Differences in biofilm development and antibiotic susceptibility among clinical *Ureaplasma urealyticum* and *Ureaplasma parvum* isolates

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**Objectives:** The aim of this work was to study the ability of clinical isolates of *Ureaplasma* spp. to form biofilms *in vitro* and to compare the antibiotic susceptibility of sessile cells and their planktonic counterparts.

**Methods:** A total of nine *Ureaplasma* spp. isolates recovered from unrelated male patients diagnosed with urethritis or chronic prostatitis and two isolates isolated from the urine of two healthy volunteers were included. *Ureaplasma* species identification was performed by 16S rDNA gene amplification and sequencing. Conventional antibiotic susceptibility tests were carried out by the broth microdilution method. Biofilm susceptibility assays were performed following the method proposed by Moskowitz using 10C urea broth medium and confirming bacterial growth by colour shift of the medium. The $\chi^2$ test was applied to analyse the statistical differences between the MIC and the minimal biofilm inhibitory concentration.

**Results:** Isolates were identified as *Ureaplasma urealyticum* serovar 7 (five isolates), *U. urealyticum* serovar 13 (four isolates) and *Ureaplasma parvum* serovar 3 (two isolates). Biofilm formation was observed in 9 out of the 11 strains studied (82%); two isolates of *U. urealyticum* serovar 13 were non-biofilm formers. Global resistance percentages of planktonic cells compared with sessile cells were different for erythromycin (0% versus 44%, $P = 0.02$), telithromycin (22% versus 77%, $P = 0.02$), ciprofloxacin (66% versus 100%), levofloxacin (0% versus 33%) and tetracycline (0% versus 33%). All nine biofilm-forming strains were fully susceptible to clarithromycin in both planktonic and biofilm types of growth.

**Conclusions:** These results indicate that biofilm formation can protect mycoplasma cells from antibiotics and host defences, favouring their persistence in chronically infected or colonized patients while increasing resistance to antimicrobial agents. Therefore, the capacity to form biofilms by *Ureaplasma* spp. isolates should be considered when antibiotic treatments are required.

**Keywords:** biofilm formation, *U. parvum*, *U. urealyticum*

**Introduction**

About 17 different species of the class *Mollicutes* have been detected that colonize or infect humans; *Mycoplasma pneumoniae*, which causes respiratory tract infections, and *Mycoplasma genitalium, Mycoplasma hominis* and *Ureaplasma* spp., which are responsible for genito-urinary tract infections, being the most important pathogens. Although these microorganisms can be intracellularly located, they usually colonize mucosal surfaces using bacterial structures that facilitate adherence to eukaryotic cells.

Biofilms are complex aggregations of sessile bacterial cells enveloped by an extracellular matrix of biopolymeric substances. Clinically, biofilm infections are notoriously difficult to treat with antimicrobial agents and disinfectants, and the host immune system is generally ineffective at clearing infection due to limited access. The biofilm mode of life conveys a survival advantage that results in persistent infections. The reduced...
permeability of biofilms usually impairs the action of antibiotics. As an exception, azithromycin has demonstrated a specific ability to avoid biofilm formation by *Pseudomonas aeruginosa* inhibiting alginate production and blocking quorum sensing signals.\(^4\) Therapeutic options for *Mycoplasma* spp. infections are limited, and macrolides are the most active agents.

The ability to form biofilms by animal *Mycoplasma* species has already been examined, showing considerable intra-species differences.\(^3\) Conversely, biofilm formation by human mycoplasmas has not been reported, although their involvement in persistent infections and their recognized adherence to mucosal surfaces firmly suggest this possibility. The aim of this work was to study the ability of clinical isolates of *Ureaplasma urealyticum* and *Ureaplasma parvum* to form biofilms in vitro and to compare the antibiotic susceptibility of sessile cells and their planktonic counterparts.

**Methods**

A total of seven *U. urealyticum* and two *U. parvum* isolates, recovered from urethral exudates and semen from unrelated male patients diagnosed with ureaplasma urethritis or chronic prostatitis, were used in this study. Two *U. urealyticum* ? (serovar 1) isolates cultured from the urine of two healthy volunteers were included as controls. All isolates were recovered during 2007 at the Microbiology Service of the Ramón y Cajal University Hospital, Madrid, Spain. Clinical samples were inoculated in 10C urea broth medium (70 mL of pleuropneumonia-like organism media (PPLO) pH 5.5, 20% horse serum, 10% yeast extract, 0.01% L-cystein hydrochloride, of pleuropneumonia-like organism media (PPLO) pH 5.5, 20% trols. All isolates were recovered during 2007 at the Microbiology Service of the Ramón y Cajal University Hospital, Madrid, Spain. Clinical samples were inoculated in 10C urea broth medium (70 mL of pleuropneumonia-like organism media (PPLO) pH 5.5, 20% horse serum, 10% yeast extract, 0.01% L-cystein hydrochloride, 0.04% urea, 0.5% FILDES (Difo), Phenol Red, ampicillin and GHL tripeptide). A positive growth was considered when a colour shift due to urea hydrolysis resulting in concomitant alkalization of the broth medium was observed and confirmed with the growth of typical brown colonies after subculture on A7 agar. *Ureaplasma* biovars were determined by 16S rDNA gene amplification and sequencing using primers and conditions previously described.\(^9\)

Conventional MIC susceptibility testing with erythromycin, clari-thromycin, telithromycin, tetracycline, ciprofloxacin, levofloxacin and linezolid was performed by the broth microdilution method following the Mycoplasmal Chemotherapy Working Team Guidelines.\(^7\) All antibiotics were supplied by their corresponding manufacturers or purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA). Microdilution panels were aerobically incubated overnight at 37°C after inoculation. All tests were performed in duplicate and results expressed as the mean value of both experiments.

Biofilm susceptibility assays were performed as previously described with some modifications.\(^8\) Briefly, 96-well microtitre plates (Alpha Laboratories LTD, Hampshire, UK) were inoculated with 100 μL of a 1/100 dilution of a *Ureaplasma* overnight culture in 10C urea broth medium. Bacterial biofilms were formed by immersing the pegs of a modified polystyrene microtitre lid (catalogue no. 445497; Nunc TSP system Roskilde, Denmark) into this biofilm growth plate incubated at 37°C until the medium changed from orange to fuchsia (due to pH shift occurring between 24 and 36 h). Lids were then washed three times in sterile PBS to eliminate the planktonic organisms and placed on another microtitre plate containing serial dilutions of the corresponding antibiotic and incubated again until the medium colour changed again. The biofilms formed in the lids were transferred to the microtitre wells by centrifugation at 3000 rpm for 10 min. The lid was rejected and replaced by another clean lid and the plate was reincubated for another 24 h. An adequate biofilm growth of the positive growth control well (without antibiotic) was defined by the colour change of the broth medium (from orange to fuchsia). MIC and minimal biofilm inhibitory concentration (MBIC) are defined as the lowest concentration of antimicrobial that prevents a colour change of the planktonic and of the sessile cell cultures, respectively, at the time when the positive growth control shows its initial colour change. Comparison between MBIC\(_{90}\)–MIC\(_{90}\) and MBIC\(_{90}\)–MIC\(_{90}\) values was carried out only for biofilm-forming strains.

Statistical analysis of the results was performed using the \(\chi^2\) test.

**Results**

*Ureaplasma* species identification by the 16S rDNA gene sequence is shown in Table 1. Biofilm formation was observed in 9 out of the 11 strains studied (82%). The two *U. urealyticum* serovar 13 strains recovered from urethral exudates were not able to form biofilms under our experimental conditions. Antibiotic susceptibility results are shown in Table 1. In general, MBICs were one or two dilutions higher than MICs, although some exceptions were observed. These exceptions comprise results found for telithromycin, levofloxacin and tetracycline in the case of four strains (numbers 1, 7, 66711 and 151302) in which one or more of these antimicrobials exhibited greater activity against sessile cells than against planktonic cells. Nevertheless, when comparing the global MIC with the MBIC values for all biofilm-former strains, MBICs were higher than MICs for all antibiotics.

Considering antibiotic susceptibility and resistance concepts, most *Ureaplasma* spp. isolates were susceptible to all antibiotics tested when conventional MICs were determined. However, resistance rates considerably increased when antibiotic susceptibility tests were carried out for sessile cells. Thus, global resistance percentages in planktonic cells compared with sessile cells were lower for erythromycin (0% versus 44%, \(P = 0.02\)), telithromycin (22% versus 77%, \(P = 0.02\)), ciprofloxacin (66% versus 100%), levofloxacin (0% versus 33%) and tetracycline (0% versus 33%). All nine biofilm-forming strains were fully susceptible to clarithromycin, in both planktonic and biofilm types of growth. At present, no breakpoints are available for linezolid against *Ureaplasma* spp.; however, the results obtained appear to indicate that this antibiotic is not effective against these microorganisms. However, it is of note that strain number 1 has an MIC value of 0.5 mg/L and strain number 7 exhibited a significantly lower MBIC value (0.06 mg/L) than the MIC value (8 mg/L) of this compound (Table 1).

**Discussion**

At least 17 well-documented *Mycoplasma* and *Ureaplasma* species either colonize or infect humans, 11 of them being considered as normal microbiota. Conversely, *M. pneumoniae*, *M. hominis*, *Mycoplasma fermentans*, *M. genitalium*, *U. urealyticum* and *U. parvum* are considered pathogenic species.\(^1\) In recent years, molecular techniques have improved the detection of these fastidious microorganisms in view of the fact that their in vitro culture remains difficult, mainly due to their slow growth and to their complex nutritional requirements.\(^5\) In order to identify the *Ureaplasma* isolates included in this work, the 16S rDNA coding gene was fully sequenced. At present, the two *Ureaplasma*
Table 1. Antibiotic susceptibility and percentage of resistance to different antibiotics of planktonic and sessile cells of the *Ureaplasma* spp. studied strains

| Strain Origin         | Identification          | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC | MIC | MBIC |
|----------------------|-------------------------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|-----|------|
| U. urealyticum       | 10 healthy volunteer    | 0.01| 0.25| 0.25| 1    | 0.06| 4    | 1   | 4    | 0.25| 1    | 0.06| 1    | 8   | 16   | 16  | 16   | 16  | 16   | 16  | 16   |
| U. urealyticum       | 1 healthy volunteer    | 0.03| 0.03| 0.12| 0.5  | 2   | 2    | 4   | 1    | 0.5 | 0.25 | 1   | 0.5  | 8   | 16   | 16  | 16   | 16  | 16   | 16  | 16   |
| U. urealyticum       | semen                   | 0.03| 0.25| 0.25| 1    | 0.12| 2    | 1   | 2    | 0.25| 0.5  | 0.12| 1    | 16  | 32   | 32  | 32   | 32  | 32   | 32  | 32   |
| U. urealyticum       | 150067 semen            | 0.05| 0.25| 0.25| 1    | 0.06| 4    | 1   | 2    | 0.25| 0.5  | 0.12| 1    | 16  | 32   | 32  | 32   | 32  | 32   | 32  | 32   |
| U. urealyticum       | 152050 semen            | 0.06| 0.5 | 0.12| 0.5  | 0.5 | 4    | 0   | 2    | 0.5 | 0.25 | 0.5 | 0.25 | 16  | 32   | 32  | 32   | 32  | 32   | 32  | 32   |
| MIC<sub>50</sub>     | 0.01 0.25 0.25 0.5 0.12 4 1 2 0.25 0.5 0.25 1 8 16 | 16  |
| MIC<sub>90</sub>     | 0.03 0.5 0.25 1 2 8 2 4 0.5 1 0.5 4 16 32 | 32  |
| Resistance (%)       | 0 0 0 44 27.3 77 72.7 100 9 33 9 33 — — | 32  |

CLA, clarithromycin; ERY, erythromycin; TEL, telithromycin; CIP, ciprofloxacin; LEV, levofloxacin; TET, tetracycline; LZD, linezolid; NB, non-biofilm-forming strain.

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Transparency declarations

None to declare.

References


