Correlation between Ratio of Serum Doxycycline Concentration to MIC and Rapid Decline of Antibody Levels during Treatment of Q Fever Endocarditis

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Endocarditis is the major clinical manifestation of chronic Q fever. Although doxycycline along with hydroxychloroquine remains the mainstay of medical therapy for Q fever endocarditis, there are wide variations in the rapidity of the patient’s decline of antibody levels during such therapy. We undertook a retrospective examination of whether there was any correlation between the ratio of serum concentration to MIC of doxycycline and response to treatment in patients with Q fever endocarditis. Included herein are 16 patients from whom Coxiella burnetii was isolated from cardiac valve materials. Serology and measurement of doxycycline and hydroxychloroquine serum levels were performed and recorded after 1 year of treatment. The MIC of doxycycline for C. burnetii was determined using the shell vial assay in a real-time quantitative PCR assay. At the completion of a yearlong therapy with doxycycline-hydroxychloroquine, all those that showed a low decline of antibody levels (n = 6) (i.e., <2-fold decrease in antibody titer to phase I C. burnetii antigen) had a ratio of serum doxycycline concentration to MIC between 0.5 and 1. In contrast, those having a ratio of ≥1 showed a rapid decline of phase I antibody levels (n = 9; P < 0.05). The only patient who had had a serum doxycycline-to-MIC ratio of <0.5, and the isolate of C. burnetii cultured from this patient was resistant to doxycycline (MIC = 8 μg/ml). The ratio of serum doxycycline concentration to MIC should be monitored during the course of therapy in patients with Q fever endocarditis.

Q fever is a worldwide zoonosis caused by Coxiella burnetii, a strict intracellular bacterium that is considered a potential biological weapon (9, 11). C. burnetii is very fastidious, and very few clinical isolates have been reported except from our lab using the shell vial method (11, 16). In humans, the main clinical form of chronic Q fever is endocarditis. Q fever endocarditis is invariably fatal if not treated properly. Q fever endocarditis is associated with very high titers of anti-phase I immunoglobulin G (IgG) and IgA antibodies. These antibodies are not protective but rather predictive of the evolution of the disease, since the antibody titers fall slowly with treatment (11, 13). A decrease of more than two titers of these antibodies after 1 year of treatment is considered a favorable response (17). In vivo, C. burnetii multiplies in monocytes and macrophages within a lysosome-fused acidic vacuole, and most antibiotics are drastically inhibited at such an acidic pH (10). In vitro, it has been demonstrated that alkalinization of the C. burnetii-containing vacuoles with a lysosomotropic agent such as chloroquine results in bacterial growth inhibition and improvement of the bactericidal activity of doxycycline (10). These in vitro results have been corroborated by the demonstration of in vivo efficacy for the combination of doxycycline and hydroxychloroquine (14). This regimen allowed a reduction in the duration of therapy to 18 months for many patients and also reduced the relapse rate to less than 5% (8, 14). This regimen is effective clinically, but there is a heterogeneity in the rapidity of the biological response to treatment depending on the levels of doxycycline in serum (17). Few C. burnetii strains were tested for antibiotic susceptibility in vitro with MICs of doxycycline ranging from 1 to 4 μg/ml (6, 7, 15, 18, 19). The shell vial technique was the most widely used method (7, 15). Real-time PCR testing allowed us to test isolates more rapidly (1, 2) and makes it possible to test clinical isolates during the time of the treatment (18 to 36 months); therefore, it should be of clinical interest. We, therefore, undertook an examination of whether there is any correlation among the MIC of doxycycline, serum levels of doxycycline, and outcome of treatment in patients treated for Q fever endocarditis with a doxycycline-hydroxychloroquine combination.

MATERIALS AND METHODS

Patients. In all patients included in the study, the definite diagnosis of Q fever endocarditis was established using the Duke criteria modified for C. burnetii (4). In all cases, C. burnetii was cultured from cardiac valve materials (5).

Isolation of strains and MIC determination. A shell vial assay was used to isolate C. burnetii from the clinical specimens as mentioned above (16). The bactericidal effect of doxycycline against C. burnetii isolates was determined using the shell vial assay in a real-time quantitative PCR assay (1). Briefly, 30% infected P32 cells were cultured at 37°C in a 5% CO2 atmosphere in 24-well microplates at final volume of 2 ml. Doxycycline (0.5 to 8 μg/ml) was added after 2 days of incubation. Antibiotic-free infected cultures served as positive growth controls, whereas noninfected cell cultures served as negative controls. All experiments were performed in duplicate and repeated twice to confirm results. Samples were collected into aliquots every 5 days for 15 days of the experiment. The aliquots were frozen and stored at −70°C before the PCR assay. Total genomic DNA from cell cultures was extracted from aliquots using the QiAamp blood kit (QIAGEN, Hilden, Germany) as described by the manufacturer. PCR was performed using the LightCycler instrument (Roche Biochemicals, Mannheim, Germany) to amplify a 220-bp fragment of the superoxide dismutase gene (1). The PCR mixture included 2 μl of DNA master SYBR Green (DNA Master
SYBR Green I kit [Roche Diagnostic], 2.4 \mu M of 3 mM MgCl₂, 1 \mu M (10 pmol) of each primer, 11.6 \mu M of distilled H₂O, and 2 \mu l extracted DNA. Each PCR mixture included distilled sterile water and uninfected cells as negative controls. The amplification conditions were as follows: an initial denaturation step at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 68°C for 1 min, with fluorescence acquisition in single mode. The amplification was completed by holding for 10 min at 68°C to allow complete extension of PCR products. Specific standard curves have been generated by using 10-fold serial dilutions of a known C. burnetii inoculum. The number of DNA copies of each sample transcript was then calculated from the standard curve using the LightCycler software. For each isolate, the numbers of DNA copies at day 0 and at day 15 were recorded and compared using a Student \( t \) test. The MIC was defined as the first antibiotic concentration allowing inhibition of growth of the bacterium as measured by comparison of the number of DNA copies at day 15 with antibiotic versus those at day 0 and day 15 without antibiotic. The ratio of the serum doxycycline concentration to its MIC was calculated after completion of a year of antibiotic treatment and rounded off to one decimal place.

### Treatment and follow-up
After informed consent was obtained, an information set was provided (http://ifr48.timon.univ-mrs.fr/), and all patients received oral doxycycline (200 mg daily) along with hydroxychloroquine (600 mg daily). During the course of therapy, all but one study subject turned up for follow-up consultation at least once every 3 months. During each follow-up consultation, serologic tests for C. burnetii were performed (21) and the levels of doxycycline and hydroxychloroquine in serum were measured (17). Wherever necessary, doses of hydroxychloroquine were adjusted to keep the serum concentrations at low, the dosage of doxycycline was increased to 400 mg/day for all statistical analyses. Statistical significance was defined as a \( P \) value of <0.05.

### RESULTS
Thirteen men and 3 women from whom a C. burnetii isolate was obtained from a cardiac valve between 1996 and 2003 were included in the study. Clinically, all patients responded except one. The mean age of the subjects was 59.3 years (range, 36 to 82 years). Seven of 16 patients received a course (duration, 2 weeks to 3 months) of doxycycline prior to valvectomy. The aortic and mitral valves were involved in 11 and 5 cases, respectively. Table 1 gives a summary of the results and outcome of treatment. There was no statistically significant difference among groups A and B in terms of mean age and frequency of antibiotic treatment prior to valvectomy. Doxycycline MICs ranged from 1 to 8 \mu g/ml, with MICs of <4 \mu g/ml for 7 isolates and MICs of ≥4 \mu g/ml for 9 isolates (Table 1). The MICs of doxycycline were significantly lower for group A (9 patients) than those observed for group B (6 patients) (\( P < 0.05 \) (Table 1). The mean serum concentration of doxycycline was significantly higher in group A patients than that observed in group B patients (4.6 ± 1.6 \mu g/ml versus 3.0 ± 0.8 \mu g/ml; \( P < 0.05 \)).

The clinical and paraclinical criteria used for the evaluation of response to antimicrobial therapy included regression of such signs and symptoms as fever, splenomegaly, echocardiographic abnormalities, and changes in antibody titers to the phase I antigen of C. burnetii (14).

Patients were said to have responded well to antimicrobial treatment (group A) if the clinical signs and symptoms, indicated above, had regressed along with a >2-fold decrease in antibody titer (IgG and/or IgA) to phase I antigen at the completion of a yearlong course of treatment. Group B patients included those in whom the regression of clinical signs and symptoms was not associated with a fall in antibody titer to phase I antigen that would have otherwise qualified them to be included in group A. The only patient who did not qualify to be included in either group A or B is discussed separately.

#### Statistical analyses
Data were analyzed using Student’s \( t \) test and chi-square test as appropriate. Standard statistical software (Epi Info, version 6.0) was used for all statistical analyses. Statistical significance was defined as a \( P \) value of <0.05.

### Table 1: Serum doxycycline concentration, MIC, and outcome for 16 patients with Q fever endocarditis

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>No. of dilution decrease</th>
<th>Serum doxycycline level (\mu g/ml) after 1 yr of treatment</th>
<th>Doxycycline MIC (\mu g/ml)</th>
<th>Serum/MIC ratio</th>
<th>Outcome</th>
<th>Duration of treatment (mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>3.8 ± 0.4</td>
<td>4</td>
<td>1.0</td>
<td>Cured</td>
<td>22</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>2.5 ± 0.1</td>
<td>4</td>
<td>0.6</td>
<td>Cured</td>
<td>44</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>3.9 ± 0.5</td>
<td>4</td>
<td>1.0</td>
<td>Cured</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>2.2 ± 0.1</td>
<td>4</td>
<td>0.6</td>
<td>Cured</td>
<td>36</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>2.1 ± 0.2</td>
<td>4</td>
<td>0.5</td>
<td>Cured</td>
<td>60</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>2.7 ± 0.4</td>
<td>4</td>
<td>0.7</td>
<td>Still under therapy</td>
<td>24</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>4.0 ± 0.4</td>
<td>4</td>
<td>1.0</td>
<td>Cured</td>
<td>30</td>
</tr>
<tr>
<td>8</td>
<td>4</td>
<td>5.9 ± 0.6</td>
<td>2</td>
<td>3.0</td>
<td>Cured</td>
<td>35</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>3.9 ± 0.4</td>
<td>2</td>
<td>2.0</td>
<td>Cured</td>
<td>18</td>
</tr>
<tr>
<td>10</td>
<td>4</td>
<td>4.0 ± 1.4</td>
<td>2</td>
<td>2.0</td>
<td>Cured</td>
<td>29</td>
</tr>
<tr>
<td>11</td>
<td>4</td>
<td>3.2 ± 0.6</td>
<td>2</td>
<td>1.6</td>
<td>Cured</td>
<td>25</td>
</tr>
<tr>
<td>12</td>
<td>5</td>
<td>4.7 ± 0.8</td>
<td>2</td>
<td>2.4</td>
<td>Cured</td>
<td>25</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>8.3 ± 1.1</td>
<td>4</td>
<td>2.1</td>
<td>Dead</td>
<td>19</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>2.9 ± 0.1</td>
<td>2</td>
<td>1.5</td>
<td>Cured</td>
<td>25</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>4.5 ± 0.7</td>
<td>1</td>
<td>4.5</td>
<td>Still under therapy</td>
<td>30</td>
</tr>
<tr>
<td>16</td>
<td>0</td>
<td>3.5 ± 0.6</td>
<td>8</td>
<td>0.4</td>
<td>Dead</td>
<td>14</td>
</tr>
</tbody>
</table>

\( a \) These patients died without suffering an evolving Q fever.

\( b \) Patients 1 to 6 had decreases of IgG and/or IgA phase I antibody titers of ≥2 dilutions (group B). Patients 7 to 15 had decreases of IgG and/or IgA phase I antibody titers of ≥2 dilutions (group A). Patient 16 died during the course of treatment.
without any improvement. The patient died in an intensive care unit of multivisceral failure in May 2003.

**DISCUSSION**

In this study, we have evaluated the MIC of doxycycline for 16 clinical isolates of *C. burnetii* obtained from patients with Q fever endocarditis. Our results were reproducible, and experiments were carried out two times to confirm the results of MICs. Within the constraints of a small sample size, the MIC of doxycycline for the clinical isolates of *C. burnetii*, as we observed, were higher than those previously reported (6, 7, 15). However, we and others have previously demonstrated that real-time PCR assay is more sensitive than the standard shell vial assay (1, 2). We describe our first clinical isolate resistant to doxycycline (MIC = 8 μg/ml) from a patient who died from Q fever endocarditis during the course of the treatment. Before this, one goat isolate was described as resistant, in a chicken embryo model (20). This resistance is worrisome, as doxycycline is the reference treatment. Because antibiotic pressure on *C. burnetii* in humans is believed to be low, it is possible that decreased susceptibility to doxycycline was linked to antibiotic pressure in livestock (12) and/or in plant agriculture (22). This may select resistant strains, and alternative antibiotic treatment may be important. Horizontally acquired DNA integrated into a natural isolate of *Chlamydia suis*, a pathogen of pigs, has been recently reported, suggesting that this phenomenon may occur within any other obligate intracellular bacterium (3). The mechanism of resistance for our isolate is not known. Since there were no mutations in the 16S RNA gene sequence nor in the tet gene in the genome of *Coxiella burnetii* (unpublished data), further studies are needed to understand the exact molecular support of resistance. Particularly, multidrug efflux transporters present in the genome of *C. burnetii* could be evaluated in the future. This resistance explains why this patient was resistant to the therapy and died during the course of the treatment. For 10 years, one of us (D.R.) has treated 105 patients with the doxycycline-hydroxychloroquine combination without clinical failure. Our data highlight the importance of monitoring serum levels of doxycycline and MIC while patients with Q fever endocarditis are on doxycycline therapy. We demonstrate in this study a clear association between a high ratio of doxycycline to MIC and a rapid decline of antibody levels.

Patients who had a rapid decline of antibody levels all had a serum doxycycline level-to-MIC ratio of >1 (Fig. 1). In 10 patients, the serum concentration of doxycycline ranged between 2.5 and 4 μg/ml. Even in this subgroup, a MIC of doxycycline of ≥4 μg/ml was shown to be indicative of a delay in the decrease of antibody levels during therapy.

The lower mean serum concentration of doxycycline in group B patients could be due to drug interactions, lack, or compliance or differences in individual rates of gastrointestinal absorption (17). The only clinical failure shown in this report was in a patient infected with a resistant strain and the lowest ratio.

In conclusion, doxycycline along with hydroxychloroquine remains the mainstay of medical therapy for Q fever endocarditis. However, the dose of doxycycline may need to be adjusted during the course of therapy. Whenever possible, the MIC of doxycycline for the *C. burnetii* isolate should be determined, since determination of the ratio influences the delay in the decrease of antibody levels.

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D.R. has obtained a contractual agreement with pharmaceutical company Hoescht-Marion-Roussel to evaluate the efficacy of telithromycin against *Coxiella burnetii.*

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