Frequent topoisomerase IV mutations associated with fluoroquinolone resistance in *Ureaplasma* species

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This study aimed to investigate the role of quinolone resistance-determining regions (QRDRs) of DNA gyrase (encoded by *gyrA* and *gyrB*) and topoisomerase IV (encoded by *parC* and *parE*) associated with fluoroquinolone resistance. A total of 114 *Ureaplasma* spp. strains, isolated from clinical female patients with symptomatic infection, were tested for species distribution and susceptibility to four fluoroquinolones. Moreover, we analysed the QRDRs and compared these with 14 ATCC reference strains of *Ureaplasma* spp. serovars to identify mutations that caused antimicrobial resistance. Our study indicated that moxifloxacin was the most effective fluoroquinolone against *Ureaplasma* spp. (MIC range: 0.125–32 μg ml⁻¹). However, extremely high MICs were estimated for ciprofloxacin (MIC range: 1–256 μg ml⁻¹) and ofloxacin (MIC range: 0.5–128 μg ml⁻¹), followed by levofloxacin (MIC range: 0.5–64 μg ml⁻¹). Seven amino acid substitutions were discovered in GyrB, ParC and ParE, but not in GyrA. Ser-83→Leu/Trp (C248T/G) in ParC and Arg-448→Lys (G1343A) in ParE, which were potentially responsible for fluoroquinolone resistance, were observed in 89 (77.2 %) and three (2.6 %) strains, respectively. Pro-462→Ser (C1384T), Asn-481→Ser (A1442G) and Ala-493→Val (C1478T) in GyrB and Met-105→Ile (G315T) in ParC seemed to be neutral polymorphisms, and were observed and occurred along with the amino acid change of Ser-83→Leu (C248T) in ParC. Interestingly, two novel mutations of ParC and ParE were independently found in four strains. These observations suggest that amino acid mutation in topoisomerase IV appears to be the leading cause of fluoroquinolone resistance, especially the mutation of Ser-83→Leu (C248T) in ParC. Moxifloxacin had the best activity against strains with Ser-83→Leu mutation.

**INTRODUCTION**

*Ureaplasma* spp. is a member of the class *Mollicutes* and one of the smallest self-replicating micro-organisms. *Ureaplasma* spp. commonly colonizes in the urogenital tract of humans, yet it is also implicated in diverse types of infection, including non-gonococcal urethritis, inflammation, infertility, adverse pregnancy outcomes, chorioamnionitis and bronchopulmonary dysplasia in neonates (Heggie et al., 2001; Katz et al., 2005; Waites et al., 2005; Salmeri et al., 2012). Based on differences of phenotype and genotype, 14 *Ureaplasma* spp. serotypes can be classified into two species, *Ureaplasma parvum* (UPA) and *Ureaplasma urealyticum* (UUR). UPA contains four relatively small genomic serovars (0.75 and 0.78 Mbp), UPA1, UPA3, UPA6 and UPA14, while UUR comprises the remaining ten serovars (0.84 and 0.95 Mbp), UUR2, UUR4, UUR5, UUR7, UUR8, UUR9, UUR10, UUR11, UUR12 and UUR13 (Glass et al., 2000; Robertson et al., 2002; Paralanov et al., 2012). Since *Ureaplasma* spp. lacks a cell wall, antibiotic agents that target the cell wall are ineffective (i.e. glycopeptides and β-lactams) and antimicrobials for infection treatment are confined primarily to agents that inhibit DNA replication (i.e. fluoroquinolones) and protein synthesis (i.e. macrolides and tetracyclines). Acquired resistance rates to all three antibiotic classes, especially fluoroquinolones, have been documented in clinical *Ureaplasma* spp. isolates and are rising rapidly (Xie & Zhang, 2006; De Francesco et al., 2013). However, there have been only a limited number of studies on surveillance for antimicrobial resistance in clinical *Ureaplasma* strains, which is crucial for the management of the ureaplastic infections.

**Abbreviations:** QRDR, quinolone resistance-determining regions; UPA, *Ureaplasma parvum*; UUR, *Ureaplasma urealyticum*.
It is generally accepted that unique amino acid substitution variants are potential molecular mechanisms for antibiotic resistance. Single point mutations of the type II topoisomerase DNA gyrase (encoded by gyrA and gyrB) and topoisomerase IV (encoded by parC and parE) are responsible for resistance to fluoroquinolones in a wide types of bacterial species, including ureaplasmas (Azmi et al., 2014; Lu et al., 2014; Singh et al., 2015). Amino acid substitutions of Asp-82→Asn, Ser-83→Leu and Ala-88→Gly in the ParC and Gln-100→Arg and Gln-104→Lys in the GyrA and others, all located in the quinolone resistance-determining regions (QRDRs), are commonly described and proposed to account for the increasing fluoroquinolone resistance in Ureaplasma (Beeton et al., 2009). However, substitutions of Asp-112→Glu in GyrA, and Thr-125→Ala and Thr-136→Ala in ParC, previously thought to be related to fluoroquinolone resistance, are testified as species-specific or serovar-specific polymorphisms (Beeton et al., 2009).

In this study, we analysed the species distribution and the fluoroquinolone susceptibilities of 114 Ureaplasma spp. isolates from the urogenital tracts of Chinese females. Furthermore, we amplified and sequenced the QRDRs of clinical Ureaplasma spp. strains and compared these with 14 serovars of ATCC reference strains. The MIC values of four fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) were determined for all the 114 clinical Ureaplasma spp. strains. Summaries of the MIC values of the tested four fluoroquinolones were used for isolating genome DNA as described in our previous study (Zhang et al., 2014a).

Identification of Ureaplasma species. The genome sequences of 14 ATCC Ureaplasma spp. serovars were retrieved from GenBank, described as follows: ABES00000000.1 (UPA1), ABFL00000000.2 (UUR2), CP000942.1 (UPA3), AAY00000000.2 (UUR4), AAZR00000000.1 (UUR5), AAZQ00000000.1 (UUR6), AAYP00000000.1 (UUR7), AAYN00000000.2 (UUR8), AAYQ00000000.2 (UUR9), CP001184.1 (UUR10) AAZ50000000.1 (UUR11), AAZT00000000.1 (UUR12), ABEV00000000.1 (UUR13) and ABER00000000.1 (UUR14). A total of 47 UPA-specific genes and 45 UUR-specific genes were found by using Blast program. Of these, UU295 and UUR10_0588 showed great conservative property in each species and tested suitably for identification of Ureaplasma spp. by using 14 reference strains. Primers to distinguish UPA from UUR were UU295-F (5’-GCCAGAAGACATTTATG-CGT-3’) and UU295-R (5’-CTGATATTGTGGCTGGTAC-3’) for UPA and UUR10_0588-F (5’-AAATGTTAAGACTTCTTGAG-3’) and UUR10_0588-R (5’-AATTGGAATATGGCCTTGTG-3’) for UUR (Zhang et al., 2014).

Amplification of the QRDR region and DNA sequencing. PCR primers for amplification of the QRDRs from gyrA, gyrB, parC and parE were gyrA-F (5’-TTGGTCTTCTGCCAAAGCG-3’) and gyrA-R (5’-CTGATGTTAACAATCCTTG-3’) for amplification of gyrA; gyrB-F (5’-CTGGTAAATTAGCTGAGT-3’) and gyrB-R (5’-CTG- GAGATTATGCTGGCATC-3’) for amplification of gyrB; parC-F (5’-AAGCAAATGTTAGTGG-3’) and parC-R (5’-CAGATCAAATCTGGGAG-3’) for amplification of parC; and parE-F (5’-TGGGCGGAAAATTAACGC-3’) and parE-R (5’-CTGGTAGTGAAGCCTC-3’) for amplification of parE (Bebear et al., 2000). Amplifications were carried out according to the Taq DNA Polymerases (Takara) protocol. The PCR amplification was initiated for 5 min at 94 °C, followed by 35 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min and with a final 5 min extension period at 72 °C, then cooling to 10 °C. The purification of amplicons was carried out by adding two volumes of 95% ethanol and 0.1 volumes of 2.5 M NaCl and then kept at −20 °C for 1 h. The mixture was centrifuged and then the remaining pellet was washed with 70% ethanol. ABI 3730x DNA analyser was used for sequencing the purified PCR products, according to the manufacturer’s instructions.

Comparison of nucleotide and amino acid alterations. Nucleotide and amino acid changes derived from gyrA, gyrB, parC and parE mutations in the QRDRs in 114 clinical Ureaplasma spp. strains were compared with 14 serovars of ATCC reference strains.

RESULTS

Ureaplasma species distribution

UU295 and UUR10_0588 showed great conservative property in each species and allowed the ability to distinguish a sample consisting of both UPA and UUR. Of the 114 clinical isolates examined, 87 (76.32%) identified as UPA, and 13 (11.4%) comprised both UPA and UUR.

Fluoroquinolone susceptibility in Ureaplasma spp.

The MIC values of four fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) were determined for all the 114 clinical Ureaplasma spp. isolates. Summaries of the MIC values of the tested four fluoroquinolones

METHODS

Bacterial strains and clinical specimens. A total of 114 Ureaplasma spp. strains were isolated from clinical samples of female patients with symptomatic infection in Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, China. Commercially available Mycoplasma IST2 (bioMérieux) was used for isolation of Ureaplasma species.

Antimicrobial agents. Four fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) were all purchased from Sigma-Aldrich. All the antibiotic powders were dissolved and diluted according to the manufacturer’s instructions and the stock solutions were prepared fresh on the day of using.

Determination of MIC value. The accurate MIC values of antibiotics (ciprofloxacin, ofloxacin, levofloxacin and moxifloxacin) were determined by a broth microdilution method with 96-well microtitre plates (Waites et al., 2012). Briefly, all the antibiotics were twofold serial diluted in 10B broth. The antibiotic gradient was ranged from 256 mg l⁻¹ to 0.125 mg l⁻¹ for ciprofloxacin and 128 mg l⁻¹ to 0.0625 mg l⁻¹ for ofloxacin, levofloxacin and moxifloxacin. Clinical Ureaplasma spp. strains of 10⁴ to 10⁵ colour-changing units were inoculated from a frozen stock culture of known concentration and incubated for 1 h at 37 °C. After inoculation, the microtitre plates were incubated at 37 °C for 16 to 18 h. The MIC was defined as the lowest concentration of antibiotics that inhibited a colour change at the time the growth control displayed a colour change. The breakpoints were designated by the Clinical and Laboratory Standards Institute (CLSI) and Cumitech 34 guidelines (Waites et al., 2001, 2012). According to breakpoints designated by the CLSI, the breakpoints were ≥4 μg ml⁻¹ for levofloxacin, and ≥4 μg ml⁻¹ for moxifloxacin.

DNA extraction. To prepare the template for PCR, a total of 0.5 ml of Ureaplasma spp. broth culture of each Ureaplasma spp. strain was used for isolating genome DNA as described in our previous study (Zhang et al., 2014a).
against UPA and UUR are shown in Table 1. Moxifloxacin was the most active fluoroquinolone tested, with relatively low MICs for *Ureaplasma* spp. (MIC range: 0.125–32 μg ml⁻¹). For the 87 UPA clinical strains, the moxifloxacin MIC at which 90% of isolates were inhibited (MIC₉₀; 8 μg ml⁻¹) was 16-fold lower than that of ciprofloxacin, eightfold lower than that of ofloxacin and fourfold lower than that of levofloxacin. For the 14 UUR strains, the moxifloxacin MIC₉₀ (8 μg ml⁻¹) was 32-fold more active than that of ciprofloxacin, 32-fold lower than that of ofloxacin and fourfold lower than that of levofloxacin. For the 13 coinfections with UPA and UUR, the moxifloxacin MIC₉₀ (4 μg ml⁻¹) was 32-fold more active than that of ciprofloxacin, eightfold lower than that of ofloxacin and twofold lower than that of levofloxacin. On the basis of breakpoints designated by CLSI, the resistance rates to levofloxacin were higher (75.9% for UPA, 71.4% for UUR and 76.9% for coinfection with both UPA and UUR), while that of moxifloxacin was considerably lower (25.3% for UPA, 50% for UUR and 23.1% for both UPA and UUR). Interestingly, the rates of resistance to moxifloxacin for single UPA infection and coinfection with UPA and UUR were almost half of that of UUR.

**Molecular characterization of QRDRs**

The nucleotide and deduced amino acid sequences of the QRDRs of gyrA, gyrB, parC and parE for all the 114 clinical *Ureaplasma* spp. strains were compared with that of 14 ATCC strains of *Ureaplasma* spp. serovars and thus species-specific or serovar-specific polymorphisms could be avoided. Table 2 displays MICs of the tested four fluoroquinolones and the corresponding amino acid changes in the QRDRs. In this study, species-specific or serovar-specific polymorphisms were observed at position 112 of GyrA protein, 59 and 125 of ParC and 417 of ParE, which were reported as fluoroquinolone-resistant mutations in previous studies.

No amino acid mutations were found in the gyrA sequences from the clinical *Ureaplasma* spp. strains. For the gene fragment amplified from gyrB, three new nucleotide substitutions that resulted in amino acid changes were revealed: Pro-462→Ser (C1384T, two strains), Asn-481→Ser (A1442G, one strain) and Ala-493→Val (C1478T, one strain). Analysis of ParC sequences revealed three nucleotide variations associated with fluoroquinolone resistance in parC, including one previously reported mutation of Ser-83→Leu (C248T, 88 strains) and two novel mutations of Ser-83→Trp (C248G, one strain) and Met-105→Ile (G315T, one strain). The ParC Ser-83→Leu substitution resulted in an increase in the MIC of up to 256-fold against tested quinolones, Moxifloxacin had the best activity against the Ser-83→Leu mutation strains. Sequence analysis of the parE gene uncovered a new nucleotide mutation resulting in an amino acid change of Arg-448→Lys (G1343A, three strains). Several double substitutions existed in the 114 clinical strains analysed, including Pro-462→Ser, Asn-481→Ser or Ala-493→Val in GyrB along with Ser-83→Leu in ParC and Met-105→Ile along with Ser-83→Leu in ParC.

Of the 114 clinical strains, 89 clinical strains harboured at least one mutation in ParC, including four strains owing to Ser-83→Leu in ParC along with one mutation in GyrB, one strain owing to Ser-83→Leu along with Ser-83→Trp in ParC, one strain owing to a single mutation of Ser-83→Trp in ParC and 83 strains owing to a single mutation of Ser-83→Leu in ParC. For the above clinical strains, the MICs of the four fluoroquinolones were considerably higher than that of strains with no mutation in the QRDR region. For the five strains harbouring double mutations, the MICs of the four fluoroquinolones were comparable to the strains with single mutation of Ser-83→Leu in ParC. It is notable that nine clinical strains, with levofloxacin MICs of 1 to 2 μg ml⁻¹, harboured the Ser-83→Leu in ParC. A novel mutation, Arg-448→Lys in ParE, was identified in two single UPA infections and one coinfection with UPA and UUR (ciprofloxacin MIC range, 32–256 μg ml⁻¹; ofloxacin MIC range, 16–128 μg ml⁻¹; levofloxacin MIC range, 4–64 μg ml⁻¹; moxifloxacin MIC range, 1–8 μg ml⁻¹).

**DISCUSSION**

As described before, most of the clinical *Ureaplasma* spp. strains thus far isolated from the urogenital tracts of women were characterized as UPA, while only a small portion were characterized as UUR (Waites *et al.*, 2005;...
Table 2. Genetic alterations of *Ureaplasma* spp. with elevated fluoroquinolone MICs

CIP, Ciprofloxacin; OFL, ofloxacin; LEX, levofloxacin; MOX, moxifloxacin.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Species (n)</th>
<th>CIP MIC (μg ml⁻¹)</th>
<th>OFL MIC (μg ml⁻¹)</th>
<th>LEX MIC (μg ml⁻¹)</th>
<th>MOX MIC (μg ml⁻¹)</th>
<th>Genetic alterations*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Range 50 % 90 %</td>
<td>Range 50 % 90 %</td>
<td>Range 50 % 90 %</td>
<td>Range 50 % 90 %</td>
<td>gyraseCparCparE</td>
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<tr>
<td>UU6, UU7</td>
<td>UPA (n=2)</td>
<td>32–256 16–128</td>
<td>4–64 1–8</td>
<td>G1343A (R448K)</td>
<td>UG1343A (R448K)</td>
<td></td>
</tr>
<tr>
<td>UU110</td>
<td>UPA + UUR (n=1)</td>
<td>128 32 16</td>
<td>OFL 32 16</td>
<td>UG1343A (R448K)</td>
<td>C1384T (P462S)</td>
<td></td>
</tr>
<tr>
<td>UU1</td>
<td>UPA (n=1)</td>
<td>16 8 4 1</td>
<td>UG1343A (R448K)</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
<td></td>
</tr>
<tr>
<td>UU2, UU3</td>
<td>UPA (n=2)</td>
<td>64–128 16–32</td>
<td>8–16 2–4</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
<td></td>
</tr>
<tr>
<td>UU4</td>
<td>UPA (n=1)</td>
<td>128 64 16</td>
<td>UG1343A (R448K)</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
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</tr>
<tr>
<td>UU5</td>
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<td>32 8 4 1</td>
<td>C1384T (P462S)</td>
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<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
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<td>16–128 16 128</td>
<td>C1384T (P462S)</td>
<td>G315T (M105I)</td>
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<td>UU73-87</td>
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<td>0.5–8 0.25–2 1</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
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<tr>
<td>UU88-96</td>
<td>UUR (n=9)</td>
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<td>4–128 16 128</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
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<tr>
<td>UU97-101</td>
<td>UUR (n=5)</td>
<td>8–64 16 64</td>
<td>2–8 1–4 2 4</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
<td></td>
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<tr>
<td>UU102-109, UU112-113</td>
<td>UPA + UUR (n=10)</td>
<td>8–128 32 64</td>
<td>8–128 16 32</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
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<tr>
<td>UU111, UU114</td>
<td>UPA + UUR (n=2)</td>
<td>16–64 8 2–4</td>
<td>1–2</td>
<td>C1384T (P462S)</td>
<td>C248T (S83L)</td>
<td></td>
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</tbody>
</table>

*Parentheses indicate amino acid substitutions that occurred as a result of DNA point mutations. Boldface indicates two novel mutations found in this study.*
Bayraktar et al., 2010; Hunjak et al., 2014). In this study, the incidence of UPA was significantly higher than UUR in women with symptomatic infection and a small portion of clinical strains were characterized as both UPA and UUR. This probably reflects the fact that UPA is isolated more frequently than UUR and that coinfection with both organisms sometimes occurs.

In the present study, high MICs were observed for the two older fluoroquinolones (ciprofloxacin and ofloxacin). Analysis of the two newer fluoroquinolone (levofloxacin and moxifloxacin) MICs showed that moxifloxacin was effective for treating most *Ureaplasma* spp. infections, while levofloxacin resistance was relatively high, with about 75% for UPA and UUR. However, Xiao et al. showed that the rate of resistance to levofloxacin was only 5% for 257 clinical isolates of *Ureaplasma* spp. isolated in the United States. These regional differences in antibiotic susceptibilities might be the result of variations in antibiotic use policies.

Amino acid substitutions in DNA gyrase and topoisomerase IV, testified to cause fluoroquinolone resistance in many types of bacteria, are supposed to be responsible for fluoroquinolones resistance in *Ureaplasma* spp. Discounting the species-specific or serovar-specific polymorphisms, Pro-462→Ser (C1384T), Asn-481→Ser (A1442G) and Ala-493→Val (G1478T) in GyrB, Ser-83→Leu (C248T)/Trp (C248G) and Met-105→Ile (G315T) in ParC and Arg-448→Lys (G1343A) in ParE were discovered. In addition to the previously reported mutations in the fluoroquinolone-interacting proteins, we have identified two novel mutations resulting in amino acid substitutions in ParC and ParE proteins, all of which are potentially contributory to fluoroquinolone resistance in *Ureaplasma* spp. Moreover, a recently published study investigated the *in vitro* activity of five quinolones and the QRDR of *gyrA*, *gyrB*, *parC* and *parE* in clinical *Ureaplasma* spp. strains in Japan (Kawai et al., 2015). The novel mutation of Ser-83→Trp of ParC was also independently found in one strain and the homology modelling analysis suggested this ParC mutation in the side chain of the DNA-topoisomerase complex may interfere with the proper binding of quinolones via steric hindrance. Our study further confirmed the positions of novel mutations associated with quinolone resistance showed highly increased MICs of quinolones in *Ureaplasma* spp.

In previous studies of fluoroquinolone resistance in *U. urealyticum*, a lot of fluoroquinolone-resistant isolates harboured the mutation Ser-83→Leu in ParC and the same mutation was identified in some other bacteria with fluoroquinolone resistance (Bébéar et al., 2003; Beeton et al., 2009). Our results displayed 80 out of 86 levofloxacin-resistant and 31 out of 32 moxifloxacin-resistant strains contained the mutation of Ser-83→Leu in ParC, indicating that quinolone-resistant *Ureaplasma* spp. are already widespread in China. This result is in line with previous studies showing that Ser-83→Leu in ParC is the most common mutation contributing to fluoroquinolone resistance of *Ureaplasma* spp. Two novel mutations of Ser-83→Trp in ParC and Arg-448→Lys in ParE were discovered in the present study that were potentially responsible for fluoroquinolone resistance. Moreover, three novel mutations in *gyrB* and one mutation of Met-105→Ile in ParC were observed and occurred when an amino acid substitution existed in ParC. Compared to strains harbouring a single mutation in ParC, the above mutations had no additional effect on fluoroquinolone resistance and seemed to be neutral polymorphisms. No amino acid changes were observed in GyrA in this study, even though mutations have been reported by other researchers and have always been related to fluoroquinolone resistance in some bacteria (Beeton et al., 2009; Xie et al., 2014). However, 60/82 (73.2%) moxifloxacin-susceptible clinical strains harbouring mutations in the QRDRs, which suggests that the spatial structure of moxifloxacin renders it resistant to these mutations and allows antibacterial effectiveness. It is reasonable to believe that fluoroquinolone resistance in *U. urealyticum* is mainly due to mutations in topoisomerase IV and the most common mutation was the amino acid change of Ser-83→Leu. Although mutations in topoisomerase IV occurred in 84 (97.7%) levofloxacin-resistant strains, the molecular mechanism of the remaining two clinical *Ureaplasma* spp. isolates with levofloxacin MICs of ≥4 μg ml⁻¹ is still unknown. Some other mechanisms, such as overexpression of efflux pumps, presence of fluoroquinolone-inactivating enzymes or existence of plasmid-encoded Qnr protein might be responsible for resistance in these strains.

In conclusion, the widespread general use of fluoroquinolones has led to decreased antibiotic sensitivity and induced a high level of fluoroquinolone resistance in *Ureaplasma* spp. Amino acid alterations in topoisomerase IV are observed commonly and may be the leading cause of fluoroquinolone resistance, especially the mutation of Ser-83→Leu (C248T) in ParC. Our investigation also provides the first surveillance data for fluoroquinolone resistance of *Ureaplasma* spp. in China.

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