



# Antimicrobial Resistance in Clinical *Ureaplasma* spp. and *Mycoplasma hominis* and Structural Mechanisms Underlying Quinolone Resistance

Ting Yang,<sup>a,b</sup> Lianlian Pan,<sup>a</sup> Ningning Wu,<sup>a</sup> Lin Wang,<sup>c</sup> Zhen Liu,<sup>a</sup> Yingying Kong,<sup>a,b</sup> Zhi Ruan,<sup>a,b</sup> Xinyou Xie,<sup>a,b</sup> Jun Zhang<sup>a,b</sup>

<sup>a</sup>Department of Clinical Laboratory, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People's Republic of China

<sup>b</sup>Biomedical Research Center, Sir Run Run Shaw Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, People's Republic of China

<sup>c</sup>Shanghai Institute for Advanced Immunochemical Studies, ShanghaiTech University, Shanghai, People's Republic of China

**ABSTRACT** Antibiotic resistance is a global concern; however, data on antibiotic-resistant *Ureaplasma* spp. and *Mycoplasma hominis* are limited in comparison to similar data on other microbes. A total of 492 *Ureaplasma* spp. and 13 *M. hominis* strains obtained in Hangzhou, China, in 2018 were subjected to antimicrobial susceptibility testing for levofloxacin, moxifloxacin, erythromycin, clindamycin, and doxycycline using the broth microdilution method. The mechanisms underlying quinolone and macrolide resistance were determined. Meanwhile, a model of the topoisomerase IV complex bound to levofloxacin in wild-type *Ureaplasma* spp. was built to study the quinolone resistance mutations. For *Ureaplasma* spp., the levofloxacin, moxifloxacin, and erythromycin resistance rates were 84.69%, 51.44%, and 3.59% in *U. parvum* and 82.43%, 62.16%, and 5.40% in *U. urealyticum*, respectively. Of the 13 *M. hominis* strains, 11 were resistant to both levofloxacin and moxifloxacin, and five strains showed clindamycin resistance. ParC S83L was the most prevalent mutation in levofloxacin-resistant *Ureaplasma* strains, followed by ParE R448K. The two mutations GyrA S153L and ParC S91I were commonly identified in quinolone-resistant *M. hominis*. A molecular dynamics-refined structure revealed that quinolone resistance-associated mutations inhibited the interaction and reduced affinity with gyrase or topoisomerase IV and quinolones. The novel mutations S21A in the L4 protein and G2654T and T2245C in 23S rRNA and the *ermB* gene were identified in erythromycin-resistant *Ureaplasma* spp. As fluoroquinolone resistance in *Ureaplasma* spp. and *Mycoplasma hominis* remains high in China, the rational use of antibiotics needs to be further enhanced.

**KEYWORDS** antibiotic resistance, molecular dynamics simulations, *Mycoplasma hominis*, resistance mechanism, *Ureaplasma* spp.

*Ureaplasma* spp. and *Mycoplasma hominis* are both genital mycoplasmas; 40% to 80% of sexually mature women have *Ureaplasma* spp. in their vagina/cervix and 20% to 50% of sexually mature women have *M. hominis* there (1). They are implicated in nongonococcal urethritis, infertility, chorioamnionitis, adverse pregnancy outcomes, and neonatal diseases (2–4). Antibiotics active against these mycoplasmas are limited to agents that interfere with protein synthesis and DNA replication via their unique physiological properties, such as fluoroquinolones, tetracyclines, and macrolide (5). The potential toxicities of quinolones and tetracyclines decrease the availability of therapeutic options for infants, children, and people allergic to tetracyclines and quinolones.

A rise of antibiotic resistance in *Ureaplasma* spp. and *M. hominis* has been reported in France, leading to global concerns (6). Studies of different regions or populations were conducted in succession. A high level of fluoroquinolone resistance in these

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Address correspondence to Xinyou Xie, [scottxie@zju.edu.cn](mailto:scottxie@zju.edu.cn), or Jun Zhang, [jameszhang2000@zju.edu.cn](mailto:jameszhang2000@zju.edu.cn).

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organisms has been observed in China (7, 8), and high levels of tetracycline and erythromycin (ERY) resistance have been reported in *Ureaplasma* species isolated from South African women (9). Investigators agreed that ongoing monitoring to determine the susceptibility of these microbes to potentially active antibiotics was necessary (10, 11).

Bacterial resistance to fluoroquinolone is related to genetic alterations in DNA gyrase (GyrA and GyrB) and/or the topoisomerase IV complex (ParC and ParE) (12). The resistance of *Ureaplasma* spp. is mainly associated with the mutation of C248T (Ser83Leu) in the *parC* gene (13, 14). Acquired resistance to macrolides has rarely been reported in *Ureaplasma* spp., and this resistance can be linked to substitutions in domain II or V of 23S rRNA and/or in the ribosomal proteins L4 and L22 (15). However, *M. hominis* is intrinsically resistant to 14- and 15-membered macrolides (16).

Previous studies concerning antibiotic resistance in *Ureaplasma* spp. were mainly conducted before 2016. Furthermore, few studies have evaluated antibiotic resistance in China, and the commercial IST kit was the prevalent method for detecting resistance (17, 18). However, this method can lead to inaccurate reporting of antibiotic resistance, such as overestimations of antibiotic resistance (10, 11). In contrast, broth microdilution is an internationally accepted technique used to evaluate antibiotic resistance and is also recommended by the Clinical and Laboratory Standard Institute (CLSI) guidelines. In this study, we used the microdilution method to evaluate resistance to five antibiotics in 492 *Ureaplasma* spp. and 13 *M. hominis* strains isolated in China from 1 September to 30 December 2018. Meanwhile, a dynamic refined molecular structure of the topoisomerase IV (ParC-ParE) complex from wild-type *Ureaplasma* spp. bound to levofloxacin (LVX) was built to study the mechanisms underlying quinolone-resistant mutations.

## RESULTS

**Prevalence of resistance in *Ureaplasma* spp. and *Mycoplasma hominis*.** Of the 492 *Ureaplasma* spp. tested, 84.69% (354/418) of *Ureaplasma parvum* samples were LVX resistant, and 82.43% (61/74) of *Ureaplasma urealyticum* samples exhibited LVX resistance, with similar trends for MIC<sub>50</sub> and MIC<sub>90</sub> values in the two species. The resistance rate for moxifloxacin (MXF) ranged from 51.44% in *U. parvum* to 62.16% in *U. urealyticum*, but similar MIC<sub>50</sub> values (4 µg/ml) were exhibited by the two species. MXF-resistant isolates were also resistant to LVX. The majority of *Ureaplasma* spp. had a MIC for ERY ranging from 1 µg/ml to 4 µg/ml. ERY resistance was observed in 15 (3.59%) *U. parvum* and 4 (5.40%) *U. urealyticum* strains. Since no CLSI breakpoint value for doxycycline (DOX) was available, DOX resistance in *Ureaplasma* spp. was unclear. The MIC<sub>90</sub> values for DOX in *U. parvum* and *U. urealyticum* were both 1 µg/ml. Of the 13 *M. hominis* strains tested, 84.62% (11/13) of the strains showed resistance to both LVX and MXF, with MIC<sub>50</sub> values of 16 µg/ml for LVX and of 4 µg/ml for MXF. Five strains (38.46%) were resistant to clindamycin (CLI), and MICs ranged from 1 to >32 µg/ml. Ten *M. hominis* strains showed MICs of 0.125 µg/ml for DOX (Table 1).

**Molecular mechanism of quinolone resistance.** Of the 354 LVX-resistant *U. parvum* and 61 LVX-resistant *U. urealyticum* strains, 322 *U. parvum* and 46 *U. urealyticum* strains presented a single mutation within ParC, Ser83L. The majority of resistant strains had a MIC value of 8 µg/ml, which was twice the breakpoint of LVX. Nine *U. parvum* and three *U. urealyticum* strains showed double mutations, one was the prevalent mutation, and the other occurred at GyrA (Q104K or Q100L), GyrB (N481S, P462S, E482D, D443A, or N486Y), or ParE (A481T or A481V). The MICs for these strains ranged from 8 µg/ml to >32 µg/ml. There were two amino acid substitutions at the 83 position in ParC other than S83L: S83W and S83A. Two *Ureaplasma* strains harbored the S83W substitution, with MICs >4-fold increased against LVX. One *U. parvum* harbored the S83A mutation, which increased the MIC up to 2-fold. Moreover, some mutations occurred at the ParC codon for Glu87: E87K and E87Q. Two *U. parvum* strains had the E87K mutation, with a 4-fold increase in the MIC for LVX. One *U. parvum* strain carried E87Q, with a 1-fold increase in the MIC for LVX. In addition to the two loci in ParC, a mutation at ParC

**TABLE 1** MIC distributions of *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *Mycoplasma hominis*

Antibiotic (MIC $\mu\text{g/ml}$ ) <sup>a</sup>	No. of isolates with an MIC ( $\mu\text{g/ml}$ ) of:										MIC ( $\mu\text{g/ml}$ )		Resistance (n [%])
	0.125	0.25	0.5	1	2	4	8	16	32	>32	50%	90%	
<i>U. parvum</i> (418 strains)													
LVX ( $\geq 4$ )	0	4	4	22	34	49	141	82	48	34	8	32	354 (84.69)
MXF ( $\geq 4$ )	3	7	28	57	108	105	64	30	11	5	4	16	215 (51.44)
ERY ( $\geq 6$ )	7	3	30	114	122	85	42	9	4	2	2	8	15 (3.59)
DOX	200	88	80	37	6	4	1	1	0	1	0.25	1	NA <sup>b</sup>
<i>U. urealyticum</i> (74 strains)													
LVX ( $\geq 4$ )	0	0	1	1	11	11	22	16	10	2	8	32	61 (82.43)
MXF ( $\geq 4$ )	0	2	2	14	10	19	20	5	2	0	4	8	46 (62.16)
ERY ( $\geq 16$ )	1	0	1	5	24	25	14	4	0	0	4	8	4 (5.40)
DOX	16	18	19	15	5	1	0	0	0	0	0.5	1	NA
<i>M. hominis</i> (13 strains)													
LVX ( $\geq 2$ )	0	0	1	1	0	2	2	1	6	0	16	32	11 (84.62)
MXF ( $\geq 0.5$ )	2	0	2	1	1	1	2	4	0	0	4	16	11 (84.62)
CLI ( $\geq 0.5$ )	8	0	0	1	1	1	0	1	0	1	0.125	16	5 (38.46)
DOX	10	0	2	0	1	0	0	0	0	0	0.125	0.5	NA

<sup>a</sup>LVX, levofloxacin; MXF, moxifloxacin; ERY, erythromycin; DOX, doxycycline; CLI, clindamycin.

<sup>b</sup>NA, not applicable (no CLSI breakpoint).

Ala136 (A136S) was detected in one less-resistant *U. parvum* strain with a MIC of 4  $\mu\text{g/ml}$ . Although quinolone resistance mutations occurred most frequently in ParC, one substitution was responsible for LVX resistance in ParE (R448K), and six *U. parvum* strains contained this mutation, exhibiting MICs ranging from 4  $\mu\text{g/ml}$  to >32  $\mu\text{g/ml}$ .

For *M. hominis*, the double substitution ParC K144R/ParE V417I was identified in 2 LVX-sensitive and 11 LVX-resistant isolates. For the resistant mutations, one strain harbored the S91I mutation in ParC and had a MIC of 8  $\mu\text{g/ml}$  for LVX. Two strains contained the double mutations ParC S91I/GyrA S153A, with MICs for LVX ranging from 8 to 16  $\mu\text{g/ml}$ . Five strains had the double mutation ParC S91I/GyrA S153L and a similar MIC of 32  $\mu\text{g/ml}$  for LVX. Moreover, three mutations were identified in one strain, ParC S91I, GyrA S153L, and GyrB A473V. This strain demonstrated a similar LVX-resistant profile as that for the five strains, while the MICs for MXF in this strain were lower (8  $\mu\text{g/ml}$ ) than those of the five strains (16  $\mu\text{g/ml}$ ). The combination of GyrA S153L and ParE R447K was observed in a strain with MICs of 4  $\mu\text{g/ml}$  for LVX and MXF (Table 2).

Based on our analysis, there were still 22 *Ureaplasma* strains and 1 *M. hominis* strain without genetic alterations in their quinolone resistance-determining regions (QRDRs).

**The MD-refined structure of topoisomerase IV bound to LVX in wild-type *Ureaplasma* spp.** Figure 1A shows the alignment of amino acid sequences of ParC and GyrA and of ParE and GyrB. Conservation of amino acids at specific positions is associated with the height of the symbol within each stack. High sequence conservation was observed within the QRDR region of ParC and GyrA. Because the majority of the previously mentioned mutations occurred in a relatively conserved region, some hot spots were identified between different subunits (GyrA and ParC) or species (*Ureaplasma* spp. and *M. hominis*). For example, the mutation at ParC Ser83 in *Ureaplasma* spp. was equivalent to the alteration at GyrA Gln100 and corresponded to mutations in *M. hominis*, ParC Ser91 and GyrA Ser153. A similar corresponding relationship was observed for GyrA Gln104 and ParC Glu87 of *Ureaplasma* spp., for ParE Arg448 of *Ureaplasma* spp., and for ParE Arg447 of *M. hominis*.

A molecular dynamics (MD)-refined structure of the wild-type topoisomerase IV (ParC-ParE) complex bound to LVX in *Ureaplasma* spp. was made to identify the structural importance of the mutated amino acids. The analyses showed that ParC Ser83, ParC Glu87, ParE Asp427, ParE Arg448, and ParE Glu467 were the important residues involved in the binding pocket. The hydroxy residues of Ser83 (ParC) directly bound to the LVX C-3 carboxylic acid via hydrogen bonding. The ParC Glu87 formed a

**TABLE 2** MICs and genetic alternations of fluoroquinolone-resistant *Ureaplasma parvum*, *Ureaplasma urealyticum*, and *Mycoplasma hominis*

No. of resistant strains	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>																			
	LVX		MXF		ERY/CLI		DOX		QRDR <sup>b</sup>											
	50%	Range	50%	Range	50%	Range	50%	Range	GyrA	GyrB										
<i>U. parvum</i>																				
322	8	4 to >32	4	0.5 to >32	2	0.125 to >32	0.25	0.125 to >32												
3	>32	32 to >32	32	8 to >32	1	(1 to 8)	0.125	0.125 to 0.5	C310A (Q104K)											
2	32	32 to >32	4	(4 to 16)	2	(2 to 4)	0.25	(0.25 to 0.5)		A1442G (N481S)										
1	16		8		4		0.5			C1384T (P462S)										
1	16		4		2		0.25			<b>A1446C (E482D)</b>										
1	>32		16		0.5		0.125			<b>A1328C (D443A)</b>										
1	32		8		0.5		0.125													
1	16		8		2		0.25													
1	8		2		1		0.125													<b>G1441A (A481T)</b>
2	16	16 to 32	4	(4 to 8)	2	(2 to 2)	0.125	0.125 to 4												
1	4		2		8		0.125													
1	4		4		8		1													
5	4	4 to >32	4	(1 to 16)	4	(0.25 to 32)	0.25	(0.125 to 1)												
1	8		4		2		0.125													
11	8	4 to >32	2	(1 to 16)	4	(1 to 16)	1	(0.125 to 1)	N	N										
<i>U. urealyticum</i>																				
46	8	4 to >32	4	(1 to 32)	4	0.125 to 16	0.5	0.125 to 8												
1	>32		32		8		0.5													
1	32		8		2		0.125													
1	32		16		8		0.5		<b>A299T (Q100L)</b>											
1	8		4		4		0.25			<b>A1456T (N486Y)</b>										
11	4	(4 to 32)	2	(1 to 8)	4	(1 to 16)	0.5	0.125 to 1	N	N										
<i>M. hominis</i>																				
2 <sup>c</sup>	1	(0.5 to 1)	0.125	0.125 to 0.125	0.125	0.125 to 0.125	0.125	0.125 to 0.125												
1	8		0.5		0.125		0.125													
2	16	(8 to 16)	2	0.5 to 2	16	(0.125 to 16)	0.5	0.125 to 0.5												
3	32	32 to 32	16	(8 to 16)	0.125	(0.125 to >32)	0.125	(0.125 to 0.5)												
2	32	32 to 32	16	(16 to 16)	1	(0.125 to 1)	0.125	0.125 to 0.125												
1	32		8		4		2													
1	4		4		0.125		0.125													
1	4		1		2		0.125													

<sup>a</sup>LVX, levofloxacin; MXF, moxifloxacin; ERY, erythromycin; CLI, clindamycin; DOX, doxycycline.

<sup>b</sup>N, no mutation detected. Numbers and letters in parentheses refer to amino acid changes based on *Ureaplasma* spp. or *Mycoplasma hominis*. Novel mutations are highlighted in boldface font. Mutations without association with fluoroquinolones resistance are underlined.

<sup>c</sup>Two strains of *M. hominis* were sensitive.



**TABLE 3** The MICs and genetic alternations of macrolide-resistant *Ureaplasma parvum* and *Ureaplasma urealyticum*

No. of macrolide-resistant strains	MIC ( $\mu\text{g/ml}$ ) <sup>a</sup>								Macrolide <sup>b</sup>		
	LVX		MXF <sup>b</sup>		ERY <sup>b</sup>		DOX <sup>b</sup>		L4	L22	23S rRNA
	50%	Range	50%	Range	50%	Range	50%	Range			
<i>U. parvum</i>											
3	>32	>32 to >32	32	16 to >32	16	16 to 16	0.5	1 to >32			G361T (A121S) & C422T (T141I)
1	>32		>32		>32		4		<b>T61G</b>		G361T (A121S) & C422T (T141I)
1	32		16		16		1				G196A (D66N)
1	16		8		32		1				<b>T2245C</b> ( <i>E. coli</i> numbering 2234T)
9	32	4 to >32	16	2 to >32	16	16 to >32	1	0.5 to >32	N	N	<b>N</b>
<i>U. urealyticum</i>											
1	16		4		16		2				<b>G2654T</b> ( <i>E. coli</i> numbering 2643G)
3	16	(8 to 32)	8	(8 to 16)	16	(16 to 16)	2	(1 to 8)	N	N	N

<sup>a</sup>LVX, levofloxacin; MXF, moxifloxacin; ERY, erythromycin; DOX, doxycycline.

<sup>b</sup>N, no mutation detected. For L4 and L22, numbers and letters in parentheses refer to amino acid changes based on *Ureaplasma* spp. For 23S rRNA, numbers and letters in parentheses are the corresponding numbers and letters in *E. coli*. Novel mutations are highlighted in boldface font.

“-”; K, “+”; Q, neutral [N]). Moreover, the K and L mutations prevented the formation of the hydrogen bond, consequently disrupting the water-magnesium ion bridge. For the mutations in ParE Arg448 (corresponding to ParE Arg447 in *M. hominis*) and ParE Asp427 (corresponding to GyrB Asp443 in *Ureaplasma* spp.), the former prevented the formation of hydrogen bonds and the latter weakened the ionic bond between the two amino acids (D443A: D, -; A, “N”). All of the above-mentioned mutations reduced the affinity between quinolone and gyrase or topoisomerase IV. Furthermore, mutations GyrB E482D and P462S and mutations occurring outside the quinolone binding pocket, which include ParC 136, GyrB 486, ParE 481, and GyrB 473 (in *M. hominis*), appeared to be similar to the differences in single nucleotide polymorphisms.

**Molecular mechanism for the macrolide resistance of *Ureaplasma* spp.** Three ERY-resistant *U. parvum* strains contained two mutations in L22, A121S and T141I, with similar MICs of 16  $\mu\text{g/ml}$  for ERY. One resistant strain harbored three substitutions, including two previously mentioned mutations and one S21A mutation in L4. A higher MIC for ERY was observed in this strain (>32  $\mu\text{g/ml}$ ). The D66N substitution in L22 was detected in one strain with a MIC of 16  $\mu\text{g/ml}$  for ERY. Furthermore, two mutations within 23S rRNA, T2245C and G2654T, were detected in one strain with a MIC of 16  $\mu\text{g/ml}$  (Table 3).

Nine *U. parvum* and three *U. urealyticum* strains did not reveal any mutations in their 23S rRNA and/or ribosomal proteins L4 and L22. Among them, one *U. parvum* strain was detected with *ermB*, which is a subtype of the *erm* rRNA methyltransferase gene.

**DISCUSSION**

The rise of antibiotic resistance in *Ureaplasma* spp. and *M. hominis* is a global concern due to limited therapeutic options. Continued surveillance of the antibiotic susceptibility of these urogenital mycoplasmas has been recommended by many research studies (5, 10, 11). In previous studies, we evaluated the quinolone resistance of *Ureaplasma* spp. from 1999 to 2014, which indicated a high level of quinolone resistance in China (7, 19). In this study, we assessed the prevalence of antibiotic resistance among *Ureaplasma* spp. and *M. hominis* from September to December 2018. Moreover, the mechanisms of the quinolone resistance were also studied.

Fluoroquinolones have been extensively used to treat bacterial infections in respiratory, gastrointestinal, and urinary tracts over the past few decades in China, thereby resulting in serious antimicrobial resistance in many bacterial pathogens, i.e., *Mycobacterium tuberculosis*, *Neisseria meningitidis*, and *Riemerella anatipestifer* (20–22). High

levels of fluoroquinolone resistance in *Ureaplasma* spp. were also observed with LVX resistance rates of 75.9% for *U. parvum* and 71.4% for *U. urealyticum* and with MXF resistance rates of 25.3% for *U. parvum* and 50% for *U. urealyticum* (7). Compared with that in the previous reports, an increasing trend of fluoroquinolone resistance has been observed in the present study, in which >80% of *Ureaplasma* spp. showed LVX resistance and >50% of *Ureaplasma* spp. were MXF resistant. The resistance rate in *Ureaplasma* spp. varied from country to country. In the United States, 6.4% of *U. parvum* and 5.2% of *U. urealyticum* strains were LVX resistant (23). In Switzerland, 19.4% and 9.7% of strains were resistant to ciprofloxacin (CIP) and ofloxacin (OFL), respectively (using a commercial kit) (24). In France, the LVX and MXF resistance rates of *Ureaplasma* spp. were 1.2% and 0.1%, respectively (11). Moreover, a high level of fluoroquinolone resistance has been observed in Japan (16/28 [57.14%] of *Ureaplasma* spp. for LVX resistance) and Korea (50.6% for OFL and 27.4% for CIP in *Ureaplasma* spp., using a commercial kit), which are both near China geographically (14, 17). In Italy, a CIP resistance rate of 77.1% was reported using a commercial kit (25). It is worth noting that commercial kits may lead to an overestimation of antibiotic resistance (11). For *M. hominis*, 84.62% of strains were LVX or MXF resistant. This finding is consistent with a previous report in Sichuan, China, where 85.7% of strains were LVX resistant and 73.8% of strains were MXF resistant (8). However, the resistance rates for *M. hominis* were 2.7% for LVX and 1.6% for MXF in France (11). Considering the levels of fluoroquinolone resistance in different reports, the resistance rates of *Ureaplasma* spp. and *M. hominis* in China is extremely high. The overuse and misuse of quinolones cause severe quinolone-resistance in China. Recent studies have shown that fluoroquinolones are still commonly used antibiotics in China (26–28), and even the concentrations of quinolones in livestock and environments are extremely high (29–32). Compared with the high levels of ERY resistance by *Ureaplasma* spp. (80%) in South Africa (9), the ERY resistance was not serious in China, observed in 3.59% of *U. parvum* and 5.4% of *U. urealyticum* strains.

Sequence analysis of the QRDRs in LVX-resistant *Ureaplasma* strains revealed that the ParC codon for Ser83 was a hot spot of mutation, with three amino acid substitutions (S83L, S83W, and S83A). Previous studies reported that the S83L substitution was the most frequent mutation (7, 13, 24); 93.50% of LVX-resistant *U. parvum* and 80.33% of LVX-resistant *U. urealyticum* carried this mutation in the present study. The S83W mutation has been reported in two *Ureaplasma* strains (7, 33), while the S83A substitution was first observed in *Ureaplasma* spp. The corresponding amino acid change has been reported in quinolone-resistant *Escherichia coli* (GyrA S83A) and *Staphylococcus aureus* (GyrA S84A) (34, 35). The second most frequent mutation was ParE R448K, which was first found in China (7). The previously reported mutations, ParC E87K and E87Q, were also observed in three *U. parvum* strains (23, 36). Moreover, a new substitution, ParC A136S, was observed in one *U. parvum* with low-level quinolone resistance. Nine mutations in GyrA, GyrB, or ParE were found along with ParC S83L. GyrA Q100R has been reported in a quinolone-resistant *Ureaplasma* strain (37); however, a new amino acid change (Q100L) was found in the present study. GyrA Q104K has been associated with MXF resistance (11); GyrB P462S and GyrB N481S appeared to act as neutral polymorphisms (7, 38). The remaining substitutions in GyrB (E482D, D443A, and N486Y) and ParE (A481T and A481V) were novel mutations, first identified in this study. For *M. hominis*, the ParC K144R mutation was observed in two susceptible strains, suggesting that this mutation did not contribute to fluoroquinolone resistance (8, 39). Six (54.55%) *M. hominis* strains carried the two mutations, ParC S91I and GyrA S153L, which were proved to be the most frequent genetic alterations in fluoroquinolone-resistant *M. hominis* (8, 11, 39). GyrA S153L substitution was associated with a high level of resistance (40). A novel mutation was identified in the same position, GyrA S153A. The previously reported R447K in ParE was detected in one strain (41). Moreover, a novel mutation, GyrB A473V, was observed in *M. hominis*.

DNA gyrase shares homology with topoisomerase IV (42). The alignment results revealed that the novel mutation GyrA S153A in *M. hominis* corresponds to the new

alteration ParC S83A in *Ureaplasma* spp., and ParE Arg447 in *M. hominis* is consistent with ParE R448K in *Ureaplasma* spp. The MD-refined structure revealed that the fluoroquinolone-resistant mutations were aimed to disrupt the stable contacts within ParC (or GyrA) and fluoroquinolone. Disrupting the direct interaction was the most effective alteration to drug resistance. Consequently, ParC Ser83 in *Ureaplasma* spp. and ParC Ser91 and GyrA Ser153 in *M. hominis*, which shared a similar direct binding mode with LVX, were all mutation hot spots. The ParC Glu87, GyrA Gln100, and GyrA Gln104 in *Ureaplasma* spp. shared a similar binding mode to ParC Ser84 and Glu88 in *Acinetobacter baumannii* in a water-Mg<sup>2+</sup>-ligand fashion (43). An interaction between ParE Arg448 and ParE Asp427 was first found in *Ureaplasma* spp. The interactions between these codons and LVX are relatively weak, and mutations occurred at these codons have a low level of quinolone resistance. Moreover, even if the mutations occur in the same position, the level of resistance to quinolone varies with the different substituted amino acids.

The mechanism of macrolide resistance in clinical *Ureaplasma* spp. is less well characterized, since macrolide-resistant *Ureaplasma* spp. are uncommon at the international level (10, 24). A G2057A/U (*E. coli* numbering) substitution was detected in mutants of *U. parvum* selected *in vitro* with ERY, and this substitution was also associated with intrinsic resistance of *M. hominis* to 14- and 15-membered macrolides and with ERY-resistant *Chlamydomonas reinhardtii*, cutaneous propionibacteria, and *E. coli* (15, 44–47). The aforementioned mutation G2057A/U (*E. coli* numbering) was not detected in the 19 macrolide-resistant *Ureaplasma* strains in the present study; however, two novel alterations occurring in the 23S ribosome, T2245C and G2654T, were identified. Moreover, double alterations (A121S and T141I) or a single mutation (D66N) that occurred in L22 was observed within five strains, including one sample harboring an S21A substitution in L4 at the same time. These three mutations detected in the L22 protein were reported in macrolide-resistant *U. parvum* that carried five amino acid insertions in its L4 protein. However, the potential resistance function of these mutations has been ignored because of the polymorphisms at these positions within 14 serovars of *Ureaplasma* spp. (15). Notably, 13 serovars are both Asp at position 66 for the L22 protein; in contrast, serovar 1 (ATCC 27813) has an Asn at this position. The same phenomenon has been observed in serovar 14 (ATCC 33697), with Ser at L22 position 122 (others have Ala) and with Ile at L22 position 141 (others have Thr). Unfortunately, we do not have ATCC 27813 and ATCC 33697 strains in our laboratory, and their MICs to ERY and susceptibility/resistance to macrolide are unknown. S21A is a novel mutation in the L4 protein; there are nine *U. parvum* strains and three *U. urealyticum* isolates that did not reveal any mutations associated with macrolide resistance. Macrolide resistance is acquired in bacteria via three mechanisms, including drug inactivation, active drug efflux pumps, and modification of the target site by methylation or mutation (48, 49). Among the nine *U. parvum* strains, one strain was detected with a subtype of the *erm* rRNA methyltransferase gene, *ermB*. Lu et al. first observed the *ermB* gene in 21 *Ureaplasma* strains (50). The encoded protein of this gene can modify an analogous adenine residue in a highly conserved region of the 23S rRNA to yield macrolide-lincosamide-streptogramin B (MLS) resistance (51).

In conclusion, high levels of quinolone resistance in *Ureaplasma* spp. and *M. hominis* still exist in China, and the resistance showed an increasing trend in *Ureaplasma* spp. in 2018. The rational use of antibiotics needs to be further enhanced, and ongoing monitoring of antibiotic resistance is necessary.

## MATERIALS AND METHODS

**Clinical samples and strains.** Over a 4-month period (1 September to 30 December 2018), 492 clinical *Ureaplasma* species (418 *Ureaplasma parvum* and 74 *Ureaplasma urealyticum*) and 13 clinical *M. hominis* samples were obtained in the present study. Among these strains, 350 isolates were from female urogenital tracts, and 155 isolates were isolated from male urogenital tracts at Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University.

**Determination of antibiotic resistance.** The antibiotics LVX, MXF, DOX, ERY, and CLI were selected and used at concentrations ranging from 0.125 µg/ml to 32 µg/ml. MICs were evaluated in duplicates by broth microdilution according to the CLSI guidelines. The breakpoints of antibiotics were interpreted,



except for DOX, for which there is no current CLSI breakpoint. The antibiotics were purchased from Sigma-Aldrich (St. Louis, MO, USA), and *Ureaplasma*-selective culture broth was purchased from Hebei Zhonggaisheng Biological Engineering Company (Hebei, China).

**Identification of genetic mechanisms of resistant strains.** Amplifications of the QRDR of LVX-resistant *Ureaplasma* strains and all *M. hominis* strains were conducted using previously reported primers, and the sequences were then mapped to the corresponding sequences of *U. parvum* ATCC 700970, *U. urealyticum* ATCC 33699, or *M. hominis* ATCC 23114 (40, 52, 53). PCR and analysis of the sequences of 23S rRNA, L4, and L22 in ERY-resistant *Ureaplasma* strains were performed as previously described (44). Moreover, genes involved in other potential resistance mechanisms associated with macrolide resistance, including *erm* rRNA methyltransferase genes, macrolide modification enzyme genes, and efflux pump genes, were screened in ERY-resistant *Ureaplasma* strains as previously described (15).

**Multiple-sequence alignment analysis.** BLASTp was performed for protein sequences of ParC and ParE in *Ureaplasma* spp. using NCBI BLAST web services (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The nonredundant matched sequences were downloaded, with 6,570 sequences (containing GyrA) for ParC and 20,007 sequences (containing GyrB) for ParE. A subsequent multiple-sequence alignment analysis was performed using MAFFT software. Based on the *Ureaplasma* spp. coordinates, the amino acid sequences of ParC from residues 61 to 140 and of ParE from residues 405 to 484 were retrieved to generate the sequence logos in WebLogo (<http://weblogo.berkeley.edu>).

**Refined molecular dynamics structure of topoisomerase IV in *Ureaplasma* spp.** A three-dimensional (3D) structure of topoisomerase IV in *Ureaplasma* spp. was built by homology modeling based on the quinolone-DNA cleavage complex of type IIA topoisomerases from *Streptococcus pneumoniae* (PDB code 3K9F) using Prime of Schrödinger Suite 2019-1 (54). The bound mode of the quinolone-DNA cleavage complex of *Acinetobacter baumannii* (PDB code 2XKK) was chosen for LVX. System equilibration was performed with the default parameters in Desmond. Fifty-nanosecond MD simulations were then carried out using the Desmond Molecular Dynamics module of Schrödinger 2019-1 (54).

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We declare no conflict of interests.

T.Y., L.P., N.W., Z.L., and Y.K. performed the experiments. T.Y. analyzed the sequence data and drafted the manuscript. L.W. built the model of the topoisomerase IV complex bound to levofloxacin in wild-type *Ureaplasma* spp. Z.R., X.X., and J.Z. reviewed and edited the text. X.X. and J.Z. designed the study. All authors read and approved the final manuscript.

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