

## NOTES

# Serine-to-Asparagine Substitution in the GyrA Gene Leads to Quinolone Resistance in Moxifloxacin-Exposed *Chlamydia pneumoniae*

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**Quinolone resistance of *Chlamydia pneumoniae* has not been described previously. Serial subcultures of *C. pneumoniae* under increasing moxifloxacin concentrations (0.0125 to 6.4 mg/liter) resulted in a 256-fold MIC increase compared to moxifloxacin-naive strains. GyrA gene sequencing revealed a novel point mutation with a Ser→Asn substitution. Subcultures under rifalazil and macrolides did not alter the respective MICs.**

The 8-methoxy-fluoroquinolone moxifloxacin (BAY 12–8039) has been proved as an efficient drug against all kinds of respiratory pathogens, including *Streptococcus pneumoniae*, *Haemophilus* sp., and *Moraxella catarrhalis*, but also atypical pathogens like *Mycoplasma pneumoniae* and *Chlamydia (Chlamydophila) pneumoniae* (12). Emergence of resistance to the newer class of fluoroquinolones in the above-mentioned pathogens is relatively uncommon, currently occurring in approximately 1% of clinical isolates in North America (6). However, *C. pneumoniae* resistance mutations against quinolones, namely in the *gyrA* quinolone-resistance-determining region, have not been described in vivo or in vitro so far (5).

We wanted to know whether serial passages of *C. pneumoniae* in subinhibitory concentrations of moxifloxacin would induce chlamydial resistance to quinolones. Thus, we chose the *C. pneumoniae* coronary artery isolate CV-6, which was made available to the Centers for Disease Control and Prevention *Chlamydia* strain collection in 2000, recovered from a chronically infected atheromatous lesion of a 68-year-old male from Northern Germany in 1996 during routine restenosis surgery (7). Susceptibility testing of *C. pneumoniae* was carried out as shown previously in cell culture with HEp-2 cells (ATCC CCL-23) grown in six-well plates with glass coverslips. After a centrifugation step ( $1,700 \times g$  for 1 h, 37°C) with  $10^5$  inclusion-forming units per ml, the medium was replaced by minimal essential medium containing cycloheximide (1  $\mu\text{g/ml}$ ) and moxifloxacin (Bayer Vital GmbH, Leverkusen, Germany) in serial twofold dilutions, starting with the 0.5-fold and 1-fold MIC. Visualization of chlamydial inclusions was performed after incubation at 37°C for 72 h with a fluorescein-conjugated monoclonal anti-*Chlamydia* lipopolysaccharide antibody (Dako, Hamburg, Germany). Moxifloxacin proved to be highly

active against the coronary artery isolate CV-6, showing a MIC of 0.025 mg/liter in a previously described assay (4). Previous studies have shown potent effects of moxifloxacin against standard reference and wild-type *C. pneumoniae* strains, with MICs ranging from 0.031 to 1.0 mg/liter (8, 10, 13). To induce quinolone resistance, chlamydiae were cultured in six-well plates under increasing doses of moxifloxacin (0.0125 to 6.4 mg/liter; initial inoculum,  $10^6$  inclusion-forming units/well) over a total of 40 passages (at 37°C and 5% CO<sub>2</sub> for 72 h), splitting the chlamydial inoculum in one culture at the same concentration and one at the twofold moxifloxacin concentration whenever more than 50% of the cell monolayer was infected. Within this prolonged culture period the MIC increased 256-fold (0.025 to 6.4 mg/liter) compared to that for the moxifloxacin-naive CV-6 strain (Fig. 1), which is in contrast to a recent study which found rapid development of fluoroquinolone resistance in subinhibitory concentrations of ofloxacin or ciprofloxacin in *Chla-*

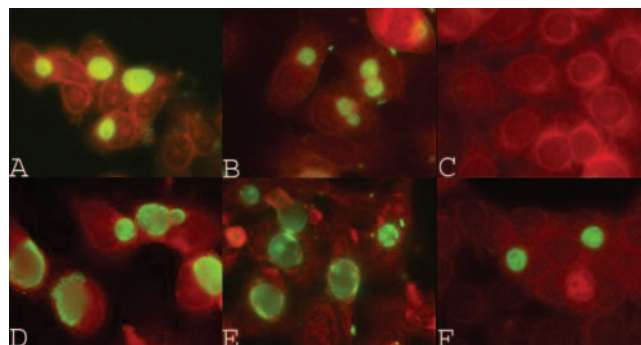


FIG. 1. Immunofluorescence staining with monoclonal anti-*Chlamydia* lipopolysaccharide antibody of HEp-2 cells infected with the *C. pneumoniae* coronary artery isolate CV-6 (A–C) and the *gyrA* mutant CV-6 strain (D–F). Growth of untreated chlamydiae is observed without moxifloxacin and at 0.5-fold MIC (A/B) but not at the MIC (0.025 mg/liter) (C). In the *gyrA* mutant CV-6 strain growth of chlamydiae can still be observed with moxifloxacin concentrations at 64-fold (1.6 mg/liter) and 256-fold (6.4 mg/liter) MIC (E/F).

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TABLE 1. Activities of different antibiotics against the vascular *C. pneumoniae* strain CV-6 and the *gyrA* mutant strain<sup>a</sup>

Antimicrobial agent	MIC <sub>90</sub> (mg/liter) <sup>b</sup> for:	
	<i>C. pneumoniae</i>	<i>gyrA</i> mutant <i>C. pneumoniae</i> strain
Moxifloxacin	0.025	6.4
Rifalazil	0.00025	0.00025
Azithromycin	0.04	0.04
Clarithromycin	0.04	0.04
Doxycycline	0.03	0.03
Levofloxacin	0.4	3.2
Ciprofloxacin	1.0	8.0

<sup>a</sup> After 40 passages in subinhibitory concentrations of moxifloxacin.

<sup>b</sup> MIC<sub>90</sub>, MIC at which 90% of the strains are inhibited.

*mydia trachomatis* but not in *C. pneumoniae* with McCoy cells (11). The propensity of *C. pneumoniae* to develop drug resistance was investigated in the same manner for rifalazil (ActivBiotics, Lexington, Mass.; MIC, 0.00025 mg/liter), a novel rifamycin, and the macrolides azithromycin (Pfizer, Groton, Conn.; MIC, 0.04 mg/liter) and clarithromycin (Abbot, Wiesbaden, Germany; MIC, 0.04 mg/liter). These drugs did not evoke an increase of their respective MICs.

To assess the underlying resistance mechanism for moxifloxacin, we sequenced the *gyrA* gene of moxifloxacin-naïve and mutant CV-6 strains as described previously (1). A point mutation (G→A) was found leading to a serine-to-asparagine substitution at position 83 (*Escherichia coli* numbering) in the corresponding gyrase protein. This mutation introduced the recognition site 5'-A<sup>▽</sup>CGT-3' (the inverted triangle indicates the cutting site of the enzyme) for the restriction endonuclease HpyCH4IV (New England Biolabs, Frankfurt, Germany), which was used to confirm the sequence data by demonstrating digestion of a 500-bp PCR amplicon (forward, GAT ATC CGG GAT GGA CTC AA; reverse, TGT GGA TTT GCA AGC AGA AG) of the *gyrA* gene of the mutant but not of the moxifloxacin-naïve CV-6 strain.

To analyze the stability of this mutation, the moxifloxacin-resistant CV-6 strain was cultured for more than 10 passages without moxifloxacin. Subsequent *gyrA* sequencing, restriction enzyme digestion, and MIC determination showed the mutant genotype and phenotype to persist. *C. pneumoniae* carrying the Ser→Asn substitution showed an eightfold increased MIC for levofloxacin (Aventis Pharma, Bad Soden, Germany) and ciprofloxacin (Bayer Vital GmbH), whereas MICs for rifalazil, azithromycin, clarithromycin, and doxycycline (Sigma-Aldrich, Munich, Germany) remained unchanged (Table 1).

Previously, high-level resistance to fluoroquinolones was observed in *C. trachomatis* L2 reference strains exposed to subinhibitory concentrations of ofloxacin (0.5 mg/liter) and sparfloxacin (0.015 mg/liter) within four rounds of selection. In these quinolone-resistant strains, a point mutation was found in the *gyrA* quinolone-resistance-determining region leading to a Ser→Ile substitution at position 83 (2), the amino acid also most commonly mutated in quinolone-resistant *Enterobacteriaceae* (3). Regarding emergence of antibiotic resistance in vitro

as a multidimensional event, discrepancies in the mutation rate of *C. pneumoniae* and *C. trachomatis* (11) may be based on host-specific (antibiotic-cell interactions) and pathogen-specific (adaptive mutation, bacterial fitness) differences (9). Augmented use of fluoroquinolones as first-line drugs in community-acquired pneumonia and the necessity for recurrent antibiotic treatment in respiratory infections of patients with underlying lung diseases (chronic obstructive pulmonary disease, lung fibrosis, etc.) may promote the development of quinolone-resistant strains of *C. pneumoniae* in vivo. Further studies are needed to evaluate differences in the disposition of vascular (CV-6) and respiratory (CWL029) chlamydial strains to acquire quinolone resistance mutations, with respect to current clinical studies investigating antibiotic treatment in coronary artery disease patients. The data observed here report for the first time the induction of quinolone resistance in a vascular strain of *C. pneumoniae* due to a stable point mutation in the *gyrA* gene featuring a target for endonuclease digestion.

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