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 chlamyidal 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 × 10^9 inclusion forming units (IFUs) of each serovar for rifampicin and 2.66 × 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 ≥ 0.2 mg/L) did not differ significantly between serovars L2 and D (5.7 × 10^-7 versus 6.3 × 10^-7). In contrast, the occurrence of moxifloxacin-resistant mutants (MIC ≥ 0.6 mg/L) was determined to be 2.0–2.2 × 10^-9 for the serovar L2 isolate and less than 2.66 × 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. Briefly, chlamydial strains 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 chlamyoidal plaques were identified after 12–13 days by reverse light microscopy or alternatively by neutral red staining of the monolayer. For isolation of resistant chlamyoidal plaques, the agarose above the plaque was stamped out with a pipette, infected cells were aspirated 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 × 10^9 IFUs of both serovar clones. The plaque assay for moxifloxacin resistance was conducted with a total of 2.66 × 10^9 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 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 in an indirect fluorescent antibody assay with a chlamyoidal 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, Düren, Germany). In moxifloxacin-resistant clones, QRDRs of gyrA, gyrB, parC and parE were amplified according to Dessus-Babus et al., with the primers CT3/4, 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. 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 × 10^9 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 × 10^8 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 × 10^9 organisms and a second clone of serovar D showing no moxifloxacin resistance originating from 2.66 × 10^9 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 × 10^7 bacteria) and 32 resistant mutants of serovar D (1 mutant per 6.3 × 10^7 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>
<thead>
<tr>
<th>Variant</th>
<th>Nucleotide change</th>
<th>Position in E. coli</th>
<th>MIC (mg/L)</th>
<th>No. of clones</th>
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<tr>
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<tr>
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<td>16–32</td>
<td>6</td>
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<td>AGT→CGT (Ser-83→Arg)</td>
<td>83</td>
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<td><strong>Rifampicin</strong></td>
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<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>
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<tr>
<td>D-3</td>
<td>GCA→GTA (Ala-467→Val)</td>
<td>522</td>
<td>4</td>
<td>3</td>
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<tr>
<td>L2-1</td>
<td>CAG→AAG (Gln-458→Lys)</td>
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<td>&gt;128</td>
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<tr>
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Chlamydia trachomatis mutation frequency

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. 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, 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 chlamydial 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 × 10−7 in both serovars and, thus, within the expected range as, for example, determined for Escherichia coli. Binet and Maurelli determined a very similar rpoB mutation frequency for Chlamydophila psittaci 6BC (7 × 10−7) and C. trachomatis L2 (2 × 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 × 10−8 in serovar L2, but we were not able to isolate a resistant clone of serovar D (mutation frequency <2.66 × 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→Ile mutant was selected in two other studies on L2 using a stepwise method with increasing drug concentrations before, the Ser→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. Binet and Maurelli 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


