Variation in the mutation frequency determining quinolone resistance in *Chlamydia trachomatis* serovars L2 and D

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**Objectives:** Quinolone resistance of chlamydiae is supposed to be extremely rare. To assess the risk for the emergence of chlamydial quinolone resistance, we analysed the occurrence of resistant mutants in a quantitative perspective.

**Methods:** Infectious elementary bodies of *Chlamydia trachomatis* serovar L2 (ATCC VR-902B) and D (ATTC VR-885) clones were purified on density gradients, and mutants resistant to moxifloxacin and rifampicin were selected by a plaque assay. Plaque assays were conducted with $2 \times 10^9$ inclusion forming units (IFUs) of each serovar for rifampicin and $2.66 \times 10^9$ IFUs for moxifloxacin. Resistant clones were analysed for mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes, and respective MICs were determined by titration experiments.

**Results:** Mutation frequencies for rifampicin (MIC $\geq 0.2$ mg/L) did not differ significantly between serovars L2 and D ($5.7 \times 10^{-7}$ versus $6.3 \times 10^{-7}$). In contrast, the occurrence of moxifloxacin-resistant mutants (MIC $\geq 0.6$ mg/L) was determined to be 2.0–2.2 $\times 10^{-8}$ for the serovar L2 isolate and less than $2.66 \times 10^{-9}$ for the serovar D isolate. Moxifloxacin resistance of all serovar L2 clones depended on single-nucleotide point mutations in the quinolone resistance-determining region of the *gyrA*, whereas no additional mutations were found in the *gyrB*, *parC* or *parE* genes.

**Conclusions:** *C. trachomatis* isolates have the potential to present with clinically relevant antibiotic resistance in future. Serovar-specific differences in the occurrence of spontaneous mutations should be taken into account to predict quinolone resistance in different chlamydial diseases.

Keywords: plaque assay, intracellular pathogens, *C. trachomatis*

**Introduction**

The obligate intracellular bacterium *Chlamydia trachomatis* is a frequent cause of ocular and sexually transmitted infections. Infections with genital serovars D to K mostly manifest as urethritis, prostatitis or epididymitis in men and as cervicitis, urethritis, salpingitis or endometritis in women. Serovar L2 is able to disseminate into the local lymph nodes and causes lymphogranuloma venereum. Tetracyclines and macrolides are antibiotics of first choice, but quinolones have proven effective in the treatment of pelvic inflammatory disease and other genital chlamydial infections. Quinolones exert their antibacterial effects by inhibition of DNA gyrase and topoisomerase IV, two enzymes that consist of two different subunits, GyrA/GyrB and ParC/ParE. Quinolone resistance most often arises after point mutations in the quinolone resistance-determining regions (QRDRs) of the subunit genes. *In vitro* studies have shown that quinolone resistance resulting from mutations in the *gyrA* gene can be induced by prolonged exposure to subinhibitory fluoroquinolone concentrations. Clinical isolates with increased MICs have been described, but phenotypic resistance to quinolones could not be unequivocally attributed to mutations in the QRDR. Thus, contrary to *Parachlamydia acanthamoebae* and to other chlamydia-related organisms that are naturally resistant to quinolones, quinolone resistance *in vivo* is supposed to be extremely rare for Chlamydiaceae. In order to assess the risk for chlamydial quinolone resistance in the future, we analysed the occurrence of resistance mutations in a quantitative perspective. Resistant clones *in vivo* are most likely to occur as a result of a single, spontaneous point mutation followed by selective enrichment as a result of antimicrobial therapy. We developed a novel *in vitro* assay, mimicking this situation, and were able to determine the mutation frequency in the QRDR of *gyrA* for *C. trachomatis* serovars L2 and D.

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Materials and methods

Plaque assay for antibiotic-resistant clones

*C. trachomatis* serovars D (ATCC VR-885) and L2 (ATCC VR-902B) were cloned by a previously described plaque assay in order to obtain genetically homogenous, susceptible populations.\(^5,6\) Briefly, chlamydial plaques were cultured for 12–13 days in HeLa-229 cell monolayers in 6-well cluster plates containing a solid 1.1% agarose (SeaKem Me agarose; FMC BioProducts, Rockland, Maine, USA) overlay and a liquid DMEM medium (Invitrogen, Karlsruhe, Germany) with 10% fetal calf serum (PAA, Coelbe, Germany) and 0.2 mg/L rifampicin or 0.6 mg/L moxifloxacin, respectively. The medium was replaced every 3–4 days by fresh medium, and chlamydial plaques were identified after 12–13 days by inverse light microscopy or alternatively by neutral red staining of the monolayer. For isolation of resistant chlamydial plaques, the agarose above the plaque was stamped out with a pipette, infected cells were resuspended in 100 μL of medium, homogenized by glass beads, and chlamydiae were re-cultivated. The plaque assay for rifampicin resistance was conducted with a total of 2 × 10⁸ IFUs of both serovar clones. The plaque assay for moxifloxacin resistance was conducted with a total of 2.66 × 10⁹ IFUs of one serovar L2 and D clone and confirmed with a second clone of both serovars.

MIC determination for antibiotic-resistant clones

The MIC values for both *C. trachomatis* serovars L2 and D were determined as 0.0025 mg/L for rifampicin and 0.025 mg/L for moxifloxacin. Resistant clones were propagated for three passages before determination of the MIC was determined in HeLa-229 cells with moxifloxacin or rifampicin in 2-fold dilutions (0.5–128 mg/L). The MIC was determined as 0.0025 mg/L for rifampicin and 0.025 mg/L for moxifloxacin. Resistant clones were propagated for three passages before the MIC was determined in an indirect fluorescent antibody assay with a chlamydial anti-LPS antibody (Dako, Hamburg, Germany).

Analysis of the QRDR and rpoB

DNA of chlamydial clones was extracted after three passages by NucleoSpin Tissue (Macherey-Nagel, Duren, Germany). In moxifloxacin-resistant clones, QRDRs of *gyrA*, *gyrB*, *parC* and *parE* were amplified according to Dessus-Babus et al.\(^7\) with the primers CTA3/4, CTB4/5, CTC3/4, CTE4/5. In rifampicin-resistant clones, a 656 bp product flanking clusters I and II on the central portion of the *rpoB* gene was amplified by the primers rpoB-US and rpoB-DS, according to Dreses-Werringloer et al.\(^8\) Amplicons were purified by NucleoSpin Extract II and sequenced according to standard procedures.

Results

Isolation of *C. trachomatis* clones resistant to moxifloxacin and rifampicin

Having screened 2.66 × 10⁹ organisms originating from a single clone of serovar L2, we isolated 12 mutants resistant to moxifloxacin at MIC ≥0.6 mg/L (1 mutant per 2.2 × 10⁸ organisms). The same number of organisms of serovar D did not yield any resistant mutant under the same conditions. The obtained results were confirmed in another clone of serovar L2 with a mutation frequency of one mutant per 2.0 × 10⁸ organisms and a second clone of serovar D showing no moxifloxacin resistance originating from 2.66 × 10⁹ organisms in the plaque assay. To validate our assay for other antibiotics, we selected rifampicin-resistant mutants from both serovars. Similar mutation frequencies of both serovars were detected, showing 35 mutants of serovar L2 resistant to ≥0.2 mg/L rifampicin (1 mutant per 5.7 × 10⁸ bacteria) and 32 resistant mutants of serovar D (1 mutant per 6.3 × 10⁸ bacteria).

Analysis of the RDRs of moxifloxacin and rifampicin

In total, 12 moxifloxacin-resistant mutants of serovar L2, 30 rifampicin-resistant mutants of serovar L2, and 15 rifampicin-resistant mutants of serovar D were cultivable for further sequence analysis of mutations and MIC determination (Table 1). All the 12 moxifloxacin-resistant clones of serovar L2 had a single point mutation in the QRDR of *gyrA*. Two different mutations at position 83 in the *gyrA* gene were detected, conferring Ser→Ile or Ser→Arg transversions. Both mutations resulted in almost identical MICs of 16–32 mg/L. No additional mutations were found in the QRDR of *gyrB*, *parC* or *parE* genes.

Table 1. Nucleotide changes and MICs for *C. trachomatis* serovar L2 and D resistant clones

<table>
<thead>
<tr>
<th>Variant</th>
<th>Nucleotide change</th>
<th>Position in <em>E. coli</em></th>
<th>MIC (mg/L)</th>
<th>No. of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moxifloxacin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L2-1</td>
<td>AGT→ATT (Ser-83→Ile)</td>
<td>83</td>
<td>16–32</td>
<td>6</td>
</tr>
<tr>
<td>L2-2</td>
<td>AGT→CGT (Ser-83→Arg)</td>
<td>83</td>
<td>32</td>
<td>6</td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D-1</td>
<td>CAC→AAC (His-471→Asn)</td>
<td>526</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>D-2</td>
<td>CAC→TAC (His-471→Tyr)</td>
<td>526</td>
<td>32–64</td>
<td>9</td>
</tr>
<tr>
<td>D-3</td>
<td>GCA→GTA (Ala-467→Val)</td>
<td>522</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>L2-1</td>
<td>CAG→AAG (Gln-458→Lys)</td>
<td>513</td>
<td>&gt;128</td>
<td>15</td>
</tr>
<tr>
<td>L2-2</td>
<td>CAC→AAC (His-471→Asn)</td>
<td>526</td>
<td>8–16</td>
<td>8</td>
</tr>
<tr>
<td>L2-3</td>
<td>CAC→TAC (His-471→Tyr)</td>
<td>526</td>
<td>16–128</td>
<td>2</td>
</tr>
<tr>
<td>L2-4</td>
<td>GCA→GAA (Ala-467→Glu)</td>
<td>522</td>
<td>&gt;128</td>
<td>1</td>
</tr>
<tr>
<td>L2-5</td>
<td>TCA→TTA (Ser-476→Leu)</td>
<td>531</td>
<td>32</td>
<td>1</td>
</tr>
<tr>
<td>L2-6</td>
<td>ATT→CTT (Ile-517→Leu)</td>
<td>572</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>L2-7</td>
<td>CAG→CTG (Gln-458→Leu)</td>
<td>513</td>
<td>&gt;128</td>
<td>2</td>
</tr>
</tbody>
</table>
In rifampicin-resistant mutants of serovar L2, seven different mutations at six loci of the \( rpoB \) gene with MICs ranging from 4 to >128 mg/L were detected. The Gln-458→Lys mutation was the most prevalent one in serovar L2 with 15 of 30 clones. Two clones with identical mutations at His-471→Tyr differed in their MICs (16 and 128 mg/L). We assume that the latter one had an additional mutation outside the sequenced fragment resulting in increased resistance. Of note, five of seven mutations in serovar L2 were a transversion accounting for 90% of the clones. In rifampicin-resistant mutants of serovar D, three different \( rpoB \) mutations at two loci were detected. The His-471→Tyr mutation was most prevalent. Clones with identical mutations had the same MIC ranging from 4 to 64 mg/L or did not differ in more than one dilution. His-471→Asn and His-471→Tyr were detected in rifampicin-resistant clones of both serovars. One of the three mutations in serovar D was a transversion, accounting for 20% of the clones.

**Discussion**

The ability of *C. trachomatis* to escape from antibiotic pressure has been shown in vitro before,\(^3,7,8\) but little is known about the occurrence of spontaneous resistance mutations in vivo. The knowledge of the mutation frequency is mandatory to assess a potential risk for chlamydiae to acquire antibiotic resistance in the future. As chlamydiae are obligate intracellular bacteria, cultivation is cumbersome and clones of different characteristics cannot be easily isolated. Thus, we modified chlamydia cell culture in order to apply selective pressure (above 16-fold MIC for rifampicin and moxifloxacin) and to determine resistant clones in a quantitative manner. Using this method, we showed that quinolone-resistant mutants of serovar L2 occurred three to four times less frequently than rifampicin-resistant mutants and that the mutation frequency in the QRDR of the serovar D isolate was more than 10 times lower than in the serovar L2 isolate, whereas the \( rpoB \) mutation frequency was the same. Mutation frequency conferring rifampicin resistance was about \( 6 \times 10^{-7} \) in both serovars and, thus, within the expected range as, for example, determined for *Escherichia coli*.\(^5\) Binet and Maurelli\(^10\) determined a very similar \( rpoB \) mutation frequency for *Chlamydia psittaci* 6BC \((7 \times 10^{-7})\) and *C. trachomatis* L2 \((2 \times 10^{-7})\) using a plaque assay comparable to ours. In contrast, we determined the mutation frequency resulting in quinolone resistance to be \( 2.0–2.2 \times 10^{-8} \) in serovar L2, but we were not able to isolate a resistant clone of serovar D (mutation frequency \(<2.66 \times 10^{-9}\)). The resistance rate of serovar L2 falls in the range described for *E. coli* \((10^{-9}–10^{-10})\) but the mutation frequency in serovar D is one of the lowest when compared with other bacteria. All resistant clones of serovar L2 carried one of the two different mutations (Ser→Ile or Ser→Arg) both at the amino acid position 83 (*E. coli* numbering). Although the Ser-83→Ile mutant was selected in two other studies on L2 using a stepwise method with increasing drug concentrations before,\(^3,7\) the Ser-83→Arg substitution has never been reported so far in chlamydiae. One possible explanation for differences in the mutation frequencies between serovars and antibiotic drugs could lie in the reduced bacterial viability of clones with acquired mutations in the resistance-determining regions and a higher tolerability of serovar L2 for transversions resulting in viable resistant clones.

Emerging antibiotic resistance is of increasing concern, although *in vivo* resistance of chlamydiae remains rare. *C. trachomatis* develops *in vitro* resistance against various antibiotics, most rapidly against rifampicin.\(^8\) Binet and Maurelli\(^10\) stated that resistance to antibiotics targeting the ribosome correlates with the number of rRNA operons, resulting in lower mutation frequencies in *C. trachomatis* and higher mutation frequencies in *C. psittaci* and *Chlamydia pneumoniae*. They speculated that the absence of significant clinical resistance is due to a loss of fitness of mutated clones.

In this study, we showed that the mutation frequency of serovar L2 determining quinolone resistance is comparable with that of *E. coli*, a bacterium in which quinolone resistance is of great concern. The lack of *in vivo* resistance seems to depend on the restrained usage of quinolones and on the relatively low bacterial load in chlamydial infections. This would reduce the likelihood of drug-induced selection of a resistant clone in chlamydial infections. Nevertheless, it seems to be a matter of time before quinolone resistance will become clinically obvious. On the basis of our data, the risk for the occurrence of future quinolone resistance could depend on the chlamydial serovar and, thus, on the entity of the chlamydial infection.

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**Transparency declarations**

None to declare.

**References**


