

## Investigation of *Ureaplasma urealyticum* biovars and their relationship with antimicrobial resistance

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### Abstract

**Purpose:** To develop Taqman fluorescence quantitative polymerase chain reaction (PCR) method for investigating the characteristics of the distributions of *Ureaplasma urealyticum* (UU) biovars and to explore the relationship between UU biovars and antimicrobial resistance. **Materials and Methods:** By the method of culture, *Ureaplasma* species were detected. Taqman fluorescence quantitative PCR for detecting UU biovars were developed and UU clinical isolates were detected to distinguish biovars. The broth micro-dilution susceptibility testing methods were used to determine UU susceptibility. **Results:** By Taqman PCR method, UU biovars was successfully detected. Of 126 samples, biovar 1 was found in 73 (57.94%). There was a statistical difference between genital-urinary tract infection group and asymptomatic group ( $P<0.05$ ). In the region, UU biovar 1 to 9 kinds of agents kept higher susceptibility rates, but biovar 2 maintained higher susceptibility rates only to tetracyclines. Compared with biovar 1, UU biovar 2 resistance rates to 7 kinds of agents were higher ( $P<0.05$ ). **Conclusions:** (1) Our new established Taqman PCR method is a useful tool for screening UU biovars. (2) UU biovar 1 predominated in asymptomatic population; whereas in genital-urinary tract infection population UU biovar 2 had a higher proportion. (3) The characteristics of drug resistance were different between UU biovars. Overall, both two biovars remained higher susceptibility rates to tetracyclines. A majority of biovar 1 strains were sensitive to macrolides and quinolones; while only a small number of biovar 2 strains kept sensitive to roxithromycin and quinolones, a large proportion of biovar 2 strains were found in intermediate ranges.

**Key words:** Antimicrobial susceptibility; biovar; Taqman fluorescence quantitative PCR; *Ureaplasma urealyticum*.

### Introduction

*Ureaplasma urealyticum* which belongs to genus *Ureaplasma*, can produce urease, IgA protease and phospholipase enzymes<sup>[1]</sup> and colonize through the adhesion of respiratory tract or urinary tract epithelial cell surface receptors.<sup>[2]</sup> Due to lack of cell wall, UU resists penicillin naturally. Presently, *U. urealyticum* is divided into two biovars—biovar 1, *Ureaplasma Parvum*, parvo and biovar 2, *Ureaplasma urealyticum*, T960, a total of 14 serovars.<sup>[3]</sup> Previous studies confirmed that *U. urealyticum* is involved in various diseases including non-gonococcal urethritis,<sup>[4]</sup> prostatitis,<sup>[5]</sup>

testicular inflammation,<sup>[6]</sup> urinary stones,<sup>[7]</sup> gynecological diseases,<sup>[8]</sup> infertility,<sup>[9]</sup> neonatal pneumonia,<sup>[10-12]</sup> and neonatal respiratory distress syndrome.<sup>[13]</sup> Recent studies showed that *U. urealyticum* biovars were associated with its clinical pathogenicity. However, it is unknown what the relationship between *U. urealyticum* biovars and antimicrobial resistance is. This study aims to investigate the distribution of UU biovars, and further to explore the relationship of *U. urealyticum* biovars with antimicrobial resistance.

### Materials and Methods

#### *Collection of clinical specimens and Ureaplasma urealyticum identification*

The important criterion for inclusion was that the participants should not have taken any antibiotics in the past 7 days. In females, the experimental specimens were consecutively collected from cervix epithelial cells with sterile swabs; and in males, from urethral secretions. By the method of culture, we checked the samples of 389 patients attending the Department of Sexually Transmitted Disease and the Department of Obstetrics and Gynecology for treatment or physical check-up between January 1, 2009 and June 1, 2010. Finally, 126 cases including 79 females and 47 males were confirmed to be *U. urealyticum* positive. Based on whether genital-urinary tract infections or not, the 126 cases were divided into two groups—the genital-urinary tract infection group (55 cases) and the asymptomatic group (71 cases). All these patients were treated in accordance

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with the Helsinki Declaration on the participation of human subjects in medical research. The study was approved by the Bioethics Committee, Soochow University and a written informed consent was obtained from each of participants.

#### Standard strains

*U. urealyticum* serovar 1-14, ATCC27813-27826 were taken as experimental quality control standard strains. The strains were kindly provided by the Capital Institute of Pediatrics, Beijing, China.

#### Antimicrobial susceptibility testing of *Ureaplasma urealyticum* species

Kits were kindly provided by Lizhu Bio-Company (Zhuhai, China) and Bio-Merieux (Marcy-l'Etoile, France). The collected swabs were inoculated in liquid broth mediums at 37°C for 48 hours. According to the kit instruction manual, antimicrobial susceptibility testing was carried out. In brief, the inoculated R1 medium was vortexed rapidly and 3 ml was added to the growth R2 medium, containing 1 ml broth. After reconstitution and mixing, 50 µL was dispensed into each of the 22 test wells on the strip. Two drops of mineral oil were added to each well. The inoculated strips were then incubated at 37°C and observed for the results at 24 and 48 hours. Due to an increase in pH caused by UU growth, an indicator (phenol red) in media could display a color change. According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), the results of resistance or susceptibility to antimicrobial agents were judged. The CLSI breakpoints for the antimicrobials are as follows (mg/L):<sup>[14]</sup> Doxycycline (DOX)  $S \leq 4$ ,  $R \geq 8$ ; minocycline (MIN)  $S \leq 1$ ,  $R \geq 4$ ; josamycin (JOS)  $S \leq 2$ ,  $R \geq 8$ ; roxithromycin (ROX)  $S \leq 1$ ,  $R \geq 4$ ; clarithromycin (CLA)  $S \leq 1$ ,  $R \geq 4$ ; azithromycin (AZI)  $S \leq 0.12$ ,  $R \geq 4$ ; ofloxacin (OFL)  $S \leq 1$ ,  $R \geq 4$ ; levofloxacin (LEV)  $S \leq 1$ ,  $R \geq 4$ ; sparfloxacin (SPA)  $S \leq 1$ ,  $R \geq 4$ .

#### Deoxyribonucleic acid extraction

Swabs collected from the participants, with 400 µl sterile saline, were centrifuged at 15,000 revolutions per minute (rpm) for 5 minutes, and discharged the supernatant; precipitated by adding 50 µl lysis (Ethylenediaminetetraacetic acid - EDTA 0.05 M, sodium hydroxide - NaOH 0.05 M, Triton 1.0% (V/V), Tris - Hydrochloride (pH 8.0) 10 mmol/L), mixed and heated at 100°C for 10-15 minutes, centrifuged at 15,000 rpm for 5 min.

#### Detection of *ureaplasma urealyticum* biovars

Detection of UU biovar were performed by Taqman fluorescence quantitative PCR. After retrieving UU1-14 multiple banded antigen (MBA) sequences on the NCBI, by DNAMAN software (Lynnon Biosoft Company, Vaudreuil-Dorion, Quebec Canada), multiple sequences alignment was performed. After

finding out conserved and specific biovar sequence, primers and Taqman probes for detecting UU biovars were designed using Primer Express 3.0 software. Primers and Taqman probes for detecting UU biovar 1: F: 5' -ACATCAG(T/C)TGAAAACAAAACAG-3' ; R: 5' -GTTTTGGTTCACGAGGTAAT-3' ; Probe: 5' -FAM-AAAAGGTCATTTG(G/A)TTGGTG AAAAA-TAMRA-3' . Primers and Taqman probes for detecting UU biovar 2: F: 5' - CACTTACTTAACAA(A/G)AAAATC(T/C)ACGT - 3' ; R: 5' -GCAGTTTTTAGTAA(T/G)CCACCTT-3' ; Probe: 5' -FAM-AAATTACCACGTGAACCAAAAGCTAAA-TAMRA-3' .

Taqman fluorescence quantitative PCR buffer system for biovar 1: 25 µl reaction system contained 2.5 U Taq enzyme,  $Mg^{2+}$  2.5 mmol/L, each of the upstream and downstream primers 0.1 pmol/L, Taqman probe 0.2 pmol/L, the template DNA 2 µl. Optimum buffer system of biovar: 25 µl reaction system contains: 2.5 U Taq enzymes,  $Mg^{2+}$  2.5 mmol/L, each of the upstream and downstream primers 0.2 pmol/L, Taqman probe 0.3 pmol/L, the template DNA 2 µl. PCR reaction conditions: step 1: 95°C, 2 min; step 2: 95°C, 10 s; 55°C (detection point of fluorescent signal), 20s, 40cycles. The instrument used iCycler iQ™. Real-Time PCR Detection System manufactured by Bio-Rad Laboratories, Inc. (Hercules, California USA).

To verify the reliability of the method, all the standard strains available (serovar 1-14) were assayed with both the biovar specific primers and probes, the positive and negative controls were set in the experiments. In addition,  $1.0 \times 10^{1-8}$  copies/ml *U. urealyticum* nucleic acid was detected to determine the linear range and sensitivity.

#### Statistics

The data were analyzed by Statistical Package for the Social Sciences (SPSS) 11.5. Comparison among the rates of multiple rows used contingency table chi-square test.

#### Results

##### Detection of *Ureaplasma urealyticum* biovars

*U. urealyticum* serotypes 1, 3, 6 and 14 were successfully detected using biovar 1 primers and probe; meanwhile, all the other serotypes could be identified with biovar 2 primers and probe. The results of the controls were acceptable. Within  $1.0 \times 10^{3-8}$  copies/ml, the method showed a good linear relationship between gradient diluted concentration and the cycle threshold (CT) value. Minimum detection limit for two biovars was 100-200 copies/ml.

The results of biovars tests showed that overall biovar 1 slightly predominated in the region [Table 1]. There was no difference between genders in the distributions of UU biovars ( $P > 0.05$ ); whereas UU biovars

had a statistical difference between genital-urinary tract infection group and asymptomatic group and the former was higher in the distribution of *U. urealyticum* biovar 2 ( $P < 0.05$ ).

#### *The relationship between ureaplasma urealyticum biovars and antimicrobial susceptibilities*

*U. urealyticum* biovars susceptibilities to 9 antimicrobial agents were shown in Table 2. In the region, UU biovar 1 to 9 kinds of antimicrobial agents kept higher susceptibility rates (above 90%); but biovar 2 maintained higher susceptibility rates only to DOX and MIN (above 95%), and the remaining were less than 80%. Compared with biovar 1, the resistance rates of UU biovar 2 to JOS, AZI, ROX, CLA, OFL, LEV and SPA were higher ( $P < 0.05$ ); while to DOX and MIN the differences in the resistance rates between the two biovars were not statistically significant ( $P > 0.05$ ).

## Discussion

### *Detection of ureaplasma urealyticum biovars*

At present, it is accepted widely that *U. urealyticum* is divided into two groups (*Ureaplasma Parvum*, biovar 1, parvo) and (*Ureaplasma urealyticum*, biovar 2, T960) including a total of 14 serotypes, biovar 1 included serotypes 1, 3, 6 and 14, and the remaining 10 serotypes belonged to biovar 2. At present, the target sequences that can detect UU biovar include: MBA genes,<sup>[15]</sup> 16SrRNA,<sup>[16]</sup>

16S-23S Ribosomal ribonucleic acid (rRNA) gene spacer region<sup>[17]</sup> and urease gene.<sup>[18]</sup> MBA is one of the major antigens that may be an important virulence factor with species-specific.<sup>[15]</sup> MBA genes have about 1,200 coding gene bases transcribing 409 amino acid residues. 1/3 of N terminal of MBA genes is a conservative area, which can be taken as biovar sequence tags. This study took UU MBA genes as the target sequences of detecting UU biovar. Via the multiple sequences alignment of UU MBA genes, we found that two biovars reveal distinctly different classifications. The results showed that our new established Taqman fluorescent PCR method for detecting UU biovars, which had a wide linear dynamic range, high sensitivity and specificity, could be used to quickly test UU biovars.

Previous studies showed that *U. urealyticum* biovars were associated with pathogenicity.<sup>[19,20]</sup> A study confirmed that biovar 2 was more associated with the loss of lactobacilli in women than biovar 1, indicating that biovar 2 was more likely to cause vagina infection.<sup>[20]</sup> This study demonstrated that *U. urealyticum* biovar 2 occurrence in genital-urinary infection group was 58.18% (32/55), which was higher than that in asymptomatic group (29.58%, 21/71), indicating that UU biovar 2 may be more closely related to genital-urinary tract infectious diseases, that biovar 1 seems to be inclined to colonize in urogenital tract and doesn't cause noticeable symptoms. However, according to our investigation, genders don't exert influence on the distributions of *U. urealyticum* biovars.

**Table 1: Distribution of *Ureaplasma urealyticum* biovars in 126 cases during the cases**

Groups		n	Biovar 1 (%)	Biovar 2 (%)	$\chi^2$	P
Gender	Males	47	27 (57.45)	20 (42.55)	0.218	0.640
	Females	79	42 (53.16)	37 (46.84)		
Symptom	Genital-urinary infection group	55	23 (41.82)	32 (58.18)	10.405	0.001
	Asymptomatic group	71	50 (70.42)	21 (29.58)		
	Total	126	73 (57.94)	53 (42.06)		

**Table 2: Comparison of antimicrobial susceptibilities between *Ureaplasma urealyticum* biovars during the study**

Antimicrobials	UU# biovar1 (n=73)			UU# biovar 2 (n=53)			$\chi^2$	P
	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)		
DOX	1 (1.37)	0 (0.00)	72 (98.63)	1 (1.89)	0 (0.00)	52 (98.11)	0.053	0.819
MIN	0 (0.00)	1 (1.37)	72 (98.63)	1 (1.89)	0 (0.00)	52 (98.11)	2.104	0.349
JOS	1 (1.37)	0 (0.00)	72 (98.63)	5 (9.43)	8 (15.09)	40 (75.47)	17.065	0.000
CLA	1 (1.37)	1 (1.37)	71 (97.26)	6 (11.32)	9 (16.98)	38 (71.70)	17.222	0.000
ROX	2 (2.74)	2 (2.74)	69 (94.52)	19 (35.85)	30 (56.60)	4 (7.55)	94.367	0.000
AZI	0 (0.00)	1 (1.37)	72 (98.63)	2 (3.77)	10 (18.87)	41 (77.36)	15.073	0.001
OFL	2 (2.74)	5 (6.85)	66 (90.41)	6 (11.32)	44 (83.02)	3 (5.66)	89.674	0.000
LEV	0 (0.00)	2 (2.74)	71 (97.26)	1 (1.89)	48 (90.57)	4 (7.55)	102.583	0.000
SPA	1 (1.37)	1 (1.37)	71 (97.26)	1 (1.89)	42 (79.25)	10 (18.87)	83.972	0.000

# UU= *U. urealyticum*

### *The relationship between Ureaplasma urealyticum biovars and antimicrobial susceptibilities*

Currently, tetracyclines including MIN and DOX, quinolones including OFL, LEV and SPA, and macrolides including AZI, JOS, CLA and ROX are commonly used to the clinical treatment for Mycoplasma infections. It is upset that some UU strains have produced drug resistance to the above drugs. Mechanisms of drug resistance of UU are more complex. Blocking the synthesis of Mycoplasma protein peptide chain at the step of extension is tetracyclines mechanism against U urealyticum. But the study found that, by transposon Tn916, U urealyticum can capture exotic tetM genes from other microorganisms encoding the resistance to tetracyclines and form resistance.<sup>[22]</sup> Macrolides can bind with UU ribosome and inhibit protein synthesis, but macrolides also may be used as the inducer of methylase, which causes the methylation of the large 50S ribosomal subunit, resulting in UU resistance to macrolides.<sup>[23,24]</sup> Previous study suggested that UU resistance to quinolone is mainly due to the mutants of target enzyme-DAN helicase,<sup>[25]</sup> which is a topoisomerase II, including two subunits (A, B subunits) that were encoded by the gyrA and gyrB respectively. Of which, the residues 68 to 107 areas are the quinolone-resistant areas (quinolones regions of drug-resistance, QRDR).

Presently, a higher U urealyticum infection rate was found in STD population in this region. At the same time, there had a certain proportions of multiple drug resistant U urealyticum clinical isolates. However, our study confirmed U urealyticum resistance to drugs was uneven, being subjected to biovars. In the region, UU biovar 1 to other 9 kinds of antimicrobial agents kept high sensitivity rates (above 90%); but biovar 2, only to doxycycline and minocycline, maintained higher sensitivities (above 95%), and the remaining were less than 80%. In fact, only a small number of biovar 2 strains were sensitive to ROX, OFL, LEV and SPA and most of them were found in intermediate results. Compared with biovar 1, UU biovar 2 resistance rates to JOS, AZI, CLA, ROX, OFL, LEV and SPA were higher; and to DOX and MIN resistance rates, did not showed the significant differences. Overall, both of the U urealyticum biovars remained higher sensitivity rates to tetracyclines, UU biovar 2 was more resistant to macrolides and quinolones. The reason why the differences in the drugs resistance between the two UU biovars emerge was unknown. However, we postulated that the patients infected by UU biovar 2 were more likely to have the chance to use antibiotics for emerging clinical symptoms, resulting in increasing resistances in biovar 2.

Finally, based on the difference in genetics, pathogenicity and antimicrobial susceptibilities, we strongly propose that the two U urealyticum biovars should be reclassified into two species-*Ureaplasma Parvum* and *Ureaplasma T960*, the classification of which seems to get closer to the fact.

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