Effects of antibiotics on *Chlamydia trachomatis* viability as determined by real-time quantitative PCR

Olivia Peuchant, Jean Philippe Duvert, Maïthé Clerc, Sophie Raherison, Christiane Bébear, Cécile M. Bébear and Bertille de Barbeyrac

Correspondence

Bertille de Barbeyrac
bertille.de.barbeyrac@u-bordeaux2.fr

Laboratoire de Bactériologie EA 3671, Infections Humaines à Mycoplasmes et Chlamydiae, Centre National de Référence des Infections à Chlamydiae, Université Victor Segalen Bordeaux 2, Bordeaux, France

The objective of this study was to determine the effect of antibiotics on *Chlamydia trachomatis* viability by using a quantitative real-time PCR assay that measured DNA replication and mRNA transcription of the structural *omp1* and *omp2* genes, 16S rRNA and the *groEL1* gene with and without antibiotics. Ofloxacin, moxifloxacin, azithromycin and doxycycline were tested against the serovar D and L2 reference strains and a derivative mutant resistant to fluoroquinolones, L2-OFXR, obtained by *in vitro* selection. Using DNA quantification, the antibiotic MIC was calculated when the number of DNA copies was equal to that of the chlamydial inoculum at time zero. This method allowed the easy determination of MICs by DNA quantification of the four selected genes and gave similar results to those obtained by immunofluorescence staining without biased interpretation. By using cDNA quantification, the lowest antibiotic concentration for which no RNA was transcribed corresponded to the minimum bactericidal concentration. *C. trachomatis* still transcribed the 16S rRNA and *groEL1* genes, even at concentrations well above the MIC, showing a bacteriostatic effect for all antibiotics tested. This method allows the study of antibiotic activity on growth and viability of *C. trachomatis* by DNA and RNA quantification at the same time without additional cell-culture passaging.

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INTRODUCTION

Chlamydia trachomatis, an obligately intracellular micro-organism, is the most common bacterium responsible for sexually transmitted infections. Most of these infections are asymptomatic; however, they can lead to severe complications if not treated, especially in young women.

Evaluation of antibiotic activities against *C. trachomatis* is necessary to demonstrate the *in vitro* effectiveness of various compounds and to study antimicrobial susceptibility when repeat or persistent infections occur. Therapeutic failures have been described for *C. trachomatis* (Somani *et al.*, 2000; Wang *et al.*, 2005), and resistance to tetracyclines has been reported for the animal species *Chlamydia suis* (Dugan *et al.*, 2004; Lenart *et al.*, 2001), suggesting the need to monitor the development of antibiotic resistance in chlamydia.

A cell-culture system using immunofluorescence (IF) staining to identify chlamydial inclusions is the most common methodology employed in antimicrobial susceptibility testing of chlamydia. Interpretation of traditional IF

staining is a tedious and subjective process, and the end point used for defining the MIC is not very precise (Suchland *et al.*, 2003) for macrolides and tetracyclines. Thus, there is a need for a more reliable, rapid and objective method to evaluate chlamydial growth, such as a molecular method.

Quantitative real-time PCR assays have been used to evaluate antibiotic susceptibility of intracellular bacteria (Boulos *et al.*, 2004, 2005; Brennan & Samuel, 2003; Rolain *et al.*, 2004) and for measuring specific RNA transcripts of viable *C. trachomatis* to determine MICs (Storm *et al.*, 2005).

Here, we present a quantitative real-time PCR assay that measured DNA replication and mRNA transcription of the *omp1* and *omp2* structural protein genes, and the 16S rRNA and *groEL1* genes to determine the MICs of various antibiotics against *C. trachomatis* and to study the effects of these antibiotics on chlamydial viability.

METHODS

Chlamydial strains and cells. Three isolates of *C. trachomatis* were used in this study: the serovar D reference strain (ATCC VR-885), the serovar L2 reference strain (ATCC VR-902B) and its derivative

Abbreviations: C_t, cycle threshold; IF, immunofluorescence; i.f.u., inclusion-forming units; p.i., post-infection; MIC_{TP}, MIC transition point.

mutant resistant to ofloxacin, L2-OFXR, obtained by *in vitro* selection (Dessus-Babus *et al.*, 1998). The genome sequence was only available for the serovar D strain at the time of this study. *C. trachomatis* strains were cultured in McCoy cells in minimal essential medium (MEM; Life Technologies, Gibco-BRL) supplemented with 1 mM L-glutamine and 5% FCS.

Antibiotics. The following antibiotics were tested in serial twofold dilutions over the indicated concentration ranges: doxycycline (Sigma), 0.002–0.12 µg ml⁻¹; azithromycin (Pfizer), 0.015–0.1 µg ml⁻¹; moxifloxacin (Bayer Pharma), 0.004–0.25 µg ml⁻¹; and ofloxacin (Sanofi Aventis), 0.12–8 µg ml⁻¹. Stock solutions were prepared according to the manufacturers' instructions and stored at -20 °C until used. Working solutions were prepared as required by diluting stock solutions in supplemented MEM.

Cell culture and antimicrobial susceptibility testing. Monolayer cells in tubes were inoculated with 10⁵ inclusion-forming units (i.f.u.) *C. trachomatis* ml⁻¹, centrifuged at 1200 g for 1 h at 37 °C and incubated for 2 h at 37 °C in 5% CO₂. Supernatants were discarded and replaced with 2 ml fresh MEM supplemented with 0.1% glucose and 1 µg cycloheximide (Gibco) ml⁻¹. Tubes were incubated at 37 °C in 5% CO₂. Cell culture was stopped at several times post-infection (p.i.) according to the experiment. Each chlamydial suspension in cell culture was set up in four tubes. Two tubes were analysed by IF and the other two were analysed by molecular methods with the same chlamydial inoculum. For each test, the cell supernatant was discarded and the infected cells were fixed with methanol for IF analysis or disrupted by vortexing with sterile glass beads in 1 ml 2-SP medium (0.2 M sucrose, 15 mM K₂HPO₄, 6 mM KH₂PO₄) for molecular analysis.

The growth curve of the serovar D strain was studied at several times p.i. in antimicrobial-free growth medium. Forty-four monolayer cell tubes were infected with a 10⁵ i.f.u. bacterial suspension ml⁻¹. At 0, 3, 6, 12, 16, 21, 25, 29, 36, 45 and 53 h p.i., chlamydial growth was stopped for four tubes at each time point. Two tubes of monolayer cells were analysed by IF and the other two were resuspended in 1 ml 2-SP medium for analysis by molecular methods.

For MIC determination, serial antibiotic dilutions were added after 1 h of centrifugation and 2 h of incubation [corresponding to time zero (*t*₀)] and the MIC was determined at 36–40 h p.i.

An inoculum control was performed by inoculating a monolayer cell tube and incubating for 2 h, followed by rinsing and either fixing and staining for microscopy or freezing at -80 °C for molecular studies. A growth control was obtained from an infected monolayer cell incubated without antibiotics, and a cell monolayer tube that was not infected by *C. trachomatis* was used as a cell control.

The MIC determined by the IF assay was defined as the concentration of antibiotic that was one twofold dilution higher than the transition point (MIC_{TP}) as described by Suchland *et al.* (2003). The molecular method defined the MIC as the lowest antibiotic concentration that resulted in the same number of PCR cycles necessary to amplify DNA as required to amplify DNA from the initial inoculum. By using RNA quantification, it was possible to evaluate the bactericidal activity of an antibiotic defined as the lowest antibiotic concentration that inhibited gene transcription.

IF determination. Chlamydial inclusions were detected by IF using a *C. trachomatis* culture confirmation test (Syva Microtrak Behring) containing a fluorescein-labelled monoclonal antibody.

Nucleic acid extraction and reverse transcription. Total genomic nucleic acids were extracted from each aliquot using a High Pure PCR Template Preparation kit (Roche), as described by the manufacturer. Nucleic acids were extracted from 200 µl cell culture and stored at -20 °C.

Total RNA was extracted from each aliquot using a High Pure RNA isolation kit (Roche) containing DNase I as described by the manufacturer. RNA was extracted from 200 µl cell culture and eluted in 100 µl elution buffer. The 16S rRNA gene was amplified by PCR to verify the absence of contaminating DNA, which would require a second DNase I treatment.

Total RNA was reverse transcribed into single-stranded cDNA using a High Capacity cDNA Archive kit (Applied Biosystems), according to the manufacturer's instructions, in a final volume of 50 µl. The cDNA was stored at -20 °C.

Real-time PCR. Real-time PCR was performed using total genomic nucleic acids and cDNA obtained as described above.

Amplification, data acquisition and data analysis were performed on an ABI Prism 7000 (Applied Biosystems) using SYBR Green chemistry detection. Four target genes were chosen: the structural genes *omp1*, encoding the major outer-membrane protein, and *omp2*, encoding an elementary body-specific membrane protein, and the 16S rRNA gene and Hsp60-encoding *groEL1* gene.

The *omp1* primers (CT1 and CT2) have been described previously (Dutilh *et al.*, 1989). The *omp2*, 16S rRNA and *groEL1* primers were designed using alignment of nucleic sequences in GenBank (Table 1). Primer sequences were determined using the Primer Express software for SYBR Green chemistry.

The PCR mixture consisted of 12.5 µl 2 × qPCR Mastermix Plus for SYBR Green I (Eurogentec), 0.75 µl forward and reverse primers (10 µM) and 5 µl template DNA or cDNA, with the total reaction made up to a final volume of 25 µl with water. Each PCR assay included sterile distilled water and uninfected cells as negative controls. After 10 min at 95 °C to activate the Hot Goldstar DNA polymerase (Eurogentec), 40 PCR cycles of 15 s at 95 °C and 1 min at 60 °C were performed. At each annealing step, fluorescence was detected. The specificity of amplification was confirmed by analysis of the melting temperature (*T*_m) (Table 1).

For each PCR, the cycle threshold (*C*_t) corresponding to the cycle where the amplification curve crossed the base line was determined. This method allowed relative quantification, as *C*_t depends on the number of target copies.

RESULTS

Growth kinetic analysis of the serovar D strain of *C. trachomatis*

Fluorescence microscopy showed no inclusions before 12 h p.i., after which micro-inclusions appeared and increased in size to reach the maximal size at 36 h p.i. before bursting at 40 h p.i. (not shown).

The growth curve of the *C. trachomatis* serovar D strain is shown in Fig. 1. Using quantitative real-time PCR, the multiplication cycle showed three states for all four amplified genes: a latent state between 0 and 12 h, an exponential growth state between 12 and 29 h and a stationary state after 29 h (Fig. 1a). The multiplication factor was approximately 200, corresponding to a variation of 8 *C*_t between the inoculum DNA rate and the DNA rate at 40 h p.i. This growth curve demonstrated that *C. trachomatis* serovar D began to synthesize detectable amounts of *omp1* DNA after 6 h of infection. The first

Table 1. Primers used to amplify the four genes of interest

The *omp1* primers were described by Dutilh *et al.* (1989). Other primers were designed in this study.

Primer	Sequence (5'–3')	Amplified fragment	
		Length (bp)	T_m (°C)
<i>omp1</i>			
CT1	GCCGCTTTGAGTTCTGCTTCCTC	129	81
CT2	CCAAGTGGTGCAAGGATCGCA		
<i>omp2</i>			
<i>omp2</i> -S	CTGCAACAGTATGCGCTTGTC	51	74
<i>omp2</i> -AS	GTCCACATTTTGTAACCGAACG		
16S rRNA gene			
16S rDNA-S	GCAAGTCGAACGGAGCAATT	51	74
16S rDNA-AS	ACCCTTCGCCCACTAAACA		
<i>groEL1</i>			
<i>groEL</i> -S	AGCTCTTTCGCTCCTTTGAA	51	73
<i>groEL</i> -AS	CCTTCTTTCTCTGCGTTTGC		

doubling occurred by 6 h p.i. and doubling occurred for approximately 3–4 h over a 30 h cycle. After 30 h, the amount of DNA became stable. The levels of *omp2*, 16S rRNA and *groEL1* DNA began to increase after 16 h of infection with the same rate of increase as the *omp1* DNA.

Transcription profiles were different according to the genes of interest. The *groEL1* and 16S rRNA genes were transcribed early, the *omp1* gene was transcribed at 3 h p.i. and the *omp2* gene was transcribed at 12 h p.i. (Fig. 1b). At the t_0 inoculum, the quantity of 16S rRNA was approximately 4000-fold greater than the other genes. This increased between 3 and 21 h p.i. by a factor 200, after which the quantity remained steady.

After 30 h of growth, replication and transcription were in a stationary state for all tested genes. Antibiotic susceptibility testing was carried out at this time.

Antibiotic susceptibility determined by IF staining

Immediately after inoculation, there were no inclusions. After 30 h of incubation without antibiotics, the presence of inclusions gave evidence of bacterial growth in cells. With increasing concentrations of antibiotics, the number and size of inclusions decreased. For instance, at 0.015 mg moxifloxacin l^{-1} , the few inclusions seen were small and altered, which corresponds to the MIC_{TP} (Suchland *et al.*, 2003); thus, the moxifloxacin MIC was 0.03 mg l^{-1} . The most active antibiotic against the serovar D reference strain was doxycycline (MIC=0.015 mg l^{-1}), followed by azithromycin and moxifloxacin (MIC=0.03 mg l^{-1}) and ofloxacin (MIC=0.5 mg l^{-1}) (Table 2).

Antibiotic effect determined by real-time PCR

For each dilution of antibiotic, the four genes were amplified from DNA of each of the three strains tested. Curves were drawn representing the C_t values against

Fig. 1. Growth kinetics of the reference strain serovar D of *C. trachomatis* determined by quantification of DNA (a) and cDNA (b) of the four target genes using real-time PCR.

antibiotic concentrations. An example of the MIC determination of moxifloxacin for the *C. trachomatis* serovar D strain is shown in Fig. 2. A moxifloxacin concentration of 0.03 mg l^{-1} inhibited bacterial growth, as

Table 2. MICs against the *C. trachomatis* serovar D reference strain determined by IF and by quantification of *omp1*, *omp2*, 16S rRNA and *groEL1* DNA

Antibiotic	MIC (mg l ⁻¹)				
	IF	DNA quantification by real-time PCR			
		<i>omp1</i>	<i>omp2</i>	16S rRNA	<i>groEL1</i>
Ofloxacin	0.5	0.5	0.5	0.5	0.5
Moxifloxacin	0.03	0.03	0.03	0.03	0.03
Azithromycin	0.03	0.06	0.06	0.06	0.06
Doxycycline	0.015	0.007	0.007	0.007	0.007

the amount of amplified DNA was equal to that of the inoculum and corresponded to the MIC. For each antibiotic tested, the MICs determined by DNA quantification of each of the four target genes were identical (Table 2). The real-time PCR analysis was easy and objective. Antibiotic MICs were obtained from the IF-stained cells and real-time PCR gave comparable results (Table 2). The MICs of ofloxacin, moxifloxacin, azithromycin and doxycycline for the serovar D strain were 0.5, 0.03, 0.03 and 0.015 mg l⁻¹, respectively, using IF, and 0.5, 0.03, 0.06 and 0.007 mg l⁻¹, respectively, using real-time PCR (Table 2). For the serovar L2 strain, the MICs were 0.5, 0.03, 0.03 and 0.06 mg l⁻¹, respectively, with both IF and real-time PCR giving the same values. The ofloxacin MIC for the derivative mutant L2-OFXR, determined by either IF or real-time PCR, was 64 mg l⁻¹, as described previously (Dessus-Babus *et al.*, 1998).

Antibiotic effect determined by reverse-transcription PCR (RT-PCR)

Using cDNA quantification, the antibiotic concentration for which no RNA is transcribed corresponds to a bactericidal concentration, as RNA transcripts measure the expression of viable bacteria. Two kinds of transcriptional profiles were obtained according to the target gene

Fig. 2. Determination of the MIC of moxifloxacin for the serovar D strain by *omp1* quantitative real-time PCR.

studied. With the structural genes *omp1* and *omp2*, the inhibiting antibiotic concentrations obtained by cDNA quantification (Table 3) corresponded to the MIC determined using DNA quantification (Table 2). For the 16S rRNA gene, the antibiotic concentration that inhibited gene transcription was twice the MIC for azithromycin and 16 times the MIC for doxycycline. For the *groEL1* gene, the antibiotic concentrations that inhibited gene transcription were more than eight to 16 times the MICs of the four antibiotics tested (Table 3).

The reference strain L2 and its derivative mutant L2-OFXR did not exhibit the same behaviour with regard to expression of RNA in the presence of ofloxacin. For the reference strain, the quantity of chlamydial *groEL1* RNA was equal to that of the inoculum at a concentration of 8 mg ofloxacin l⁻¹ (16 times the MIC) (Fig. 3a), whereas, for the L2-OFXR mutant, the antibiotic concentration that gave a quantity of chlamydial *groEL1* RNA equal to that of the inoculum was 128 mg l⁻¹ (twice the MIC) (Fig. 3b).

DISCUSSION

C. trachomatis requires cell-culture systems for *in vitro* studies of antibiotic susceptibility. The objective of this study was to use real-time PCR and RT-PCR-based methods to evaluate the effects of antibiotics on *C. trachomatis* and to determine the antibiotic MICs in a more objective and less time-consuming way than a phenotypic method. MIC determination using IF staining and microscopy observation is not easy and is subjective. For concentrations near the MIC, it is difficult to differentiate small inclusions from the rest of the inoculum. This phenomenon has been observed more frequently with doxycycline and azithromycin. With these antibiotics, small, atypical inclusions persist at high antibiotic concentrations. Comparisons of MICs determined by microscopy from different laboratories are difficult. A multicentre study conducted in six laboratories with five *C. trachomatis* isolates and identical batches of antibiotics showed important variation in MICs (up to five dilutions difference), despite using an identical protocol (Peeling *et al.*, 1994). Among the variability factors, the lack of

Table 3. Antibiotic concentrations that were able to inhibit transcription of each gene in the *C. trachomatis* serovar D strain, determined by quantification of *omp1*, *omp2*, 16S rRNA and *groEL1* cDNA

Antibiotic	Antibiotic concentration (mg l ⁻¹)			
	<i>omp1</i>	<i>omp2</i>	16S rRNA gene	<i>groEL1</i>
Ofloxacin	0.5	0.5	4	>8
Moxifloxacin	0.03	0.03	0.25	>0.25
Azithromycin	0.06	0.06	0.12	>1
Doxycycline	0.007	0.007	0.12	>0.12

Fig. 3. Quantification of the *groEL1* cDNA of the L2 strain (a) and its derivative L2 OFXR mutant (b) in the presence of ofloxacin (OFX). Downward arrows indicate the antibiotic concentrations that inhibited *groEL1* transcription.

standardized methods and operator subjectivity were responsible for these differences. Given the difficulty in reading the end point for defining the MIC, Suchland *et al.* (2003) suggested reading the MIC_{TP}, where almost all inclusions are altered in size and morphology, and defining the MIC as the concentration of antibiotic that is twofold higher than MIC_{TP} (Suchland *et al.*, 2003). The study of the minimal chlamydiae concentration needs one freeze-thaw passage in antimicrobial-free medium and 48 h of additional incubation. Moreover, the failure to detect chlamydial infection in cell culture *in vitro* does not exclude a viable state that could occur after antibiotics are removed.

Real-time PCR is a quantitative molecular method that allows a measure of bacterial growth and is particularly interesting for intracellular bacteria such as chlamydia. First, we used a real-time PCR assay to monitor the accumulation of chlamydial chromosomal DNA over time for 50 h p.i. in McCoy cells infected with *C. trachomatis* serovar D. We analysed DNA replication of the *omp1*, *omp2*, 16S rRNA and *groEL1* genes using total DNA. In our experiment, levels of DNA began to increase significantly at 12 h p.i., as has been described for serovar K (Gérard *et al.*, 2001), but somewhat later than reported for serovar L2 growing in HeLa cells (Shaw *et al.*, 2000). The doubling rate of approximately of 3.4 h was longer than has been

reported for serovar L2 (2.5 h). Thus, for the MIC determination, DNA replication could be studied by using any of the four selected genes, especially the *omp1* gene.

Secondly, to determine the time p.i. at which chlamydial DNA replication-related genes were transcribed under conditions of normal growth, we looked for transcripts from the four selected genes using total RNA harvested at several times after infection. We selected the *groEL1* gene rather than *groEL2* or *groEL3* because of the results published by Karunakaran *et al.* (2003) showing that, although all Hsp60-encoding genes were expressed throughout the developmental cycle, *groEL1* has the highest level of transcription. As expected, we showed that the 16S rRNA gene and *groEL1* were typically expressed early in chlamydial development, before the *omp1* gene encoding the major outer-membrane protein was expressed. The *omp2* gene encoding the 60 kDa cysteine-rich outer-membrane protein is transcribed later during the developmental cycle, when reticulate bodies begin to differentiate back to elementary bodies. Our results were in accordance with those of Shaw *et al.* (2000) using an end-point PCR and describing three temporal classes of genes. The 16S rRNA gene was representative of early genes, which are detectable 2 h after infection. The *omp1* gene represented mid-cycle genes, which are not detected 2 h after infection but are present at 12 h p.i. The *omp2* gene represented the late genes, which are not detected until 20 h p.i. In our experiments, the order was the same but expression was earlier, as the *omp1* RNA was transcribed from 3 h p.i. and the *omp2* RNA from 12 h p.i., probably because of the higher sensitivity of the real-time PCR in detecting amplified fragments at the beginning of the PCR exponential phase. Our results showing that transcription of *groEL1* was initiated as early as 2 h p.i. were in accordance with those of Karunakaran *et al.* (2003).

The antibiotic MICs obtained by DNA quantification for the four genes tested were identical or no more than one dilution different from those obtained by IF staining, for both susceptible and resistant strains. Furthermore, the MICs determined in our study by either IF or real-time PCR corresponded to those described in the literature (Bébéar *et al.*, 2008; Donati *et al.*, 1999). This method enabled MICs to be determined rapidly, consistently and without biased interpretation. The antibiotic concentration that inhibited RNA transcription of the *omp1* or *omp2* gene was identical to the MICs determined by DNA quantification; however, the RT-PCR-based method was more expensive and more time-consuming than the DNA quantification. As described by Storm *et al.* (2005), by measuring specific RNA transcripts of *omp2* by *C. trachomatis* exposed to doxycycline, the MICs obtained from IF-stained cells and real-time PCR were comparable. Actually, our results showed that the antibiotic MIC can be determined by using only *omp1* gene DNA quantification, as *omp1* is the gene that encodes the major outer-membrane protein and is expressed early.

To study the antibiotic effect on bacterial viability, we measured specific RNA transcripts of the four target genes by an RT-PCR-based method. As described previously by Dreses-Werringloer *et al.* (2000), we demonstrated the continuous presence of *groEL1* transcripts in *C. trachomatis*-infected cells treated with antibiotics, indicating the viability of the organism. For all antibiotics tested, *C. trachomatis* still transcribed *groEL1*, even at concentrations well above the MIC. In two previous studies measuring *dnaK* transcripts using RT-PCR (Cross *et al.*, 1999; Storm *et al.*, 2005), the authors found antibiotic inhibiting concentrations two- to fourfold higher than the MICs usually described for these antibiotics. These authors used conventional PCRs, which are less sensitive than the real-time PCR assay performed in this study. Under our experimental conditions, no antibiotic tested appeared to be bactericidal *in vitro*, except when used against the quinolone-resistant mutant strain L2-OFXR. Surprisingly, this mutant strain did not exhibit the same behaviour as the parental strain with regard to expression of *groEL1* RNA. The ofloxacin concentration that inhibited the transcription of *groEL1* was only twofold higher than the ofloxacin MIC (Fig. 3a).

The antibiotics inhibited the multiplication of *C. trachomatis*, as shown by DNA or RNA quantification results for genes encoding known chlamydial membrane proteins, but were not bactericidal towards the micro-organism, according to the RNA expression levels of the *groEL1* gene. A decrease in *omp1* transcription was noted, whilst the transcription of *groEL1* was less affected. A potential problem with bacteriostatic antibiotics in the therapy of chlamydia infections is the induction of latent or persistent forms of chlamydiae, which results in apparent eradication but which may allow subclinical progression of persistent infections. In *C. trachomatis* infections treated with bacteriostatic antibiotics, the clearance of bacteria depends not only on the activity of the antibiotic but also the capacity of the host to eliminate the remaining bacteria.

In summary, we have developed a real-time PCR method allowing the easy determination of MICs by *omp1* DNA quantification and the study of viability measured by *groEL1* RNA quantification at the same time without additional cell-culture passaging. We demonstrated that all the antibiotics tested under our experimental conditions had only a bacteriostatic effect.

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