TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2015) 45: 55-59
© TÜBİTAK
doi:10.3906/sag-1307-18

Effects on quinolone resistance due to the biofilm formation activity in *Ureaplasma urealyticum*

Chunyan FENG[1], Ya HUANG[2], Yunsong YU[3], Guorong DUAN[4], Yuying DAI[1], Ke DONG[5], Qingtian LI[1,6,*]

[1]Department of Internal Medicine, Zhejiang Medical College, Hangzhou, P.R. China
[2]Center for Reproductive Medicine, Chinese PLA No. 455 Hospital, Shanghai, P.R. China
[3]Department of Infectious Diseases, Sir Run Run Shaw Hospital, College of Medicine, Zhejiang University, Hangzhou, P.R. China
[4]Department of Infectious Diseases, Zhejiang Hospital, Hangzhou, P.R. China
[5]Department of Medical Microbiology and Parasitology, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China
[6]Department of Laboratory Medicine, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, P.R. China

Received: 05.07.2013 • **Accepted:** 24.01.2014 • **Published Online:** 12.01.2015 • **Printed:** 09.02.2015

Background/aim: To detect the effects on quinolone resistance due to biofilm formation activity in *Ureaplasma urealyticum*.

Materials and methods: An improved checkerboard dilution susceptibility test was used to analyze the quinolone resistance of clinical *Ureaplasma* isolates and a postbiofilm formation susceptibility assay was used to compare the effects on the quinolone susceptibility between pre- and postbiofilm formations. Several important functional gene expressions were detected to evaluate their roles in the process of the quinolone resistance mechanism.

Results: The quinolone-resistant isolates produced more biofilms than the sensitive isolates. In both the quinolone-resistant and -sensitive groups, the minimal inhibitory concentrations after biofilm formation were higher than those before biofilm formation. The expression of the metabolism-related gene *ureC* in postbiofilm formation was higher than those in prebiofilm formation.

Conclusion: It seems that biofilm formation is quite important in the generation of quinolone resistance in *Ureaplasma*. It is very useful to detect biofilm formation activity as well as to analyze other laboratory parameters for *Ureaplasma*.

Key words: Quinolone resistance, biofilm formation activity, *Ureaplasma urealyticum*

1.Introduction

Ureaplasma urealyticum is considered a part of the normal genital flora in human beings. It is found in about 70% of sexually active humans. It has also been associated with a number of diseases, such as nonspecific urethritis (1), infertility (2), or chorioamnionitis (3). The symptoms of *U. urealyticum* infection are variable in different people and bacterial strains (4). In addition, *U. urealyticum* has some effects on other pathogen infections in the genital tract and results in more complex mixed infections (5). Host–pathogen interactions and adapted immune responses are involved in the *U. urealyticum* infection processes, and these features make for variable virulence and antibiotic resistances in different populations (6).

Quinolone antibiotics are used widely to deal with *Ureaplasma* infections. However, resistance to quinolone is an increasingly serious problem in today's clinical treatment of this pathogen (7). The most important reason for this situation is the improper uses of antibiotics. Furthermore, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), the resistance or susceptibility of *U. urealyticum* is judged by the 'color change unit'. Unfortunately, the current clinical susceptibility testing programs, based on many commercial kits such as Mycoplasma IST2 kits (bioMerieux, Marcy l'Etoile, France), cannot accurately reflect the real resistance level of *Ureaplasma*.

In 2009, an available CLSI-compliance susceptibility testing method was reported on by Beeton et al. (8). This method made it possible to test the quantity of *Ureaplasma* and the actual susceptibility of the organism. In this study, we detected the biofilm formation of different *Ureaplasma* strains, and then observed the *Ureaplasma*'s susceptibility prebiofilm formation and postbiofilm formation. Some functional gene expressions were evaluated to analyze whether there was a correlation between these gene expressions and biofilm formation in the pathogen.

* Correspondence: qingtianli@sjtu.edu.cn

2. Materials and methods

2.1. Clinical *Ureaplasma* isolates and other reagents

Sixty-nine *Ureaplasma* isolates were collected from the Department of Urology and Genecology of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine from June to November 2009. Mycoplasma IST2 kits were purchased from bioMerieux. Antibiotics ciprofloxacin and ofloxacin were sourced from Sigma Chemical Co. (St Louis, MO, USA). *Ureaplasma* media were purchased from Livzon Inc. (Zhuhai, China). Gene extraction and real-time polymerase chain reaction (PCR) kits were purchased from Tiagen Biotech Co., Ltd. (Beijing, China).

2.2. *Ureaplasma* susceptibility test

Ureaplasma susceptibility was detected by the improved checkerboard dilution method as described by Beeton et al. (8). Antibiotics ciprofloxacin and ofloxacin were used in the susceptibility test. In 96-well plates, 180 μL of ciprofloxacin and of ofloxacin was added in different wells, with concentrations ranging from 16 μg/mL to 0.25 μg/mL for ciprofloxacin and from 32 μg/mL to 0.5 μg/mL for ofloxacin, changing by 2-fold serial dilutions. Thereafter, 20-μL *Ureaplasma* samples were added to the test wells. Another dilution gradient was the *Ureaplasma* sample itself without antibiotics. This nonantibiotic control group was used to identify the initial sample sizes. Based on the minimal inhibitory concentrations (MICs) of these 2 antibiotics, the isolates were divided into 2 groups: a quinolone-sensitive group and a quinolone-resistant group. Only when the MIC was ≥2 μg/mL for ciprofloxacin and ≥4 μg/mL for ofloxacin were isolates classified as quinolone-resistant; *Ureaplasma* isolates were determined as sensitive when the MIC was ≤1 μg/mL for ciprofloxacin and ≤2 μg/mL for ofloxacin.

2.3. Biofilm formation and postbiofilm formation susceptibility assays

For the bacterial biofilm formation test, sterile Teflon membranes (9,10) were immersed into the *Ureaplasma* media and incubated at 37 °C. After the media changed from orange to fuchsia, these membranes were washed twice with sterile phosphate-buffered saline and removed to the second *Ureaplasma* culture, and total protein was analyzed. The biofilm formation ability was then determined by the second culture time (the medium color change time) and the protein volume was detected by a classical Coomassie Brilliant Blue stain method with the optical density read at 570 nm.

Postbiofilm formation susceptibility assays were performed as previously described (11) and a comparison was made between the susceptibility of pre- and postbiofilm formation based on the previously improved checkerboard dilution method. Ofloxacin was used in these postbiofilm formation susceptibility tests. All tests were performed in duplicate and results are expressed as the mean value of both experiments.

2.4. Biofilm formation and multiple functional gene expression of *U. urealyticum*

Several key gene expressions were detected by q-PCR in this study. They were the virulence-related gene *mba*, the metabolism-related gene *ureC*, and the quinolone resistance-related gene *gyrA*. The q-PCR primers were: mba (ACCTACACCAACTCCTGA, TCTTGTAATTCTTGAGTAT), ureC (AGCGTTAGATTAGGAGACAC, CATTACTTCAGCATTCCC), gyrA (ATGGCGTTAAAAAAACC, TGCTCTTCGATGTACAGG), and the control 16S rDNA (TCGTGTCGTGAGATGTTGGGTTA, GGTTTCGCTGCCCTTTGTATTGT). An Applied Biosystems ABI 7500 Fast Real-Time PCR System and the standard q-PCR procedure were used for the q-PCR amplifications in this study. The ratios of postbiofilm expression to prebiofilm expression were calculated, and ratios of >2.0 or <0.5 mean increased or decreased gene expression, respectively.

2.5. Statistical analysis

The significance of the differences between the means was analyzed by double-sided Student's t-test, and other data were analyzed by the chi-square test. $P < 0.05$ was considered significant.

3. Results

In this study, the isolates resistant to both ciprofloxacin and ofloxacin were considered as quinolone-resistant strains, and those susceptible to both antibiotics were considered as quinolone-susceptible. Of the 69 *Ureaplasma* isolates, there were 42 isolates that were determined as quinolone-resistant and 27 as quinolone-sensitive (Table 1).

The biofilm formation activity was determined by the secondary culture time to the media color change and the biofilm total protein quantitation (Figure 1). The secondary culture time of the isolates in the resistant group was significant lower than that in the sensitive group ($P < 0.05$). At the same time, the biofilm total protein volume in the resistant group was much higher than that in the sensitive group ($P < 0.05$). These results showed that the *Ureaplasma* isolates in the resistant group could produce more biofilm than the sensitive isolates.

Another classical biofilm susceptibility test (11) was performed on these 2 groups of *Ureaplasma* (Figure 2). Compared with the MIC before biofilm formation, the MIC after biofilm formation was increased significantly in the isolates in the resistant and sensitive groups. It seems that biofilm formation can improve the resistance to ofloxacin in *U. urealyticum*.

Table 1. Minimal inhibitory concentrations of ciprofloxacin and ofloxacin in 69 *Ureaplasma urealyticum* isolates.

		Number of isolates (MIC of ciprofloxacin, μg/mL)						
		≥16	8	4	2	1	0.5	≤0.25
Number of isolates (MIC of ofloxacin, μg/mL)	≥32	1						
	16	1	2					
	8		2	6	1			
	4			11	18			
	2					2		
	1					6	14	
	≤0.5						4	1

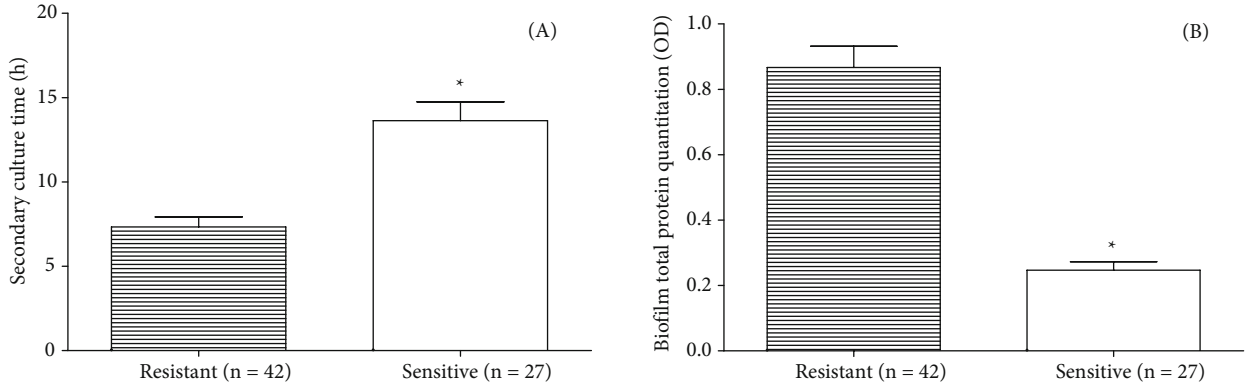

Figure 1. The biofilm formation activities of quinolone-resistant and -sensitive *U. urealyticum*. **(A)** The secondary culture time of quinolone-resistant and -sensitive *Ureaplasma*. The biofilm was produced on sterile Teflon membranes; then the free bacteria were discarded and the membranes were cultured in Mycoplasma IST2 media and the secondary culture time was calculated. **(B)** The biofilm total protein quantitation of quinolone-resistant and -sensitive *Ureaplasma*. The Teflon membranes were stained and the optical densities at 570 nm were recorded. * = Significant difference compared to resistant groups, $P < 0.05$.

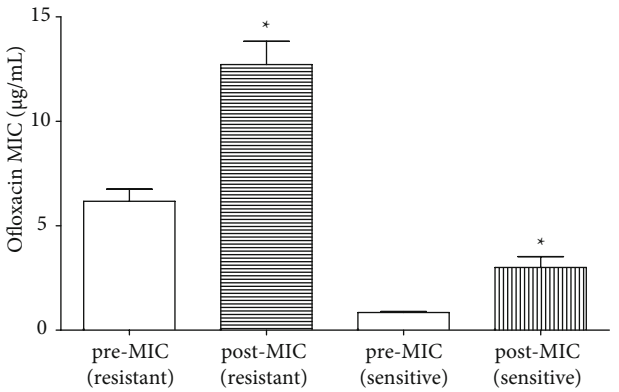

Figure 2. The ofloxacin MIC (μg/mL) of quinolone-resistant and -sensitive *Ureaplasma* before (pre-MIC) and after (post-MIC) biofilm was produced. The improved checkerboard dilution method was used to analyze the ofloxacin MIC (μg/mL). * = Significant difference compared to pre-MIC, $P < 0.05$.

The expressions of virulence-, metabolism-, and quinolone resistance-related genes *mba*, *ureC*, and *gyrA* were detected and compared between prebiofilm formation and postbiofilm formation in this study. *ureC* gene expression was increased at postbiofilm formation compared with prebiofilm formation; however, there were no significant differences in the other genes' expressions between pre- and postbiofilm formation (Table 2).

4. Discussion

Ureaplasma urealyticum is a bacterium that is related to several diseases and can be passed through sexual contact, although it is not a classic STI or STD (12,13). At present, 3 types of antibiotics are used in the treatment of *Ureaplasma*. They are tetracyclines (14), macrolides (15), and quinolones (16). The rates of resistance to fluoroquinolones (ofloxacin, ciprofloxacin) have been >50% since 1999 (17).

Table 2. Multiple functional gene expressions in quinolone resistant and sensitive *U. urealyticum*.

Gene	*mba* (ratio of postbiofilm expression to prebiofilm expression)	*ureC* (ratio of postbiofilm expression to prebiofilm expression)	*gyrA* (ratio of postbiofilm expression to prebiofilm expression)
Resistant (n = 42)	0.83 ± 0.12	4.72 ± 2.58 *	0.94 ± 0.09
Sensitive (n = 27)	1.05 ± 0.17	2.49 ± 0.84 *	1.24 ± 0.13

*: $P < 0.05$.

To date, the current clinical *Ureaplasma* susceptibility test is colorimetry using Mycoplasma IST2 produced by bioMerieux. However, the initial actual amount is not considered in this method, so this method is not in full compliance with the CLSI criteria (18).

Ureaplasma is known to produce biofilms, which could trap inflammatory products and thereby mechanically block inflammatory signaling (19). In our study, secondary culture time was used to evaluate the biofilm formation activities of isolates in 2 groups. The secondary culture time of the isolates in the resistant group was much shorter than the time of those in the sensitive group. At the same time, the optical density value of the total protein detection of the isolates in the resistant group was much higher than that in the sensitive group. These results illustrated that quinolone-resistant *Ureaplasma* isolates created more biofilms than quinolone-sensitive isolates, which means that the quinolone resistance features of *Ureaplasma* may be related to the biofilm formation of this pathogen.

After the biofilm formation, higher ofloxacin MIC values were detected in both quinolone-resistant *Ureaplasma* and quinolone-sensitive *Ureaplasma*. The barrier function may be the major reason for the higher quinolone resistance in those postformation *Ureaplasma* strains. It is noteworthy that after the biofilm formation, some of the sensitive *Ureaplasma* isolates "transformed" to resistant isolates, as determined by the improved checkerboard dilution method for *Ureaplasma* susceptibility analysis. More attention should be paid to this situation in order to improve clinical efficacy and prevent the development of drug resistance.

Higher expressions of the metabolism-related gene *ureC* were found in postbiofilm formation than in prebiofilm formation, which was not the case for the virulence- and quinolone resistance-related genes *mba* and *gyrA*. This improved ureC expression might be due to the physical barrier that disturbs the nutrient acquisition of this pathogen.

In conclusion, the biofilm formation activity in *Ureaplasma* might affect the clinical antibiotic susceptibility tests and the treatment efficacy, and even lead to the emergence and development of drug resistance. It is extremely valuable to add biofilm formation tests to the laboratory analyses for *Ureaplasma*.

Acknowledgments

This study was supported by grants from the Scientific Foundation of the Education Department of Zhejiang Province, China (Y201122483); the National Natural Science Foundation of China (81000708 and 81201334); and the National High Technology Research and Development Program of China (2011AA02A119).

References

1. Furneri PM, Roccasalva L, Fallica L, Tempera G. Efficacy of miocamycin in the therapy of non-specific genital infections (NSGI): non-gonococcal urethritis (NGU) and acute urethral syndrome (AUS). Int J Clin Pharmacol Res 1988; 8: 111–116.
2. Salmeri M, Valenti D, La Vignera S, Bellanca S, Morello A, Toscano MA, Mastrojeni S, Calogero AE. Prevalence of *Ureaplasma urealyticum* and *Mycoplasma hominis* infection in unselected infertile men. J Chemother 2012; 24: 81–86.
3. Abele-Horn M, Scholz M, Wolff C, Kolben M. High-density vaginal *Ureaplasma urealyticum* colonization as a risk factor for chorioamnionitis and preterm delivery. Acta Obstet Gynecol Scand 2000; 79: 973–978.
4. Wetmore CM, Manhart LE, Lowens MS, Golden MR, Jensen NL, Astete SG, Whittington WL, Totten PA. *Ureaplasma urealyticum* is associated with nongonococcal urethritis among men with fewer lifetime sexual partners: a case-control study. J Infect Dis 2012; 204: 1274–1282.
5. Yamazaki T, Matsumoto M, Matsuo J, Abe K, Minami K, Yamaguchi H. Frequency of *Chlamydia trachomatis* in *Ureaplasma*-positive healthy women attending their first prenatal visit in a community hospital in Sapporo, Japan. BMC Infect Dis 2012; 12: 82.

6. Leli C, Mencacci A, Bombaci JC, D'Alo F, Farinelli S, Vitali M, Montagna P, Bietolini C, Meucci M, Perito S et al. Prevalence and antimicrobial susceptibility of *Ureaplasma urealyticum* and *Mycoplasma hominis* in a population of Italian and immigrant outpatients. Infez Med 2012; 20: 82–87.

7. Shimada Y, Deguchi T, Nakane K, Masue T, Yasuda M, Yokoi S, Ito S, Nakano M, Ishiko H. Emergence of clinical strains of *Mycoplasma genitalium* harbouring alterations in ParC associated with fluoroquinolone resistance. Int J Antimicrob Agents 2010; 36: 255–258.

8. Beeton ML, Chalker VJ, Maxwell NC, Kotecha S, Spiller OB. Concurrent titration and determination of antibiotic resistance in *Ureaplasma* species with identification of novel point mutations in genes associated with resistance. Antimicrob Agents Chemother 2009; 53: 2020–2027.

9. Irving TE, Allen DG. Species and material considerations in the formation and development of microalgal biofilms. Appl Microbiol Biotechnol 2011; 92: 283–294.

10. Pandelidis K, McCarthy A, Chesko KL, Viscardi RM. Role of biofilm formation in *Ureaplasma* antibiotic susceptibility and development of bronchopulmonary dysplasia in preterm neonates. Pediatr Infect Dis J 2013; 32: 394–398.

11. Garcia-Castillo M, Morosini MI, Galvez M, Baquero F, del Campo R, Meseguer MA. Differences in biofilm development and antibiotic susceptibility among clinical *Ureaplasma urealyticum* and *Ureaplasma parvum* isolates. J Antimicrob Chemother 2008; 62: 1027–1030.

12. Chang-Tai Z, Zhong-Yi H, Chun-Lei D, Chang-Song Z, Mei-Zhen W, Yang L. Investigation of *Ureaplasma urealyticum* biovars and their relationship with antimicrobial resistance. Indian J Med Microbiol 2011; 29: 288–292.

13. Cheong KA, Agrawal SR, Lee AY. Validation of nested PCR and a selective biochemical method as alternatives for mycoplasma detection. J Basic Microbiol 2011; 51: 215–219.

14. Connell SR, Tracz DM, Nierhaus KH, Taylor DE. Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob Agents Chemother 2003; 47: 3675–3681.

15. Pereyre S, Metifiot M, Cazanave C, Renaudin H, Charron A, Bebear C, Bebear CM. Characterisation of in vitro-selected mutants of *Ureaplasma parvum* resistant to macrolides and related antibiotics. Int J Antimicrob Agents 2007; 29: 207–211.

16. Bearden DT, Danziger LH. Mechanism of action of and resistance to quinolones. Pharmacotherapy 2001; 21: 224S–232S.

17. Xie X, Zhang J. Trends in the rates of resistance of *Ureaplasma urealyticum* to antibiotics and identification of the mutation site in the quinolone resistance-determining region in Chinese patients. Fems Microbiol Lett 2006; 259: 181–186.

18. Xiao L, Crabb DM, Duffy LB, Paralanov V, Glass JI, Waites KB. Chromosomal mutations responsible for fluoroquinolone resistance in *Ureaplasma* species in the United States. Antimicrob Agents Chemother 2012; 56: 2780–2783.

19. Kallapur SG, Kramer BW, Knox CL, Berry CA, Collins JJ, Kemp MW, Nitsos I, Polglase GR, Robinson J, Hillman NH et al. Chronic fetal exposure to *Ureaplasma parvum* suppresses innate immune responses in sheep. J Immunol 2011; 187: 2688–2695.