Diagnosis of Quinolone-Resistant *Coxiella burnetii* Strains by PCR-RFLP

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A total of 12 strains of *Coxiella burnetii* (8 Greek isolates from acute Q-fever patients, two reference strains—Nine Mile and Q212—and two pefloxacin-resistant laboratory strains) were examined for the presence of point mutations in the quinolone resistance determining region (QRDR) of *gyrA* gene by direct DNA sequencing of the polymerase chain reaction (PCR)-amplified fragments. The gene sequences of all eight Greek isolates and the two reference strains Nine Mile and Q212 [minimal inhibitory concentration (MIC) ≤ 4 $\mu\text{g/ml}$] were identical. Direct DNA sequencing of the in vitro-selected resistant strains (MICs to pefloxacin, 8–32 $\mu\text{g/ml}$) re-

vealed a transition (G $\rightarrow$ A) at the corresponding codon 87 of *E. coli*. This mutation lead to the substitution of Glu (codon GAG) by Lys (codon AAG). Restriction maps of amplified *gyrA* gene sequences were determined by GCG Wisconsin PACKAGE, and the *MnI* restriction enzyme was found to cut only the sensitive strains sequences and not the resistant ones. The present PCR-RFLP analysis has proved to be a simple, rapid, and useful method for the detection of *Coxiella burnetii* and, at the same time, for the diagnosis of quinolone-resistant *Coxiella burnetii* strains. J. Clin. Lab. Anal. 14:59–63, 2000. © 2000 Wiley-Liss, Inc.

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INTRODUCTION

Coxiella burnetii is the etiological agent of human Q fever. Two major forms of the disease are known: acute and chronic. The acute form of the disease is usually a self-limiting acute febrile illness during which pneumonia or hepatitis may occur. The chronic form is, however, a severe disease in which endocarditis predominates, but in some cases, vascular, aneurysm, or bony infections may occur (1,2). For acute Q fever, bacteriostatic antibiotics are useful in helping a patient recover, but to cure a patient with chronic Q fever, a physician should use a bactericidal antibiotic regimen (2). Various antibiotic regimens, including the quinolones, have been proposed for the treatment of acute Q fever, but none of these antibiotics is bactericidal in vitro. This fact may explain failures in treating chronic Q fever infections (2,3). The optimal duration of antibiotic therapy of chronic Q fever remains undetermined, since treatment for one year as well as for indefinite periods have been suggested (2). It is very important to monitor the possible development of resistance during these long periods of treatment.

New fluoroquinolones are known as broad-spectrum antimicrobial agents (4–6). In gram-negative bacteria three mechanisms of quinolone resistance have been described, including alterations in the DNA gyrase structure, decreased outer mem-

brane permeability, and export through an active efflux system (7). Inversely, in gram-positive bacteria such as *Staphylococcus aureus* (8), and *Streptococcus pneumoniae* (9,10), GyrA mutations could be detected only when a ParC mutation was present, indicating that, in these gram-positive bacteria, topoisomerase IV was the primary target of ciprofloxacin (8–11). In *Escherichia coli* (12), *Neisseria gonorrhoeae* (13), and *Klebsiella pneumoniae* (14), DNA gyrase is thought to be the primary target of quinolones, since amino acid changes in ParC or ParE could be detected only when GyrA mutations were present (11).

Mutations in *gyrA* gene lead to alterations of the GyrA protein and usually occur between nucleotides 199 to 318 of the *gyrA* gene sequence in *E. coli* (coding for amino acids 67 to 106 of the *E. coli* GyrA protein) (15,16).

Little is known about the mechanisms of resistance to fluoroquinolones in *C. burnetii*. However, a high level of resistance to ciprofloxacin has been associated with a nucleotide mutation in *gyrA* (16).

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The identification of resistance gene mutations could be very important for a better treatment of many diseases. Various methods have been reported for the detection of single nucleotide change of genes, including direct sequencing (17), sequence-specific oligonucleotide probe hybridization (18), and nonradioisotopic single-strand conformation polymorphism (6). Although these methods have been proved to be useful, they are unsatisfactory for a routine clinical laboratory or for the performance of epidemiological studies.

For first time, we set up a PCR-RFLP analysis for the detection of point mutation in pefloxacin resistant *C. burnetii* strains. Specific primers were used to amplify a defined region of 261 bp of the *C. burnetii* *gyrA* gene in susceptible and resistant strains. Comparing the sequences of the amplified *gyrA* gene fragment for each strain, we detected a point mutation. Restriction maps were determined for each sequence and further we developed a PCR-RFLP assay, which is able to identify and discriminate between resistant and sensitive *C. burnetii* strains. This methodology can be performed routinely in diagnostic laboratories and is faster than other methods such as the ones mentioned above.

MATERIALS AND METHODS

Bacterial Strains and Cultivation

Strains CB1 to CB8 were isolated in our laboratory from patients with acute Q fever (19). *C. burnetii* Q212 and Nine Mile are reference strains for Q fever. All isolates of *C. burnetii* were grown in Vero cells. Infected cells were cultured in minimum essential medium (MEM) supplemented with 4% fetal calf serum and 2 mM glutamine at 37°C in a 5% CO₂ incubator (19). Infection was monitored by Gimenez staining (20).

The two pefloxacin-resistant *C. burnetii* strains RCB2 (MIC 32 µg/ml) and the RCB4 (MIC 64 µg/ml), were derived from pefloxacin susceptible clinical strains SCB2 (MIC 1 µg/ml) and SCB4 (MIC 4 µg/ml), respectively, by increasing concentrations of pefloxacin in the medium. Vero cells were inoculated and cultured with the medium free of antibiotic until 90% of the cells were infected. The supernatant was removed, and infected cells were harvested, and centrifuged at 1,000g for 15 min. The pellet was resuspended in fresh culture medium, and the cells were disrupted by using three freeze-thaw cycles in liquid nitrogen. After centrifugation at 1,000g for 15 min, the supernatant with the liberated bacteria was used to infect Vero cell monolayers. The inoculum was removed after 1 hr of incubation at 37°C in a 5% CO₂ atmosphere, the cells were washed with culture medium in order to eliminate the unphagocytosed bacteria, and fresh medium containing 0.2 µg of pefloxacin per ml, was added. The monolayer was incubated at 35°C in a 5% CO₂ atmosphere. The supernatant was removed after 3 days, and fresh medium containing 0.2 µg of pefloxacin per ml was added. The infected cells were harvested after one week and were

used to infect Vero cell monolayers using fresh medium containing 0.6 µg pefloxacin per ml. The same protocol was followed for the pefloxacin concentrations of 1 to 4 µg/ml (by increases of 1 µg/ml). At each change of antibiotic concentration, the presence of strict intracellular *C. burnetii* was controlled by direct immunofluorescence (19). For each isolate, a flask of infected Vero cells without antibiotic was prepared as a positive control.

Susceptibility Testing

Pefloxacin (400 mg/5 ml; Rhone Poulenc S.A., Paris, France) and Ciprofloxacin (100 mg/50 ml; Bayer AG, Leverkusen, Germany) were aliquoted and frozen at -20°C.

The pefloxacin and ciprofloxacin susceptibilities of eight Greek isolates of *C. burnetii* and the reference strains Nine Mile and Q212 were determined previously using the shell vial assay in Vero cells (21). The same protocol was followed, in this study, for the pefloxacin resistant strains (MICs ≥ 8 µg/ml).

DNA Extraction

C. burnetii isolates were purified from Vero cells by Renografin density gradient centrifugation (22). Two hundred microliters of the microorganism were incubated in the presence of 400 ng of proteinase K per ml for two hours at 55°C. Subsequently, proteinase K was inactivated by boiling for 10 min, and the solution was centrifuged at 5,000 rpm for 10 min at 22°C in Beckman GS-6R centrifuge.

PCR Amplification

The *gyrA* gene of quinolone resistance-determining region (QRDR) of *C. burnetii*, was amplified with oligonucleotide primer 118 (5'-TTGCGCATATCGCAGACGGT-3') and primer 446 (5'-TGGCGGCAAAGGATTTGG-3'), as previously described (16).

The amplification was carried out in 100 µl of reaction mixture containing 1 × reaction buffer, 1 µM of each primer, 200 µM of each deoxynucleoside triphosphate, 2 mM MgCl₂, and 0.5 U of Taq polymerase (GIBCO BRL Life Technologies, Gaithersburg, MD). Thirty-five cycles consisting of 94°C for 30 sec for denaturation, 55°C for 30 sec for annealing, and 72°C for 30 sec for extension were performed in a DNA Thermal Cycler 9600 (Pelkin-Elmer Cetus, Emeryville, CA). The PCR products were resolved in a 2% agarose gel and visualized by UV illumination.

Sequence Analysis of the GyrA Gene of *C. burnetii*

Reamplification with extended primers (118 + M13 universal [-21] and 446 + M13 reverse [-29]) was carried out for subsequent sequencing. PCR products of each strain were purified by the QIAquick Spin PCR purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruc-

tions. Direct sequencing of 5 µl of the reamplified products was performed by cycle sequencing (Thermosequenase Kit Amersham) using primers M13 universal (–21) labeled with the 700-nm fluorescent label and M13 reverse (–29) labeled with the 800-nm fluorescent label. Sequences were resolved in a LI-COR 4200 IR2 automatic DNA sequencer (LI-COR, Lincoln, NE).

Restriction Endonuclease Digestion

Thirty µl of each amplified product were digested with *MnII* (New England Biolabs) according to the manufacturer's instructions (19,22). Restricted DNA was analysed by horizontal electrophoresis on a 3% low-melting-point agarose gel (GIBCO BRL, Gaithersburg, MD).

RESULTS

In Vitro Production of Pefloxacin-Resistant *C. burnetii*

The eight Greek *C. burnetii* isolates (CB1 to CB8), the reference strains Nine Mile and Q212, and the controls of infected cells without antibiotic presented MICs of 1–4 µg/ml for pefloxacin and 4–8 µg/ml for ciprofloxacin (21). For the first

time, *C. burnetii* was converted to pefloxacin-resistant strain in vitro conditions. First-step resistant bacteria (MIC 8 µg/ml) were obtained after circa 2 weeks of passage with 2 µg/ml pefloxacin; second-step resistant bacteria (MIC 16 µg/ml) were obtained after 10 additional days of passage with 3 µg/ml pefloxacin, and third-step resistant bacteria (MIC ≥ 32) were obtained after 10 additional days of passage with 4 µg/ml pefloxacin.

Direct immunofluorescence assay performed on infected cells, cultured with pefloxacin at 4 µg/ml, confirmed the intracellular presence of *C. burnetii*. The cytopathic effect of *C. burnetii* in Vero cells was also observed (19,23).

Amplification and Sequencing of the QRDR of *C. burnetii*

PCR with primers 118 and 446 amplified a single DNA product of 261 bp, enclosing the QRDR of *C. burnetii* strains. Comparison of the obtained sequences of all the tested strains revealed a nucleotide mutation (G→A) leading to the substitution of Glu (codon GAG) by Lys (codon AAG) at the position corresponding to amino acid 87 of *E. coli* in resistant strains (MICs to pefloxacin 8–32 µg/ml). The nucleotide and the corresponding amino acid sequence of the PCR amplified products are given in Figure 1.

Fig. 1. Nucleotide and amino acid sequence of sensitive *C. burnetii* strains compared to those of the in vitro pefloxacin resistant strains (*).

PCR-RFLP Analysis

Cleavage of the amplified product of 261 bp with *MnII* restriction enzyme produced 2 fragments of 152 and 109 bp in sensitive isolates (MICs 1 to 4 µg/ml). The resistant strains (MICs 8–32 µg/ml) were not digested using *MnII* enzyme. (Fig. 2).

DISCUSSION

The quinolone resistance-determining region (QRDR) in *gyrA* of *E. coli* consists of a chromosomal region where single nucleotide changes result in quinolone resistance (6). From the knowledge gained from fluoroquinolone resistance in *E. coli*, other workers have cloned and sequenced the *gyrA* genes of several bacterial pathogens including *S. aureus* (24), *Shigella dysenteriae* (25), *K. pneumoniae* (26), *C. jejuni* (6), and *C. burnetii* (16). Using the fact that the 5' ends of *gyrA* genes from different bacteria species contain corresponding areas that are highly conserved, PCR became a suitable approach for analysis of the QRDR.

In the present study, for first time, two sensitive Greek *C. burnetii* isolates were made to be quinolone resistant with serial passages in the presence of pefloxacin. These strains presented higher MICs of pefloxacin (8–32 µg/ml) than the

respective sensitive strains (MICs 1–4 µg/ml). The resistant strains with MICs ≥ 32 µg/ml were also resistant to ciprofloxacin with MIC ≥ 64 µg/ml.

The sequence analysis of the resistant strains showed a point mutation at the corresponding codon 87 of *E. coli*. The nucleotide mutation (G→A) lead to the substitution of Glu (codon GAG) by Lys (codon AAG) in the *gyrA* aminoacid chain.

Amplification of the QRDR of *gyrA* gene using specific primers for *C. burnetii* (16), followed by RFLP analysis have shown to be useful for identification of *C. burnetii* and also for detection of the point mutation in *C. burnetii* quinolone-resistant strains. All the amplified *gyrA* genes from sensitive *C. burnetii* strains (MICs 1 to 4 mg/ml) gave a specific pattern when cleaved with the *MnII* enzyme. The products from pefloxacin resistant strains (MICs 8–32 µg/ml) were not digested using *MnII* enzyme.

Direct sequencing is the more accurate technique for the detection of nucleotide mutations, but it is expensive, time consuming, and it requires some specialized equipment and a certain degree of technical expertise. Therefore, DNA sequencing cannot be used as a routine assay for diagnosis in the clinical laboratory, when many samples should be studied. The latter-described PCR-RFLP methodology is a simple and rapid method (it can be performed within 5 hr) and it can be carried out in diagnostic laboratories as a routine assay, because, from the electrophoretic profile, it is possible to detect and identify the *C. burnetii* quinolone-resistant strains.

The proposed technique is a very important tool for the detection of quinolone-resistant *C. burnetii* strains in patients with Q fever, because accurate diagnosis will lead to better antibiotic treatment of such patients. In fact, the possible persistence of microorganisms after several months of treatment needs to be documented in those patients with chronic Q fever who are undergoing an antibiotic regimen; this may lead to improved therapeutic treatments.

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Fig. 2. Restriction endonuclease profile analysis of the 261-bp amplification products of sensitive and resistant *C. burnetii* isolates. Lane A: negative control, (PCR reaction without DNA); lane B: molecular size markers (Φ X174 cleaved with *HinfI*); lane C: reference-sensitive strain Nine Mile; lanes D and F: resistant laboratory Greek isolates; lanes E and G: sensitive Greek isolates. Lanes C–G are the RFLP patterns of the *gyrA* amplified products digested with *MnII*.

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