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Chromosomal Mutations Responsible for Fluoroquinolone Resistance in *Ureaplasma* Species in the United States

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We sequenced the full lengths of the *gyrA*, *gyrB*, *parC*, and *parE* genes in 13 fluoroquinolone-resistant *Ureaplasma* isolates (levofloxacin MICs, 4 to 32 μg/ml) and 10 susceptible isolates (MICs ≤ 2 μg/ml). Mutations were detected in all resistant isolates but in none of the susceptible isolates. The most prevalent mutation was the S83L substitution in the ParC protein. No plasmid-mediated fluoroquinolone resistance genes were detected.

Ureaplasma species colonize many healthy persons, yet they are also implicated in various types of infections in adults, such as nongonococcal urethritis, endometritis, chorioamnionitis, spontaneous abortion, arthritis, and urinary calculi, as well as prematurity and low birth weight, bacteremia, meningitis, and lung infections in neonates (14). Since ureaplasmas lack a cell wall, drugs for treatment of infections caused by these organisms are limited primarily to agents that act on protein synthesis, such as tetracyclines and macrolides, and agents that inhibit DNA replication, such as fluoroquinolones (12). Acquired resistance to all three drug classes has been reported in ureaplasmas and appears to be increasing, particularly for the tetracyclines (1–3, 5, 9, 14, 17, 20, 21). Point mutations in DNA gyrase and topoisomerase IV are responsible for fluoroquinolone resistance in many types of bacteria (6). Each enzyme is composed of two subunits that are encoded by the *gyrA* and *gyrB* genes for DNA gyrase and the *parC* and *parE* genes for topoisomerase IV. Amino acid substitutions such as D82N, S83L, and E87K in ParC and Q104K in GyrA and others that occur within the quinolone resistance-determining regions (QRDRs) as a result of DNA point mutations of the respective genes are thought to be responsible for the fluoroquinolone resistance described thus far in *Ureaplasma* spp. (1, 2, 5, 20, 21).

Very limited data are available regarding the frequency of acquired antimicrobial resistance in *Ureaplasma* isolates from the United States since organisms isolated in culture are rarely tested for antimicrobial resistance *in vitro*. This investigation was undertaken to evaluate the occurrence and molecular mechanisms of fluoroquinolone resistance in a collection of 257 nonduplicate *Ureaplasma* isolates from a variety of clinical specimens from several states that were submitted to the University of Alabama at Birmingham (UAB) Diagnostic Mycoplasma Laboratory between 1997 and 2011.

Broth microdilution antimicrobial susceptibility assays were performed as described previously (13) at the time each isolate was cultured for diagnostic purposes. All isolates were then frozen at −80°C in 10B broth (4) until further genetic analysis. Table 1 shows a summary of MICs obtained for erythromycin, tetracycline, and levofloxacin. The rate of resistance to tetracycline was greatest (34%), while the rates of resistance to erythromycin (1%) and levofloxacin (5%) were considerably less, using breakpoints recently designated by the Clinical and Laboratory Standards Institute (CLSI) (4). Among the 13 levofloxacin-resistant isolates (MIC = 4 to 32 μg/ml), one (isolate 25353) was also resistant to both erythromycin (MIC > 256 μg/ml) and tetracycline (MIC = 16 μg/ml) (Table 2). All levofloxacin MICs of ≥2 μg/ml were confirmed by retesting to make sure there were no technical errors in the procedure resulting in false MIC elevation. Clinical information and previous exposure to antimicrobial agents was not available, with the exception of two patients whose cases had been published previously (isolates 25353 and 48105) (5, 7).

TABLE 1 MIC Summary for 257 clinical isolates of *Ureaplasma* species

Drug	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	No. of resistant isolates (% resistance)[a]
Levofloxacin	0.125-32	0.5	2	13 (5)
Erythromycin	0.125-128	1	2	3 (1)
Tetracycline	0.016-128	0.5	32	87 (34)

[a] Resistance was defined using breakpoints published by the CLSI for *Ureaplasma urealyticum* (4). These breakpoints were ≥4 μg/ml for levofloxacin, ≥16 μg/ml for erythromycin, and ≥2 μg/ml for tetracycline.

To investigate molecular mechanisms of fluoroquinolone resistance, genomic DNA was purified from broth cultures using a QIAamp DNA blood minikit (Qiagen, Valencia, CA). *Ureaplasma* species designations were determined by real-time PCR as described previously (18). The full lengths of *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified using published primers, which were based on reference sequences of *Ureaplasma parvum* serovar 3 (2), and newly designed primers favoring *Ureaplasma urealyticum* (based on serovar 10 ATCC 33699) or both species (Table 3). PCR was performed using Platinum PCR supermix (Invitrogen, Carlsbad, CA) on a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA). Amplicons were purified by ExoSAP-IT (USB, Cleveland, OH). DNA sequencing was performed in the UAB Heflin Center Genomics Core Facility using the aforementioned published and newly designed sequencing primers (Table 3) (2). The assembled sequences were compared to reference sequences of 14 fluoroquinolone-susceptible ATCC *Ureaplasma* serovar type strains to identify polymorphisms (2). DNA sequence analy-

Received 11 December 2011 **Accepted** 16 February 2012
Published ahead of print 27 February 2012
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doi:10.1128/AAC.06342-11

TABLE 2 Genetic alterations of ureaplasmas with elevated levofloxacin MICs

Species and isolate	MIC (μg/ml)[a]			Genetic alteration(s)[b]			
	Levo	Ery	Tet	*gyrA*	*gyrB*	*parC*	*parE*
U. urealyticum							
25353	8	≥256	16	A2399T (N800I)	T355C (L119S)	C248T (S83L), **C1265A (A422E), A1646G (H549R)**	**G866A (S289N)**
U. parvum							
26554 Up	8	1	0.25			G259A (E87K)	
48105 Up	32	2	0.5	C310A (Q104K)		C248T (S83L)	G1369A (A457T)
48736 Up	4	1	0.5				C1409T (S470L)
48874 Up	8	2	0.5			C248T (S83L)	
49063 Up	16	2	2			C248T (S83L)	
50056 Up	8	2	0.063			C248T (S83L)	
50186 Up	4	1	32				C5T (A2V), A1280T (D427V)
50892 Up	4	1	2			C248T (S83L)	
51110 Up	8	2	0.5		C1384T (P462S)	C248T (S83L), **A2556 deletion**	
53051 Up	4	1	16			C248T (S83L), **G1342A (D448N), C1344T (D448N), G1588A (D530N), C1657A (L553I), A1708G (T570A), A2104G (I702V), G2203A (A735S), C2204G (A735S), A2229G (I743 M), C2370G (I790 M), G2380A (D794N), G2395A (D799N),**	
55576 Up	8	0.5	0.25			T8A (V3E) C248T (S83L)	
56082 Up	4	2	1			C248T (S83L)	

[a] Abbreviations: Levo, levofloxacin; Ery, erythromycin; Tet, tetracycline.
[b] Parentheses indicate amino acid substitutions that occurred as a result of DNA point mutations. Boldface indicates polymorphisms found in levofloxacin-susceptible clinical isolates but not in the 14 ATCC type strains.

sis was carried out with CLC DNA Workbench (CLC Bio USA, Cambridge, MA). Plasmid-mediated fluoroquinolone resistance genes *qnrA*, *qnrB*, *qnrS*, *aac(6′)-lb*, and *qepA* were sought by PCR using previously published primers (16). The 13 fluoroquinolone-resistant *Ureaplasma* isolates and 10 randomly selected susceptible isolates (levofloxacin MICs, ≤2 μg/ml) were tested.

Among the 13 levofloxacin-resistant isolates, 1 was *U. urealyticum* and 10 were *U. parvum*; among the susceptible isolates, 3 were *U. urealyticum*, 6 were *U. parvum*, and 1 contained a mixture of both species. This probably reflects the fact that *U. parvum* is isolated more frequently than *U. urealyticum* and that coinfection with both organisms sometimes occurs (10). Among the 4 fluoroquinolone-interacting genes, mutations were detected in one or more genes in all resistant isolates (Table 2). The most common mutation was the S83L amino acid substitution in the ParC protein, which was found in 10 of 13 isolates. This is in agreement with a prior report from the United Kingdom indicating that ParC S83L is the most frequently reported mutation in fluoroquinolone-resistant *Ureaplasma* isolates (2). This mutation is located in the QRDR and is homologous to the mutations identified in other fluoroquinolone-resistant bacteria, such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (8). It is notable that this single mutation was sufficient to cause fluoroquinolone resistance in 5 isolates. Another typical mutation in the QRDR region of the ParC protein, E87K, was detected in only one resistant isolate (26554). A new mutation, V3E, in ParC was identified in *U. parvum* isolate 55576 (levofloxacin MIC, 8 μg/ml), which also harbored the S83L substitution. The V3E mutation is outside the QRDR and is probably just a neutral polymorphism.

In the *parE* gene, five point mutations were detected in 3 resistant *U. parvum* isolates that translate into five amino acid substitutions in the corresponding protein, A2V, D427V, A457T (5), S470L, and V96I (Table 2). Similarly, two amino acid substitutions in the GyrA protein, Q104K (5) and N800I, were detected in *U. parvum* isolate 48105 and *U. urealyticum* isolate 25353. In the GyrB protein, L119S and P462S substitutions were detected in *U. urealyticum* isolate 25353 and *U. parvum* isolate 51110. There were 4 isolates (25353, 48105, 50186, and 51110) harboring multiple mutations in one or more genes. Two mutations resulting in amino acid substitutions A88G and S301G in ParC in isolate 48105 that were reported previously (5) were no longer detected after the isolate was regrown in 10B broth, suggesting that this organism can adapt to environmental changes and the possibility that those mutations result in a less robust organism in the absence of fluoroquinolone selection.

According to the sequences of the 4 ATCC *U. parvum* type strains, there are only single copies of *parC* and *parE* in the genome. However, we observed mixed peaks in multiple loci in DNA sequencing trace files of the *parC* and *parE* genes in *U. parvum* isolate 50056 and one mixed peak at position 1409 in the *parE* gene of *U. parvum* isolate 48736 (data not shown). In order to find out whether these isolates were mixed populations of mutant and nonmutant organisms or a single organism harboring two copies of the drug-interacting genes, real-time PCR was used to quantify

TABLE 3 Primers used in PCR and sequencing

Target	Primer	Sequence (5′ to 3′)	Specificity	Application
gyrB	gyrB-Uu-F	TAGAGAATGAAGTTATAGTTG	*U. urealyticum*	PCR/sequencing
	gyrB-Uu-R	CTTCTTTTGTAATTGATTGT	*U. urealyticum*	PCR/sequencing
	gyrB_Uu_743F	ACGGTGAAGAAGAAAAAGA	*U. urealyticum*	Sequencing
	gyrB_Uu_1388R	AATGGTAAAATTGCTTGGAA	*U. urealyticum*	Sequencing
gyrA	gyrA_386F	TTGATGGTGATAGTGCAG	*U. urealyticum*	Sequencing
	gyrA_Uu_1722R	TAGTAAGTCAGTGTGGGT	*U. urealyticum*	Sequencing
parE	parE-Uu-F	TGATTGAGTAAAGATTGGAA	*U. urealyticum*	PCR/sequencing
	parE_Uu_ 1104R	GGCTTGTGTTTTATTTTCTT	*U. urealyticum*	Sequencing
	parE_Uu_1193F	TAAAGAAGAACGTGGAATGG	*U. urealyticum*	Sequencing
	parE_Uu_R2	CGCTCATGGCATACAAAATA	*U. urealyticum*	PCR/sequencing
	parE_CF	CAACATGAAATTGGTCATTTAGATGC	Both species[a]	PCR/sequencing
	parE_CR	GCATAACTTTCACCAACAATGTTGTCT	Both species	PCR/sequencing
	parE_809CF	CTGTGAAAACTAGTGAAGGTGGTGT	Both species	Sequencing
	parE_1047CR	TACTGCTTCATTTGCTTCTGGTGTG	Both species	Sequencing
parC	parC-Uu-F	TGGGTAATGATGTAAGTATT	*U. urealyticum*	PCR/sequencing
	parC-Uu-R	TCATTTAGTTTTGGTGTATAG	*U. urealyticum*	PCR/sequencing
	parC_Uu_1541R	CCATCACGACTAATAACTAAA	*U. urealyticum*	Sequencing
	parC_Uu_1338F	TCAACAACAAGCTAAAACAC	*U. urealyticum*	Sequencing
	parC_CF	GGGTAATGACGTAAGTATTCGA	Both species	PCR/sequencing
	parC_856CR	CATCACGAACTTCGATTAATCCAGC	Both species	Sequencing
	parC_693CF	TGCTCACAAAACTGGTGAAGGT	Both species	Sequencing
	parC_1738CR	GTTCACCAACATCTTTTCACCGCAT	Both species	Sequencing
	parC_1375CF	TCACTTCAAGTTCGCAAAAATT	Both species	Sequencing
	parC_2269CR	CAACTAAAAAAGCTGCTAAAAC	Both species	Sequencing
	parC_2093CF	ATGAAATTAAAACCAAATGATGA	Both species	Sequencing
	parC_2672CR	TTTAAAACAACGTTGGCATAAATT	Both species	PCR/sequencing

[a] *Ureaplasma parvum* and *Ureaplasma urealyticum*.

the double-copy 16S rRNA gene and a single-copy gene for the ribosomal protein L22 (according to the genome of serovar 3, ATCC 27815), as well as *gyrB* and *parE* in isolates 48736 and 50056. The ratio of the 16S rRNA/L22 genes was about 3, while the ratios of the ParE/L22 genes and the ParE/GyrB genes were both about 1 in each isolate, indicating that these two isolates only had one copy of the *parE* gene and were indeed mixtures of two organisms. Serotyping using methods described previously (18) revealed that isolate 48736 contained serovars 1 and 3, while isolate 50056 contained serovars 1 and 6. Thus, isolate 48736 contained one mutant (S470L in ParE) and one wild-type organism, while both organisms in isolate 50056 were mutants (S83L in ParC).

Isolate 25353 carried markers of *U. urealyticum* serovars 9 and 10 as determined by multiplex PCR (18). Two copies of *parC* and *parE* were amplified in this isolate: one copy was similar to serovar 9, while the other one was similar to serovar 10. The S83L mutation in isolate 25353 was located in the serovar 10-like copy but not the serovar 9-like copy. These results indicate that horizontal gene transfer among ureaplasmas (19) and/or other species may be involved in the development of fluoroquinolone resistance in these organisms and thereby facilitate evolution of the drug-resistant phenotype.

No mutations were found in the 4 fluoroquinolone-interacting genes in 4 levofloxacin-susceptible isolates. However, some amino acid substitutions were observed in the other 6 susceptible isolates. All were outside the QRDR region and were therefore considered to be neutral polymorphisms. A total of 20 polymorphisms, 1 in GyrA, 1 in GyrB, 16 in ParC, and 2 in ParE, were identified in the levofloxacin-susceptible isolates but not in the 14 ATCC serovar type strains. Among them, 13 polymorphisms in ParC were present in 3 levofloxacin-resistant isolates (Table 2). Isolate 25353 had a polymorphism in ParE, S289N, which was only found in the levofloxacin-susceptible isolate 24318 from the same patient (7) and not in the ATCC type strains. Also, GyrA Y369H and GyrB S525L polymorphisms were identified only in the levofloxacin-susceptible isolates. No plasmid-mediated fluoroquinolone resistance genes were detected.

Our results support the breakpoint of ≥ 4 μg/ml recently published by the CLSI for designating levofloxacin resistance in *Ureaplasma* spp. (4), since QRDR mutations were detected in all 13 isolates with MICs of 4 to 32 μg/ml and no mutations within the QRDR were detected in those with levofloxacin MICs of ≤ 2 μg/ml. Among isolates with levofloxacin MICs of ≥ 4 μg/ml, 11 of 13 had mutations in the QRDR region of ParC, whereas the other 2 had mutations in ParE. It is known that DNA gyrase of Gram-negative bacteria and topoisomerase IV of Gram-positive bacteria are the preferred sites of action for fluoroquinolones (11). *Ureaplasma* spp. is proposed to have evolved from Gram-positive progenitors (15), so this is in agreement with our observation of the higher incidence of mutations within the ParC subunit of topoisomerase IV.

In addition to the previously reported mutations in the fluoroquinolone-interacting proteins, we have identified 7 new mutations resulting in amino acid substitutions in GyrA, GyrB, ParC, and ParE proteins, all of which are potentially contributory to levofloxacin resistance in *Ureaplasma*. This investigation also provides the first surveillance data for fluoroquinolone resistance in *Ureaplasma* spp. in the United States and spans much of the period in which this drug class became widely used for treatment of Gram-positive organisms, such as *S. pneumoniae.* Thus far, this acquired resistance appears to remain relatively uncommon, and in view of the multiple types of mutations represented, it is most

likely to occur as a result of antibiotic pressure and not clonal spread of organisms already resistant to these agents. Due to the small number of resistant isolates we encountered, it was not possible to make any association between the location or number of mutations and the magnitude of levofloxacin resistance. No attempt was made to evaluate the possibility of efflux-mediated fluoroquinolone resistance since all of the isolates with elevated levofloxacin MICs had one or more point mutations that could account for it.

ACKNOWLEDGMENTS

This work was supported by federal funds under grant R01A1072577 from the National Institute of Allergy and Infectious Diseases.

Technical support by Amy Ratliff is gratefully acknowledged.

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