

## Emergence of Resistance to Rifampin and Rifalazil in *Chlamydomydia pneumoniae* and *Chlamydia trachomatis*

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Although rifamycins have excellent activity against *Chlamydomydia pneumoniae* and *Chlamydia trachomatis* in vitro, concerns about the possible development of resistance during therapy have discouraged their use for treatment of chlamydial infections. Rifalazil, a new semisynthetic rifamycin with a long half-life, is the most active antimicrobial against *C. pneumoniae* and *C. trachomatis* in vitro, indicating its potential for treatment of acute and chronic *C. pneumoniae* and *C. trachomatis* infections. We investigated the effect of serial passage of two *C. pneumoniae* isolates and two serotypes of *C. trachomatis* in subinhibitory concentrations of rifalazil and rifampin on the development of phenotypic and genotypic resistance. *C. trachomatis* developed resistance to both antimicrobials within six passages, with higher level resistance to rifampin (128 to 256 µg/ml) and lower level resistance to rifalazil (0.5 to 1 µg/ml). *C. pneumoniae* TW-183 developed only low-level resistance to rifampin (0.25 µg/ml) and rifalazil (0.016 µg/ml) after 12 passages. *C. pneumoniae* CWL-029 failed to develop resistance to either drug. Two unique mutations emerged in the *rpoB* gene of rifampin (L456I) and rifalazil (D461E)-resistant *C. pneumoniae* TW-183. A single mutation (H471Y) was detected in both rifampin- and rifalazil-resistant *C. trachomatis* UW-3/Cx/D, and a unique mutation (V136F) was found in rifalazil-resistant BU-434/L<sub>2</sub>. No mutations were detected in the entire *rpoB* gene of rifampin-resistant BU-434/L<sub>2</sub>. This is the first description of antibiotic resistance-associated mutations in *C. pneumoniae* and of rifampin resistance in *C. trachomatis* not associated with mutations in the *rpoB* gene.

*Chlamydomydia pneumoniae* is a common cause of respiratory infections, and *Chlamydia trachomatis* is the most common sexually transmitted bacterial disease. In addition, persistent *C. trachomatis* and *C. pneumoniae* infections have been associated with the development of infertility, asthma, chronic obstructive pulmonary diseases, atherosclerosis, and Alzheimer's disease (3, 12, 16, 19, 20), and therapeutic trials with antimicrobial agents are in progress for some of these conditions. Yet the optimal treatment regimens for *C. pneumoniae* are unknown, and resistance to available antibiotics is beginning to emerge in *C. trachomatis* (21) and *Chlamydia suis*, a related veterinary pathogen (17). Alternative or improved therapies for acute and chronic chlamydial infections would be desirable.

Rifamycins have excellent activity against *C. pneumoniae* and *C. trachomatis* in vitro, but concerns about the emergence of resistance during therapy have discouraged their use for treatment of chlamydial infections in humans (25). Resistance to this class of antibiotics frequently occurs in other species, correlating with point mutations in their target gene, encoding RNA polymerase beta (*rpoB*) (2, 4, 14). The emergence of resistance to rifampin in vitro has been demonstrated in *C. trachomatis* (9, 15, 22, 25, 26). In contrast, no resistance has developed in *C. trachomatis* propagated in the presence of the rifamycin derivatives rifabutin and 3-azinomethyl-rifamycin under the same in vitro conditions (26, 29).

Rifalazil is a new semisynthetic rifamycin with a long half-

life (60 h) and is the most potent antimicrobial in vitro against *C. pneumoniae* and *C. trachomatis* (23, 24). Therefore, it may have potential for short- and long-term therapy of acute and chronic *C. pneumoniae* and *C. trachomatis* infections.

In this study, we investigated the effect of serial passage of *C. pneumoniae* and *C. trachomatis* in subinhibitory concentrations of rifalazil or rifampin on the development of phenotypic and genotypic resistance in vitro.

### MATERIALS AND METHODS

**Selection of resistance.** HEp-2 cell monolayers (ATCC CCL-23) were seeded into six-well plates and inoculated with two *C. pneumoniae* isolates, TW-183 (ATCC VR-2282) and CDC/CWL-029 (ATCC VR-1310), and two *C. trachomatis* strains, BU-434/L<sub>2</sub> (ATCC VR-902B) and UW-3/Cx/D (ATCC VR-885), at high multiplicities of infection. The medium was then replaced with one containing one-eighth to one-half the previously determined MIC of rifalazil or rifampin and incubated for 2 to 4 days at 35°C. Six passages for *C. trachomatis* and 12 passages for *C. pneumoniae* were performed in the presence of the same antimicrobials at the above or twofold increasing concentrations. After 6 and 12 passages, drug susceptibility testing was performed.

**Susceptibility testing.** In vitro susceptibility testing of *C. pneumoniae* and *C. trachomatis* was performed in cell culture with HEp-2 cells grown in 96-well microtiter plates. Each well was inoculated with 0.1 ml of the microorganism diluted to yield 10<sup>4</sup> inclusion-forming units per ml. The plates were centrifuged at 2,000 × g for 1 h. The wells were then aspirated and overlaid with 0.1 ml of medium containing 1 µg of cycloheximide per ml and serial twofold dilutions of rifampin or rifalazil. After incubation at 35°C for 72 h, the cultures were fixed and stained for inclusions with Pathfinder Chlamydia Culture Confirmation monoclonal antibody (Bio-Rad Laboratories, Redmond, Wash.). The MIC was defined as the lowest antibiotic concentration at which no inclusions were seen. The minimum bactericidal concentration was determined by freezing the cultures at -70°C, thawing the cultures, passaging the disrupted cell monolayers onto new cells, incubating the cells for 48 h for *C. trachomatis* and 72 h for *C. pneumoniae*, and then fixing and staining the cells as described above. The minimum bactericidal concentration was defined as the lowest antibiotic concentration that resulted in no inclusions after passage. All tests were run in triplicate.

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TABLE 1. Activity of rifampin and rifalazil against *C. pneumoniae* and *C. trachomatis* after passage in subinhibitory concentrations of drug and associated mutations

Species	Strain	Drug	MIC <sup>a</sup> (μg/ml)			Mutation	Corresponding codon in <i>E. coli</i>
			Initial	6 passages	12 passages		
<i>C. pneumoniae</i>	TW-183	Rifamycin	0.008	0.060	0.25	L456I	511
		Rifalazil	0.001	0.004	0.016	D461E	516
	CWL029	Rifamycin	0.016	0.016	0.016	None	
<i>C. trachomatis</i>	UW-3/Cx/D	Rifalazil	0.001	0.001	0.001	None	
		Rifamycin	0.008	128	ND	H471Y	526
	Rifalazil	0.001	0.5	ND	H471Y	526	
	BU-434/L <sub>2</sub>	Rifamycin	0.008	256	ND	None	
		Rifalazil	0.002	1.0	ND	V136F	146

<sup>a</sup> Values are geometric means of three parallel tests for each strain-drug combination. ND, not determined.

**PCR and sequencing.** DNA from the original and resistant *C. pneumoniae* and *C. trachomatis* cultures was extracted with the DNeasy Tissue Kit (Qiagen, Valencia, Calif.). PCR primers were designed to amplify a 593-bp and a 610-bp PCR fragment covering the predicted rifamycin resistance region of the *rpoB* genes of *C. pneumoniae* and *C. trachomatis*, respectively, based on homology to the *rpoB* sequence of *Mycobacterium tuberculosis* (6, 28). The primers used were Cpn\_rpoB\_forward, 5'-CTAATCGACGTGTCCGCTC T-3', and Cpn\_rpoB\_reverse, 5'-TTCGAAAGCT TCTCCAGCAT-3', for *C. pneumoniae* and L2\_rpoB\_forward, 5'-ATGGGCGAT GAGAAGACATC-3', and L2\_rpoB\_reverse, 5'-CCCAGCATACGGGAGTTTT A-3', for *C. trachomatis*.

PCR was performed with the ProofStart DNA polymerase kit (Qiagen, Valencia, Calif.) at 95°C for 5 min, then 40 cycles of 94°C for 30 s, 62°C for 30 s, and 72°C for 60 sec. The PCR products were purified (QIAquick PCR purification kit; Qiagen) and sequenced in both directions (GeneWiz, North Brunswick, N.J.). The sequences were analyzed with BLAST 2 (<http://www.ncbi.nlm.nih.gov/BLAST/bl2seq/bl2.html>) and compared to the reference *C. pneumoniae* TW-183 (accession number AE017157), *C. pneumoniae* CDC/CWL-029 (accession number AE001593), and *C. trachomatis* serotype D (accession number NC\_000117) sequences deposited in GenBank.

Additionally, the entire 3,759-bp *rpoB* gene of *C. trachomatis* 434/L<sub>2</sub> from the original and resistant cultures was amplified and sequenced in both directions, with overlap, with the following primer pairs: D\_whole\_rpoB\_F, 5'-TTCCTTTT ACCAGGGCTGCAT-3'; D\_whole\_rpoB\_R, 5'-TCTAGCGTCTCTGCATTG GA-3'; D\_rpoB\_1\_F, 5'-ACCATAGGGCGAACATCAA G-3'; D\_rpoB\_1\_R, 5'-CAAGGGAGTGGTTTCCAAGA-3'; D\_rpoB\_2\_F, 5'-ACCTGTCCAAGGTT CATTCG-3'; D\_rpoB\_2\_R, 5'-ACGGTTCCTCTGGTACAGA-3'; D\_rpoB\_3\_F, 5'-GACAAGCGATCCTTTCTGCT-3'; D\_rpoB\_3\_R, 5'-TCAGGAAGATGGGGT AGTCG-3'; D\_rpoB\_4\_F, 5'-CATGTTCCGGAGTTGGATCT-3'; D\_rpoB\_4\_R, 5'-GCTGGGTTTGAAGTTCGAGA-3'; D\_rpoB\_5\_F, 5'-GCACGACACGATAAGG AGTCT-3'; D\_rpoB\_5\_R, 5'-TATGCGCTTATTCTTCGATGC-3'; D\_rpoB\_6\_F, 5'-CGTCGTCTAATGGGAATCCT-3'; D\_rpoB\_6\_R, 5'-TCTCAAGTCC ACCGTCTCC-3'; D\_rpoB\_7\_F, 5'-TGTCGAAGACGCTTCTAACCA-3'; and D\_rpoB\_7\_R, 5'-TCTAGCGTCTCTGCATTGGA-3'.

**Nucleotide sequence accession number.** The sequence of the 434/L<sub>2</sub> *rpoB* gene was submitted to GenBank (accession number AY623623).

**RESULTS**

The MICs of both rifampin and rifalazil increased for *C. pneumoniae* and *C. trachomatis* within six passages in subinhibitory concentrations of both drugs (Table 1). The MICs for *C. trachomatis* UW-3/Cx/D and BU-434/L<sub>2</sub> increased 1.6- to 3.2 × 10<sup>-4</sup>- and 5 × 10<sup>-2</sup>-fold for rifampin and rifalazil, respectively. In contrast, the MICs of rifampin and rifalazil for *C. pneumoniae* TW-183 increased only 7.5- and 4-fold after six passages and 31.25- and 16-fold after 12 passages. Cross-resistance to both rifamycins was demonstrated with both species. Regardless of whether rifampin or rifalazil was used during selection, the resistant *C. pneumoniae* TW-183 had the same MICs to rifampin (both 0.25 μg/ml) and similar MICs to rifalazil (0.008 and 0.016 μg/ml). Resistant *C. trachomatis* BU-434/L<sub>2</sub> strains both had MICs of 256 μg/ml to rifampin and 1.0 μg/ml to rifalazil. *C. pneumoniae* CWL-029 did not develop resistance after passage in subinhibitory concentrations of either rifampin or rifalazil.

Two different mutations were found in the predicted rifamycin resistance region of the *rpoB* gene in rifampin- and rifalazil-resistant *C. pneumoniae* TW-183 (Fig. 1). Resistance to rifampin was associated with nucleotide changes at codon 456 resulting in an amino acid change from leucine to isoleu-

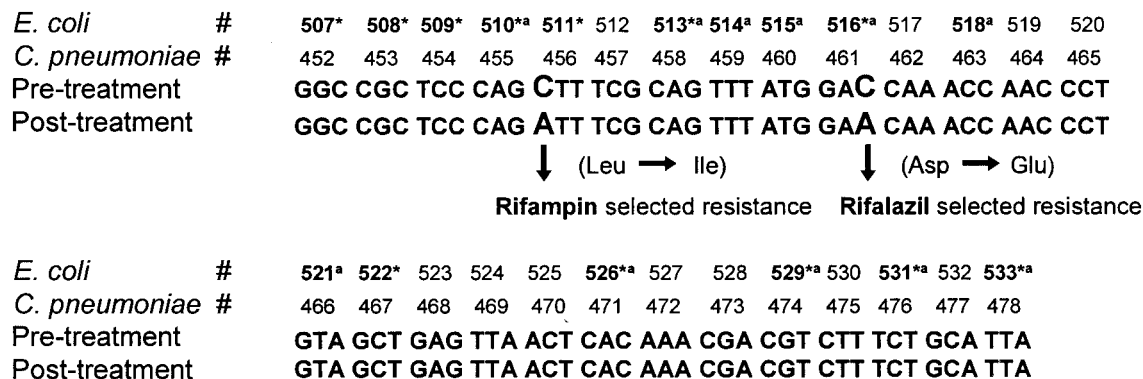


FIG. 1. Mutations in the predicted rifamycin resistance region of the *rpoB* gene of *C. pneumoniae* TW-183. Frequent genetic alteration sites reported in rifamycin-resistant *E. coli* (14) and *M. tuberculosis* (28) are marked with an asterisk and with the letter a, respectively.

<i>E. coli</i>	#	507*	508*	509*	510* <sup>a</sup>	511*	512	513* <sup>a</sup>	514 <sup>a</sup>	515 <sup>a</sup>	516* <sup>a</sup>	517	518 <sup>a</sup>	519	520
<i>C. trachomatis</i>	#	452	453	454	455	456	457	458	459	460	461	462	463	464	465
Pre-treatment		<b>AGC</b>	<b>CGT</b>	<b>TCT</b>	<b>CAA</b>	<b>TTA</b>	<b>TCT</b>	<b>CAG</b>	<b>TTT</b>	<b>ATG</b>	<b>GAT</b>	<b>CAG</b>	<b>ACA</b>	<b>AAC</b>	<b>CCT</b>
Post-treatment		<b>AGC</b>	<b>CGT</b>	<b>TCT</b>	<b>CAA</b>	<b>TTA</b>	<b>TCT</b>	<b>CAG</b>	<b>TTT</b>	<b>ATG</b>	<b>GAT</b>	<b>CAG</b>	<b>ACA</b>	<b>AAC</b>	<b>CCT</b>

<i>E. coli</i>	#	521 <sup>a</sup>	522*	523	524	525	526* <sup>a</sup>	527	528	529* <sup>a</sup>	530	531* <sup>a</sup>	532	533* <sup>a</sup>
<i>C. trachomatis</i>	#	466	467	468	469	470	471	472	473	474	475	476	477	478
Pre-treatment		<b>GTC</b>	<b>GCA</b>	<b>GAA</b>	<b>TTG</b>	<b>ACG</b>	<b>CAC</b>	<b>AAG</b>	<b>CGT</b>	<b>CGT</b>	<b>CTG</b>	<b>TCA</b>	<b>GCA</b>	<b>TTA</b>
Post-treatment		<b>GTC</b>	<b>GCA</b>	<b>GAA</b>	<b>TTG</b>	<b>ACG</b>	<b>TAC</b>	<b>AAG</b>	<b>CGT</b>	<b>CGT</b>	<b>CTG</b>	<b>TCA</b>	<b>GCA</b>	<b>TTA</b>

↓ (His → Tyr)

**Rifampin and Rifalazil selected resistance**

FIG. 2. Mutations in the predicted rifamycin resistance region of the *rpoB* gene of *C. trachomatis* D. Frequent genetic alteration sites reported in rifamycin-resistant *E. coli* (14) and *M. tuberculosis* (28) are marked with an asterisk and with the letter a, respectively.

cine. Resistance to rifalazil was associated with a nucleotide mutation at codon 461 causing replacement of aspartic acid with glutamic acid. No mutations were found in the predicted rifamycin resistance region of the *rpoB* genes in nonresistant *C. pneumoniae* CWL-029 after passaging in the presence of either rifamycin. The sequence of the rifamycin resistance region in *C. pneumoniae* CWL-029 exactly matched the sequence in the untreated *C. pneumoniae* TW-183.

The same mutations were detected at codon 471 in the *rpoB* genes of both rifampin- and rifalazil-resistant *C. trachomatis* UW-3/Cx/D, leading to a histidine-to-tyrosine change (Fig. 2). No mutations were found in the predicted rifamycin resistance regions I and II of the *rpoB* gene of resistant *C. trachomatis* BU-434/L<sub>2</sub>. However, sequencing of the entire *rpoB* gene showed a mutation upstream of that predicted resistance region at codon 136 (146, *Escherichia coli* numbering) in rifalazil-resistant *C. trachomatis* (Fig. 3). This mutation results in replacement of valine with phenylalanine. Interestingly, no mutations were found in the entire *rpoB* gene of the highly rifampin-resistant *C. trachomatis* BU-434/L<sub>2</sub>. Single-nucleotide polymorphisms of the *rpoB* gene of BU-434/L<sub>2</sub> were found at 33 loci compared to the *rpoB* gene of serotype D.

**DISCUSSION**

Rifampin and other rifamycins have been shown to be very active in vitro against *Chlamydia* spp. with MICs ranging from 0.0075 to 0.03 μg/ml (10, 11, 23). The high activity, favorable pharmacokinetics, and excellent cell penetration suggested

that this class of antimicrobials could be very effective against chlamydial infections. In a limited number of studies, treatment of *C. trachomatis* infections with rifampin has been found to be successful and as effective as treatment with tetracycline (25). No signs of emerging resistance of *C. trachomatis* to rifamycins have been reported in vivo. However, the results of several in vitro studies demonstrated that *C. trachomatis* easily and rapidly develops resistance after serial passage in subinhibitory concentrations of rifamycins in both eggs and tissue culture (9, 10, 15).

The risk of development of resistance and data on the emergence of resistance in other bacteria (2, 14, 15, 26, 28) have discouraged further evaluation of rifamycins for the treatment of human chlamydial infections. The development of a new generation of rifamycins with increased activities and enhanced pharmacokinetic properties may prove valuable in treating acute and chronic chlamydial infections (24). In fact, clinical trials utilizing new rifamycins for the treatment of non-gonococcal urethritis caused by *C. trachomatis* are under way. There are no data on the possible emergence of resistance to any rifamycin in *C. pneumoniae* in vitro or in vivo.

Rifalazil is the most potent rifamycin against *Chlamydia* spp., with MICs 10- to 1,000-fold lower than those of azithromycin and levofloxacin (23, 24). Limited experience with the use of rifalazil for treatment of pulmonary tuberculosis in animals and humans has not documented clinically significant emergence of resistance (8). However, emergence of low-level resistance in *C. trachomatis* was reported in one in vitro study

<i>E. coli</i>	#	142	143	144	145	146*	147	148	149	150	151
<i>C. trachomatis</i>	#	132	133	134	135	136	137	138	139	140	141
Pre-treatment		<b>GAG</b>	<b>AGA</b>	<b>GTC</b>	<b>GTT</b>	<b>GTT</b>	<b>TCT</b>	<b>CAA</b>	<b>GTC</b>	<b>CAC</b>	<b>CGT</b>
Post-treatment		<b>GAG</b>	<b>AGA</b>	<b>GTC</b>	<b>GTT</b>	<b>TTT</b>	<b>TCT</b>	<b>CAA</b>	<b>GTC</b>	<b>CAC</b>	<b>CGT</b>

↓ (Val → Phe)

**Rifalazil selected resistance**

FIG. 3. Mutation at the beginning region of the *rpoB* gene of *C. trachomatis* 434/L<sub>2</sub>. \*, mutation site associated with rifampin resistance in *E. coli* (18).

(R. J. Suchland, W. E. Stamm, E. Denamur and D. Rothstein. Abstr. 43rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. C1-2130, 2003).

In this study, serial passage in subinhibitory concentrations of rifalazil and rifampin selected for phenotypic resistance in both chlamydial species. However, rifalazil MICs for resistant *C. trachomatis* (0.5 to 1.0 µg/ml) were more than 250-fold lower than the rifampin MICs (128 to 256 µg/ml). Similarly, the rifalazil MIC for resistant *C. pneumoniae* TW-183 (0.016 µg/ml) was 15.5-fold lower than the rifampin MIC (0.25 µg/ml).

Remarkably, *C. pneumoniae* appeared to be less prone to the development of resistance than *C. trachomatis*. One of the two *C. pneumoniae* isolates tested, CWL-029, failed to develop resistance to either drug after 12 passages, while the other isolate, TW-183, developed a low level of resistance. Given the favorable pharmacokinetic profile of rifalazil and its derivatives (24), this drug may maintain physiological levels well above the increased MIC. Data are very limited on achievable levels of rifalazil in humans and animals. One study in humans found plasma levels of rifalazil of 0.013 to 0.026 µg/ml (8). A study in dogs and rats found plasma rifalazil concentrations of 0.21 to 0.77 µg/ml and 0.08 to 0.25 µg/ml, respectively (13). The results in rats demonstrated much higher levels of rifalazil in tissues: 0.51 to 5.3 µg/ml in lungs, 1.6 to 50.4 µg/ml in spleen, and 2.6 to 19.5 µg/ml in liver. These data suggest that rifalazil, like other rifamycins, may have good tissue penetration and intracellular levels in humans as well. If so, rifalazil might be effective even if relative resistance should occur during therapy.

Based on data from other microorganisms, rifamycin resistance is most frequently associated with genetic alterations in cluster I (codons 507 to 533, *E. coli*) or II (codons 563 to 572) of the *rpoB* gene (14, 28), a region of high amino acid homology across species, including chlamydiae.

As predicted, the resistance of *C. pneumoniae* TW-183 to rifampin and rifalazil was associated with mutations at codons equivalent to *E. coli* codons 511 and 516, respectively. Mutations at codon 526 (*E. coli*) in *C. trachomatis* were found in the *rpoB* gene of rifampin- and rifalazil-resistant UW-3/Cx/D. This mutation was also found by Dreses-Werringloer et al. (9) in rifampin-resistant *C. trachomatis* serovar K, in addition to one at codon 522. The codons involved in our mutants, especially at position 526, have been associated with rifamycin resistance in other species (2, 4, 14, 28). The mutations at 526 also led to the highest degrees of resistance. Perhaps as significant as the site of the mutation is the nature of the amino acid changes. It is quite possible that conservative changes in *C. pneumoniae* were responsible for the lower-level resistance observed, whereas more radical amino acid changes caused higher-level resistance in *C. trachomatis*.

Rifalazil-resistant *C. trachomatis* BU-434/L<sub>2</sub> demonstrated a mutation at the beginning of the *rpoB* gene at codon 146. This rare mutation has been previously reported in the *rpoB* gene of rifampin-resistant *E. coli* by Lisitsyn et al. (18). Intriguingly, although BU-434/L<sub>2</sub> developed high-level phenotypic resistance to rifampin, no genetic alterations were detected in the entire *rpoB* gene. The lack of mutations in the *rpoB* gene is not entirely unexpected. While in most cases high-level rifamycin resistance is associated with *rpoB* gene alteration (28), other

mechanisms of rifampin resistance have been identified and reported for other bacteria, including a rifampin efflux pump, rifampin glucosylation, ribosylation, and phosphorylation enzymes (1, 5, 7, 27).

This is the first description of any antibiotic resistance-associated mutations in *C. pneumoniae* and of high-level rifampin resistance in *C. trachomatis* not associated with mutations in the *rpoB* gene. The clinical significance of these findings is not clear and needs to be investigated.

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