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SHORT COMMUNICATION

Antibiotic susceptibilities and genetic variations in macrolide resistance genes of *Ureaplasma* spp. isolated in China

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SUMMARY

Macrolides are widely used for the treatment of *Ureaplasma* spp. infection. The aim of this study was to investigate possible genetic resistance determinants in *Ureaplasma* spp. isolates. A total of eleven macrolide-resistant *Ureaplasma* spp. isolates, recovered from urogenital specimens, were investigated for genetic mechanisms of macrolide resistance. The 23S rRNA operons, as well as L4 and L22 ribosomal protein genes, were amplified and sequenced. Our study identified that the mutation A2066G in the 23S rRNA and four mutations (G361T, A406G, C422T and G196A) in the L22 ribosomal protein, may be responsible for the resistance of *Ureaplasma* spp. to macrolides, in China.

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Ureaplasma spp. contribute to human microbiota but are also involved in urogenital tract infection as well as a variety of other disease states, including: nongonococcal urethritis (NGU), infertility, adverse pregnancy outcomes and chorioamnionitis (Waites *et al.,* 2005; Paralanov *et al.,* 2012; Foschi *et al.,* 2018a). The *Ureaplasma* spp. that are of medical importance are sub-classified into two distinct species; *Ureaplasma parvum* and *Ureaplasma urealyticum*. Antimicrobial options for the treatment of *Ureaplasma* spp. infection are limited. The absence of a bacterial cell wall renders *Ureaplasma* spp. intrinsically resistant to all beta-lactam and glycopeptide antibiotics. Therefore, the treatment of this type of infection is limited to three classes of antibiotics that inhibit DNA replication (i.e., fluoroquinolones) and protein synthesis (i.e., macrolides and tetracyclines). The use of these therapeutic options is further restricted in pregnant women or neonates, for whom macrolides represent the only recognized treatment option, due to the toxicity of tetracyclines and fluoroquinolones (Beeton and Spiller 2017). Since the first report of macrolide resistance in *Ureaplasma* spp. in 1989, occasional case reports of macrolide-resistant *Ureaplasma* spp. have appeared in different geographical regions and patient populations (Palu *et al.,* 1989; Karabay *et al.,* 2006; Samra *et al.,* 2011). For these species, mutations effecting either the 23S rRNA subunit or the ribosomal protein L4 and L22 genes have been associated with macrolide resistance development (Govender *et al.,* 2012). To our knowledge, studies addressing the antimicrobial susceptibility profiles and macrolide resistance mechanisms of *Ureaplasma* spp. are still scarce in China. The present study was designed to investigate antibiotic susceptibility profiles of *Ureaplasma* spp. strains isolated in China and the genetic variations of macrolide resistance genes associated with resistance development.

The 11 macrolide-resistant *Ureaplasma* spp. isolates, documented in this study, were obtained from our previous epidemiological investigation and antimicrobial susceptibility analysis of *Ureaplasma* spp. and *Mycoplasma hominis* in outpatients with genital manifestations (Song *et al.,* 2014). Only samples collected at the first isolation were considered, excluding any specimens obtained from the same patient during follow-up. The culture, detection and antimicrobial susceptibility testing of *Ureaplasma* spp. were performed using a commercially-available Mycoplasma IST2 kit (bioMérieux, Marcy l'Etoile, France), according to the manufacturer's guidelines. Briefly, clinical specimens were placed in transport medium (R1), before adding 3 mL of this mixture to the lyophilized R2 medium, followed by vortexing until the lyophilized R2 medium pellet had completely dissolved. A Mycoplasma IST2 strip was then inoculated with the rehydrated R2 growth medium. The remainder of the broth and the inoculated strip were incubated at 37°C. Color changes were subsequently noted and the results were interpreted after 48 h of incubation. The antimicrobial susceptibility testing included erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA), josamycin (JOS), ciprofloxacin (CIP), ofloxacin (OFL), doxycycline (DOX) and tetracycline (TET). The antimicrobial resistance breakpoints were interpreted according to manufacturer's guide. The breakpoints for the antimicrobials tested are given in *Table 1*. Clinical isolates set aside for further testing were stored at -80°C. The method of DNA extraction for use in the polymerase chain reaction (PCR) experiments was described in our previous study (Zhang *et al.,* 2014). To distinguish *U. parvum* from *U. urealyticum*, two pairs of primers: UMS-125/UMA226 and UMS-51/UMA-427, were used as described by Teng *et al.* (Teng *et al.,* 1994) Previously published primers were used to amplify the macrolide resistance gene in the follow-

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Ureaplasma spp., macrolide resistance; ribosomal proteins, 23S rRNA, genetic variation.

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Table 1 - *The primary epidemiological characteristics of macrolide resistant Ureaplasma spp. isolates involved in this study.*

Isolate No.	*Age*	*Gender*	*Geographic location*	*Isolation year*
N9	42	F	Jiangsu: Suqian	2012
79	34	F	Hubei: Jingzhou	2013
N77	36	F	Zhejiang: Hangzhou	2013
N23	46	F	Zhejiang: Yiwu	2012
N54	31	F	Zhejiang: Hangzhou	2013
97	36	M	Zhejiang: Hangzhou	2013
273	31	F	Zhejiang: Hangzhou	2013
92	35	M	Zhejiang: Hangzhou	2012
83	26	F	Zhejiang: Hangzhou	2013
N107	29	F	Zhejiang: Cixi	2012
N4	54	F	Zhejiang: Hangzhou	2013

ing regions: ribosomal protein L4 and L22 genes (primer pairs UPL4-U/UPL4-R and UPL22-U/UPL22-R) and two 23S rRNA operons (primer pairs UP23S-30/UP23S-OP1 and UP23S-30/UP23S-OP2) (Beeton *et al.,* 2009). Amplifications were carried out according to the Taq DNA Polymerase (Takara, Ohtsu, Japan) protocol. The ABI 3730xl DNA analyzer was used to sequence the purified PCR products, according to the manufacturer's instructions. Amino acid substitutions in L4 and L22, as well as nucleotide mutations in the 23S rRNA alleles, were identified by comparison with those of the reference strains *U. parvum* American Type Culture Collection (ATCC) 700970 and *U. urealyticum* ATCC 33699 (GenBank accession numbers AF222894 and CP001184, respectively).

The primary epidemiological characteristics provided by the study population are detailed in *Table 1*. Of the 11 macrolide-resistant *Ureaplasma* spp. isolates, *U. parvum* was found in 7 (63.6%) and *U. urealyticum* in 4 (36.4%) clinical samples. Overall, the male-to-female ratio of the *Ureaplasma* spp. positive patients was 0.22, and the positive rates in the male and female group were 18.2% and 81.8%, respectively. The highest levels of resistance were found for ciprofloxacin and ofloxacin (each contributing to 90.9% of the resistant strains). Conversely, tetracyclines (tetracycline and doxycycline) resulted in the lowest rate of resistance (0% each). Although significant discrepancies exist between countries, relating to the antimicrobial susceptibilities of specific pathogens, due to differences in antimicrobial use policies, our results are in line with several reports indicating that doxycycline, tetracycline and josamycin were the most active antimicrobial agents against *Ureaplasma* spp. (De Francesco *et al.,* 2013; Foschi *et al.,* 2018b).

Mutations in 23S rRNA or in ribosomal protein L4 and L22 genes were often associated with macrolide resistance in different bacterial species (Govender *et al.,* 2012). In this study, eleven *Ureaplasma* spp. isolates with different phenotypes of resistance to macrolides were screened for mutations in the ribosomal protein L4 and L22 genes and 23S rRNA. The genetic alterations involving the ribosomal proteins L4 and L22, as well as the 23S rRNA detected in these isolates are listed in *Table 2*. Mutations obtained from the ribosomal protein L4 were all located

Table 2 - *Genetic alterations of Ureaplasma spp. isolates with macrolide resistance.*

Isolate No.	*Antibiotics*[a,b]								*Genetic alterations(s)*[c]		
	ERY	*AZI*	*CLA*	*JOS*	*CIP*	*OFL*	*DOX*	*TET*	*L4*	*L22*	*23S rRNA*
U. parvum											
N9	R	R	S	S	R	R	S	S	A81T	G361T (A121S), A406G (I136V), C422T (T141I)	A2066G
79	R	S	R	S	R	R	S	S	A81T	G361T (A121S), A406G (I136V), C422T (T141I)	None
N77	I	R	R	S	R	R	S	S	A81G	None	A2066G
N23	I	I	R	S	R	R	S	S	A81G	None	A2066G
N54	S	R	S	S	R	I	S	S	A81G	None	A2066G
97	S	I	R	S	I	R	S	S	A81G	G196A (D66N)	None
273	S	S	R	S	R	R	S	S	A81G	None	A2066G
U. urealyticum											
92	R	R	R	S	R	R	S	S	C60T, A81G, T141C, T309C, G357A, C373T	C395T (A132V), T455C (I152T)	None
83	R	R	R	S	R	R	S	S	C60T, A81G, T309C, G357A, C373T	C395T (A132V), T455C (I152T)	None
N107	R	I	R	S	R	R	S	S	C60T, A81G, T309C, G357A, C373T	C395T (A132V), T455C (I152T)	None
N4	R	I	R	S	R	R	S	S	C60T, A81G, T141C, T309C, G357A, C373T	C395T (A132V), T455C (I152T)	None

[a]ERY, erythromycin; AZI, azithromycin; CLA, clarithromycin; JOS, josamycin; CIP, ciprofloxacin; OFL, ofloxacin; DOX, doxycycline; TET, tetracycline.
[b]R, resistant; I, intermediate; S, susceptible. The breakpoints (mg/L) according to Clinical and Laboratory Standards Institute (CLSI) are as follows: ERY S≤1, R≥4; AZI S≤0.12, R≥4; CLA S≤1, R≥4; JOS S≤2, R≥8; CIP S≤1, R≥2; OFL S≤1, R≥4; DOX S≤4, R≥8; TET S≤4, R≥8.
[c]Numbers and letters in parentheses refer to amino acid changes using standard nomenclature and abbreviations.

in the highly-conserved loop of the protein, which is a hotspot for mutations associated with macrolide resistance. No amino acid mutations (synonymous mutation) were found in the ribosomal protein L4 sequences. A previous study had indicated that the point mutation A81G in the ribosomal protein L4 gene was observed both in resistant and susceptible strains, which questioned the correlation of this polymorphism with *Ureaplasma* spp. resistance to macrolides (Xiao *et al.,* 2011). One possible explanation is that a different nucleotide observed in the same position (e.g. 81A/G) may associate with a specific serovar. This highlights the importance of selecting an appropriate reference strain when comparing sequence data, due to the existence of variant polymorphisms.

Four-point mutations (G361T, A406G, C422T and G196A), which result in four amino acid modifications (A121S, I136V, T141I and D66N) in the ribosomal protein L22, were found in three *U. parvum* isolates. In addition, two-point mutations (C395T and T455C), which result in two amino acid changes (A132V and I152T) were found in four *U. urealyticum* isolates. Govender *et al.* presented fifteen different point mutations in three macrolide resistant *U. parvum* isolates, which did not include the mutations mentioned above (Govender *et al.,* 2012). Xiao *et al.* reported three ribosomal protein L22 point mutations (A121S, T141I and D66N) in three different *U. parvum* isolates. However, these mutations are considered to be atypical polymorphisms and may not contribute to macrolide resistance (Xiao *et al.,* 2011).

In this study, we found that domain V of the 23S rRNA gene carried an A to G transition at position 2066 in four *U. parvum* isolates, resulting in phenotypes that were resistant to different macrolides. This A2066G mutation was also reported in three *U. urealyticum* isolates recovered in the US, where it was also shown to result in high-level macrolide resistance (Xiao *et al.,* 2011). Meng *et al.* also found that a different mutation, C2243N (T or C) in the 23S rRNA, may be associated with *Ureaplasma* spp. macrolide resistance in the Chinese population. However, the authors did not examine any mutations affecting the ribosomal proteins L4 and L22 (Meng *et al.,* 2008). The 23S rRNA mutation (A2066G) also corresponds to location 2058 according to the *Escherichia coli* numbering system, within the peptidyl transferase center of domain V, which is known to confer macrolide resistance when altered by methylation or mutation in other bacterial species, including *Mycoplasma pneumoniae* (Vester and Douthwaite 2001). Mutation at position 2058 in the 23S rRNA of *E. coli* can perturb the higher order structure of the 23S rRNA drug-binding pocket, thereby reducing the ability of the antimicrobials to interact with ribosomes (Douthwaite and Aagaard 1993). Whether the 23S rRNA mutation (A2066G) in *Ureaplasma* spp. confers macrolide resistance by the same mechanism needs further verification.

Although this study has extended our current knowledge of the macrolide resistance mechanisms of *Ureaplasma* spp, it has some limitations. The total number of isolates included in this study was relatively small due to the low incidence of macrolide resistance in China. Additionally, although these seldom contribute to macrolide resistance in *Ureaplasma* spp, the present study did not investigate the involvement of methylases, esterases or efflux pumps.

In conclusion, this is the first report of macrolide-resistant mutations affecting both the L4 and L22 ribosomal proteins, as well as the 23S rRNA of *Ureaplasma* spp. in China. Large-scale combined microbiological and clinical studies are warranted to address the underlying mechanisms in the near future.

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Conflicts of interest

The authors declare no conflicts of interest.

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