antimicrobial intervention, mortality rates have been reported to be as high as 24% (2). The most effective treatment of chronic Q fever includes a combination of doxycycline and chloroquine administered for at least 18 months (13). In cases of tetracycline intolerance or contraindication, alternative and often less effective antibiotics are used (13). The pursuit of new and more effective treatments for Q fever remains an area of active research. Two recent studies based on in vitro sensitivities indicated that certain macrolides and trovafloxacin were good potential candidates for the treatment of Q fever (5, 6).

One of the challenges investigators face when determining C. burnetii antibiotic sensitivities is calculating bacterial numbers. In many intracellular bacterial infection models, infectious units can be measured by plating lysates on semisolid media for CFU determination or performing an agar overlay for PFU determination. However, C. burnetii has yet to be cultivated on axenic medium, and a plating system (23) has been difficult to adapt to most laboratories. Thus, the inability to cultivate this bacterium on axenic medium prevents the use of conventional assays to test antibiotic susceptibilities. Therefore, three different systems have been evaluated to determine the antibiotic susceptibilities of C. burnetii, including an animal model (8), an embryonated egg model (9), and several cell culture models (26). These methods are time-consuming and difficult to use for testing of multiple antibiotics with multiple clinical isolates. In order to overcome this limitation, a shell vial assay was developed that used immunofluorescent-antibody (IFA) assay to determine the bacteriostatic and bactericidal activities of antibiotics (17). The disadvantages of this technique are that it is labor-intensive and results must be determined by blinded scoring because of the subjectivity of the test. Therefore, techniques based at the molecular level have the potential to be more efficient methods for determination of the antimicrobial susceptibilities of C. burnetii.

In this report, we describe the application of a SYBR Green I dye-based real-time PCR assay to antibiotic susceptibility testing of C. burnetii. This assay provides a rapid and sensitive method for determination of C. burnetii antibiotic sensitivities and eliminates the subjectivity associated with other methods.
MATERIALS AND METHODS

Oligonucleotide primers. Primers FAF216 (5’-GCACATTATTAGCCGGAACCTT-3’) and RAF290 (5’-TGAGGAGAAAACTGGATTGAGA-3’), which amplify a 74-bp fragment of the C. burnetii com1 gene (GenBank accession no. AF318146), which is highly conserved among 21 C. burnetii strains (27), were selected by using Primer Express software (PE Applied Biosystems, Foster City, Calif.). The primers were synthesized by the Gene Technologies Laboratory at Texas A&M University, College Station.

Preparation of standard curve. To construct a standard curve, total genomic DNA was purified from C. burnetii Nine Mile, phase I (RSA 493), which is considered the representative strain for acute infections (10, 18). The DNA concentration was measured spectrophotometrically and converted to genome copy numbers by using the molecular weight of DNA. Briefly, the weight of one C. burnetii genome copy (in grams) was calculated by multiplying the size of the C. burnetii genome (2.1 × 10^6 bp) by the average mass of a DNA base pair (615 Da) (20) and then multiplying this number by the weight of 1 atomic mass unit (1.67 × 10^-24 g) (22). This number was then divided into the concentration of the total genomic DNA to determine how many genome copies were in each microliter of the genomic DNA. Tenfold serial dilutions ranging from 10^7 to 10^1 genome copies were then made.

PCR assay conditions. Real-time PCR was performed with an ABI Prism 7700 sequence detector (PE Applied Biosystems) according to the empirical design of the manufacturer. Briefly, template DNA was added to a reaction mixture containing 0.3 μM each primer, 12.5 μl of 2× SYBR Green I PCR Master Mix (PE Applied Biosystems), and 8 μl of distilled H2O in a final volume of 25 μl. All reactions were carried out in 96-well plates. After initial activation of AmpliTaq Gold DNA polymerase at 95°C for 10 min, 40 PCR cycles of 95°C for 15 s and 60°C for 1 min were performed. Cycle threshold (Ct) values were determined by using cycles 3 to 9 as the baseline. Assay specificity was confirmed by subjecting the PCR products to agarose gel electrophoresis and SYBR Green I melting.

FIG. 1. Establishment of the standard curve for C. burnetii quantification. (A) Amplification plots of C. burnetii standards. Genome concentrations are (from left to right) 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1. For each dilution the normalized fluorescence signal (ΔRn) is plotted against the PCR cycle number. (B) Standard curve generated from the Ct values of the amplification plots with ABI Sequence Detection software. This curve represents the standard curve only; no unknowns are represented in this graph.
curve analysis by using three separate holds of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s, with a ramp time of 19 min and 59 s from the second to the third holding temperatures.

Extraction of *C. burnetii* DNA from infected tissue culture cells. *C. burnetii* DNA was extracted from murine macrophage-like J774.16 cells by resuspending cell pellets in 200 μl of lysis buffer (6.25 ml of 2 M Tris [pH 7.5], 1 ml of 0.5 M EDTA, 50 mg of glucose, and 200 mg of lysozyme brought to a final volume of 50 ml with distilled H₂O) plus 10 μl of 20 mg of proteinase K per ml for 60°C. Following this treatment, 21 μl of 10% sodium dodecyl sulfate was added, and the mixture was incubated for 1 h at room temperature. DNA was recovered by using the High Pure PCR template preparation kit (Roche Molecular Biochemicals, Indianapolis, Ind.).

Comparison of real-time PCR to IFA. J774.16 mouse macrophages were plated at a concentration of 10⁵ cells/ml in 24-well tissue culture plates (Becton Dickinson, Franklin Lakes, N.J.) with or without coverslips and incubated for 4 h at 37°C in 5% CO₂ for attachment. The cells were then inoculated with either 10- or 2-fold serial dilutions of *C. burnetii* Nine Mile, phase I (RSA 493), and incubated overnight at 37°C in 5% CO₂ to allow attachment. The medium was removed, and the monolayers were inoculated with *C. burnetii* Nine Mile, phase I (RSA 493), diluted 1:500 in DMEM, and incubated overnight. Infected cells were washed into wells containing coverslips. The wells were washed three times with warm Dulbecco modified Eagle medium with 1-glutamine and supplemented with 10% fetal bovine serum (DMEM; Fisher Scientific, Houston, Tex.), fixed with 2% paraformaldehyde–100% methanol–1% toluene, and washed once with phosphate-buffered saline (PBS). Five hundred microliters of rabbit anti-*C. burnetii* Nine Mile antiserum diluted 1:300 in PBS–2% normal goat serum was added, and the mixture was incubated at room temperature for 1 h, followed by three 5-min washes in PBS. Five hundred microliters of Alexa Fluor 488 goat anti-rabbit immunoglobulin G (heavy and light chains; Molecular Probes, Eugene, Oreg.) diluted 1:300 in PBS–2% normal goat serum was added, and the mixture was incubated at room temperature for 1 h. Coverslips were washed three times for 5 min each time in PBS, mounted on slides, and examined by fluorescence microscopy. The IFA result was expressed as an infection index, which is the product of the mean number of bacteria per infected cell and the percentage of infected cells multiplied by 100 (3).

Antibiotic susceptibility testing. J774.16 mouse macrophages were plated at a concentration of 10⁵ cells/ml in 24-well tissue culture plates and incubated for 4 h at 37°C in 5% CO₂ to allow attachment. The medium was removed, and the monolayers were inoculated with *C. burnetii* Nine Mile, phase I (RSA 493), diluted 1:500 in DMEM, and incubated overnight. Infected cells were washed

![Fluorescence](image-url)

**FIG. 2.** Determination of PCR specificity. (A) Melting curve analysis performed with ABI Dissociation Curve software revealed that the com1-specific primer pair amplified a single predominant product with a melting temperature of 81.3°C. (B) Agarose gel electrophoresis of the amplified product with ethidium bromide staining shows the presence of a single band, confirming the specificity of the PCR assay. Lane 1, 100-bp molecular mass marker; lane 2, amplified product from a *C. burnetii* DNA standard dilution of 10⁷; lane 3, blank; lane 4, amplified product from a 1:200 dilution of the *C. burnetii* inoculum.
SYBR Green I dye and the limits of resolution of agarose gel
decrease in the mobility of the fragment as a result of bound
and the size of the band seen in the gel is likely due to a
74-bp product. This discrepancy between the predicted size
with Primer Express software were predicted to amplify a
con of approximately 100 bp (Fig. 2B). The primers designed
(Fig. 2A). Specificity was confirmed by agarose gel elec-
tron microscopy, which demonstrated the presence of a single ampli-
clon of approximately 100 bp (Fig. 2B). The primers designed
with Primer Express software were predicted to amplify a
74-bp product. This discrepancy between the predicted size
and the size of the band seen in the gel is likely due to a
decrease in the mobility of the fragment as a result of bound
SYBR Green I dye and the limits of resolution of agarose gel
electrophoresis.

The precision of the assay was determined by measuring $C_\text{t}$
values for eight replicates of the standards (Table 1). The
results represent independent dilution series and different
PCR runs. The mean $C_\text{t}$, standard deviation, and percent CV
were calculated for each template concentration. The results
showed low variability, with CVs ranging from 1.2 to 5.6%,
indicating that the assay was efficiently reproducible.

To further validate the assay, tissue culture cells were inocu-
lated with either 10- or 2-fold dilution series of C. burnetii,
icubated overnight, and quantified by both IFA and real-time
PCR. When 10-fold dilutions from 1:100 to 1:100,000 were
used, both IFA and real-time PCR results showed strong linear
relationships ($r = 0.99$) (Fig. 3A). The repeatability of each
assay was determined by calculating the percent CV at each
dilution. The CVs ranged from 7.1 to 33% and from 10.3 to
55% for the real-time PCR and IFA, respectively (Table 2).
These results show that the repeatabilities of the real-time
PCR and IFA were similar at dilutions of 1:100 and 1:1,000,
but as the number of infectious organisms decreased, the CV
of IFA was considerably greater. The CVs for the real-time
PCR at 1:10,000 and 1:100,000 dilutions of the inoculum were
12.5 and 33%, respectively, whereas for IFA they were 44 and
53%, respectively. At twofold dilutions of the inoculum from
1:200 to 1:1,600, the linearities of both assays were again sim-
ilar ($r = 0.84$) (Fig. 3B). CVs ranged from 3.6 to 13% and from
8.8 to 26% for the real-time PCR and IFA, respectively (Table
2). Again, as the number of infectious organisms decreased,
the repeatability of IFA was not as consistent as that of the
real-time PCR assay. The CVs for the real-time PCR at 1:800
and 1:1,600 dilutions of the inoculum were 13 and 3.6%, re-

TABLE 1. Summary of eight different PCR runs performed
on eight separate dilution series

<table>
<thead>
<tr>
<th>Conc (no. of copies/µl)</th>
<th>Mean $C_\text{t}$</th>
<th>SD *</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^7$</td>
<td>10.68</td>
<td>0.35</td>
<td>3.3</td>
</tr>
<tr>
<td>$10^6$</td>
<td>13.52</td>
<td>0.16</td>
<td>1.2</td>
</tr>
<tr>
<td>$10^5$</td>
<td>17.08</td>
<td>0.21</td>
<td>1.2</td>
</tr>
<tr>
<td>$10^4$</td>
<td>20.82</td>
<td>0.61</td>
<td>2.9</td>
</tr>
<tr>
<td>$10^3$</td>
<td>24.72</td>
<td>1.39</td>
<td>5.6</td>
</tr>
<tr>
<td>$10^2$</td>
<td>28.92</td>
<td>1.63</td>
<td>5.6</td>
</tr>
<tr>
<td>$10^1$</td>
<td>31.61</td>
<td>0.84</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* SD, standard deviation of eight replicates.

three times with warm DMEM to remove noninternalized bacteria and were
then treated with antibiotics or sham treated. The concentrations of each of the
antibiotics used were as follows: chloramphenicol, 8 µg/ml; tetracycline, 4 µg/ml;
rifampin, 4 µg/ml; ampicillin, 4 µg/ml; and ciprofloxacin, 1 µg/ml. These con-
centrations are the lower critical concentrations according to the French Anti-
biogram Committee (1) and were previously reported to have bacteriostatic
effects against C. burnetii (17, 21). Fresh medium with antibiotics was added on
days 1, 2, 3, 4, 5, and 6 postinoculation. Sham-treated cells received DMEM only
and served as a reference to create growth curves for treated samples. Samples
for real-time PCR were collected on days 1, 2, 4, and 7 postinoculation.

Statistical analysis. Significant differences between methods and between
antibiotic treatment groups were assessed by the paired Student $t$ test. The
repeatability of each method (i.e., the variability of a method when repeated
measures are taken within a single experiment) was estimated by computing the
percent coefficient of variation (CV; the ratio between the standard deviation
and the mean of the repeated measurements multiplied by 100).

RESULTS

Prior to application of the real-time methodology to antimicrobial
sensitivity testing of C. burnetii, several steps were
taken to validate the assay. First, a BLAST search of the
ENTREZ database indicated that oligonucleotide primers
FAF216 and RAF290 were specific for comI. Tenfold serial
dilutions of purified C. burnetii genomic DNA were used to
construct a standard curve, from $10^7$ copies at the start to $10^1$
copies. Figure 1A presents typical amplification plots for these
standards. A standard curve was then generated from the $C_\text{t}$
of the amplification plots with ABI Sequence Detection soft-
ware (Fig. 1B). The slope of the standard curve was $-3.297$,
indicating that the efficiency of the PCR was approximately
100%, according to the equation $E = 10^{-\text{slope}} - 1$, where $E$
is the run efficiency and $s$ is the slope of the standard curve (4).

Melting curve analysis revealed that the primer pair ampli-

FIG. 3. Validation of real-time PCR by comparison to IFA. The
results are represented as the means and standard errors of three
replicates. (A) Tenfold dilution series; (B) twofold dilution series.
Circles, real-time PCR data; diamonds, IFA data.
spectively, whereas for IFA they were 26 and 25%, respectively.

Real-time PCR was used to evaluate the effectiveness of antibiotics previously shown to be bacteriostatic for *C. burnetii* Nine Mile, phase I, by the shell vial assay. Newly infected J774.16 cells were treated for 6 days with 8 μg of chloramphenicol per ml, 4 μg of tetracycline per ml, 4 μg of rifampin per ml, 4 μg of ampicillin per ml, or 1 μg of per ciprofloxacin ml, which are the lower critical bacteriostatic concentrations (1). Total DNA was extracted and antibiotic efficacies were determined. Sham-treated infected cells served as positive controls for *C. burnetii* replication. For each treatment or control group, J774.16 cell viabilities were greater than 90%. The results of *C. burnetii* antibiotic susceptibility testing are shown in Fig. 4. After 6 days of treatment, tetracycline, rifampin, and ampicillin significantly inhibited the replication of the bacteria (*P* < 0.01) while chloramphenicol and ciprofloxacin did not inhibit replication compared to the results for the sham-treated controls. Of the bacteriostatic antibiotics, rifampin was the most effective, followed by tetracycline and ampicillin.

DISCUSSION

The results of this study demonstrate that a real-time PCR assay can be used for the rapid and reliable determination of *C. burnetii* antimicrobial susceptibilities without the subjectivity associated with microscopic enumeration methodologies. The use of *com1* as a target gene allowed the specific amplification of *C. burnetii* DNA over a 7-log DNA concentration range. The specificity of the assay was confirmed by a search for primer-specific sequences with the BLAST program, melting curve analysis, and agarose gel electrophoresis of the PCR-amplified products. Comparison of the real-time PCR and IFA revealed that the real-time PCR assay was more repeatable. No statistically significant difference (*P* > 0.05) was found between the percent CVs obtained by the real-time PCR assay and those obtained by the IFA method when 10-fold dilutions were used. However, when twofold dilutions were used, the real-time PCR assay did perform significantly better (*P* < 0.05). With the 10- and 2-fold dilution series of the *C. burnetii* inoculum, the real-time assay consistently detected more bac-
We thank Laurie Davidson for technical assistance and Jon Skare, Vernon Tesh, and Renée Tsolis for critical review of the manuscript.

REFERENCES


