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Role of Biofilm Formation in *Ureaplasma* Antibiotic Susceptibility and Development of Bronchopulmonary Dysplasia in Preterm Neonates

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Abstract

BACKGROUND—*Ureaplasma* respiratory tract colonization is a risk factor for bronchopulmonary dysplasia (BPD) in preterm infants, but whether *Ureaplasma* isolates from colonized infants can form biofilms is unknown. We hypothesized that *Ureaplasma* isolates vary in capacity to form biofilms that contribute to their antibiotic resistance and ability to evade host immune responses. Study objectives were to 1) determine the ability of *Ureaplasma* isolates from preterm neonates to form biofilms *in vitro*; 2) compare the susceptibility of the sessile and planktonic organisms to azithromycin and erythromycin; and 3) determine the relationship of biofilm-forming capacity in *Urea-plasma* isolates and the risk for BPD.

METHODS—Forty-three clinical isolates from preterm neonates and five ATCC strains were characterized for their capacity to form biofilms *in vitro* and antibiotic susceptibility was performed on each isolate pre- and post-biofilm formation.

RESULTS—Forty-one (95%) clinical and 4 of 5 (80%) ATCC isolates formed biofilms. All isolates were more susceptible to azithromycin (Minimum Inhibitory Concentration, MIC₅₀ 2 µg/mL) than erythromycin (MIC₅₀ 4 µg/mL), and biofilm formation did not significantly affect antibiotic susceptibility for the 2 tested antibiotics. The MIC₅₀ and minimum biofilm inhibitory concentrations (MBIC₅₀) for *U. urealyticum* clinical isolates for azithromycin were higher than for MIC₅₀ and MBIC₅₀ for *U. parvum* isolates. There were no differences in MIC or MBICs among isolates from BPD infants and non-BPD infants.

CONCLUSIONS—Capacity to form biofilms is common among *Ureaplasma* spp. isolates, but biofilm-formation did not impact MICs for azithromycin or erythromycin.

Keywords

Ureaplasma parvum; *Ureaplasma urealyticum*; bacterial biofilms; erythromycin; azithromycin; bronchopulmonary dysplasia

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Supplemental Digital Content

Supplemental Digital Content 1: Antibiotic susceptibility to erythromycin and azithromycin of planktonic and sessile cells of *Ureaplasma* isolates from ATCC reference strains and preterm infants with and without BPD

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INTRODUCTION

The genital mycoplasmas *Ureaplasma parvum* (serovars 1, 3, 6, and 14) and *U. urealyticum* (serovars 2, 4, 5, 7-13) are common commensals in the female genitourinary tract, but colonization with these organisms during pregnancy has been increasingly associated with adverse outcomes such as infertility, stillbirth, intrauterine infection, and preterm birth.^{1, 2} Vertical transmission may occur *in utero* or at the time of birth. The organisms can be detected in amniotic fluid,³ placental tissues,⁴ cord blood,⁵⁻⁷ neonatal respiratory⁸ and gastric secretions,⁹ and cerebral spinal fluid.⁶ Detection of ureaplasma organisms in preterm infants has been associated with an increased incidence of bronchopulmonary dysplasia (BPD),^{10, 11} necrotizing enterocolitis (NEC),¹² meningitis,¹³ and abnormal cranial ultrasounds.² Although the organisms are susceptible to macrolide antibiotics *in vitro*,¹⁴ trials of erythromycin and azithromycin in women and infants have failed to demonstrate efficacy for eradication in intrauterine and neonatal lung compartments, prevention of maternal-fetal vertical transmission in humans^{15, 16} and experimental pregnancy models,¹⁷ or prevention of BPD in infected infants.¹⁸⁻²⁴ The mechanisms by which these organisms evade host defenses have not been elucidated.

Biofilms are sessile bacteria attached to a substratum encased in complex structures with an extracellular matrix of polysaccharide, lipid, DNA, and protein.²⁵ Biofilm formation increases microbial resistance to host defenses and antibiotics. Since *Mycoplasma* species have a limited genome and lack cell walls, it was unknown until recently that these species had the capacity to form biofilms.²⁵⁻²⁹ García-Castillo *et al.*³⁰ reported that seven of nine *Ureaplasma* clinical isolates from the urine of healthy men or those with urethritis or chronic prostatitis formed biofilms *in vitro*. Whether *Ureaplasma* isolates from colonized infants can form biofilms is unknown. We hypothesized that *Ureaplasma* isolates vary in capacity to form biofilms that contribute to their antibiotic resistance and ability to evade host immune responses. This study 1) determined the ability of *Ureaplasma* spp. isolates from preterm neonates to form biofilms *in vitro*; 2) compared the susceptibility of the sessile and planktonic organisms to azithromycin and erythromycin; and 3) determined the relationship of biofilm-forming capacity in *Urea-plasma* spp. isolates and the risk for BPD.

MATERIALS AND METHODS

Ureaplasma isolates

Ureaplasma isolates were selected from banked isolates derived from respiratory specimens from preterm neonates admitted to the University of Maryland Medical Center (UMMC) Neonatal Intensive Care Unit that had previously been characterized by real-time PCR for species and in some cases, serotype classification.⁸ All isolates were within 2 passages from the original patient-derived culture. Five laboratory strains representing *U. parvum* serotypes 1 (27813), 3 (700970), 6 (27818), and 14 (33697) and *U. urealyticum* serotype 8 (27618) were obtained from the American Tissue Culture Collection (ATCC, Rockville, MD). All isolates stocks were frozen at -80°C prior to experiments.

Preparation of Antibiotic Stock Solutions

Erythromycin and azithromycin (Sigma, St Louis, MO) were used in both conventional antibiotic susceptibility testing and in biofilm susceptibility testing. Stock solutions of 1.6 mg/ml antibiotic in ethanol were prepared and stored at -20 °C until ready for use. A 1:100 dilution (16 ug/mL) of this stock solution in 10B broth medium was prepared for serial dilution in microtiter plates for conventional antibiotic susceptibility testing and biofilm susceptibility testing.

Conventional Minimum Inhibitory Concentration (MIC) Susceptibility Testing

Conventional MIC susceptibility testing with erythromycin and azithromycin were performed for each isolate in quadruplicate by the broth microdilution method as previously described.³⁰ Cultures of each clinical and laboratory isolate were grown at 37°C for 15-18 hours overnight. One hundred microliter of overnight culture diluted 1:100 in 10B broth (pH 6.0) for final concentration 1×10^4 color changing units/ml was added to each well of a 96-well microtiter plate (Nunc, Thermo Fisher Scientific, Waltham, MA), prepared with serial dilutions of erythromycin and azithromycin. The plates were incubated at 37°C and observed until there was a color change indicating a pH change as a result of *Ureaplasma* growth in the wells without antibiotics. The MIC was defined as the lowest concentration preventing bacterial growth at the time that growth was observed in the positive control wells.

Biofilm Susceptibility Assay

Biofilm susceptibility assays were performed in duplicate as previously described with modifications³⁰. Briefly, 100 µL of a 1:100 dilution of an overnight culture was added to 100 µL of 10B medium (pH 6.0) in each well of a 96-well microtiter plate. For bacterial biofilm formation, pegs of a modified lid (Nunc Immuno™ TSP system, Thermo Fisher Scientific, Waltham, MA) were immersed into the wells and the plates incubated at 37°C until a color change was observed. The peg lids were washed to remove planktonic organisms by dipping the lids ten times into each of two 96-well plates containing PBS ($-Ca^{2+}$, $-Mg^{2+}$). Immediately following the second wash, the lids were placed onto another 96-well plate containing serial dilutions of both erythromycin and azithromycin, each in quadruplicate. To transfer the biofilm forming organisms to the antibiotic-containing medium, the plates were centrifuged at $2,000 \times g$ for ten minutes at 4°C. The peg lids were discarded and replaced with a sterile standard lid. The plates were incubated at 37°C and observed until color change in the positive control wells. The MBIC was defined as the lowest concentration preventing bacterial growth at the time that growth was observed in the positive control wells. Similarly, biofilm formation was determined by growth in the positive control wells of the antibiotic plate.

Clinical outcomes

The clinical characteristics of the cohort from which the banked isolates were obtained have been previously described.⁸ The respiratory specimens were obtained after parental consent was obtained for protocols approved by the University of Maryland School of Medicine Institutional Review Board. Bronchopulmonary dysplasia severity was defined according to NIH criteria.³¹ Infants receiving supplemental oxygen at 28 d were assessed at 36 wk postmenstrual age or discharge whichever came first, and were classified as mild BPD if breathing room air; moderate BPD, if receiving supplemental oxygen $<30\%$; or severe BPD, if receiving positive pressure support and/or supplemental oxygen $\geq 30\%$ at the assessment time point.

Statistical analysis

Data were analyzed by Chi square and Wilcoxon signed rank test. A p value <0.05 was considered significant.

RESULTS

The banked clinical isolates selected for biofilm-forming capacity included 26 *U. parvum* and 17 *U. urealyticum* isolates (see Table, Supplemental Digital Content 1, <http://links.lww.com/INF/B387> which summarizes MICs and MBICs for all isolates tested). Forty-

one (95%) clinical and 4 of 5 (80%) ATCC isolates tested formed biofilms under *in vitro* conditions. The three non-biofilm forming isolates included ATCC 27618, a *U. urealyticum* serovar 8 strain, and two *U. parvum* serovar 6 isolates. All isolates were more susceptible to azithromycin (Minimum Inhibitory Concentration, MIC₅₀ 2 µg/mL) than erythromycin (MIC₅₀ 4 µg/mL), and biofilm formation did not significantly affect antibiotic susceptibility for the two tested antibiotics. There was a trend towards lower conventional MICs for erythromycin (p=0.07) and statistically significant lower MICs for azithromycin (p=0.006) for the ATCC strains compared to the MICs for these antibiotics for the clinical isolates (Fig. 1). No tested isolate was resistant to azithromycin. Seven of 48 isolates (14.5%) were resistant to erythromycin (MIC₅₀ 16 µg/ml) as planktonic cells (see Table, Supplemental Digital Content 1, <http://links.lww.com/INF/B387>). Of these, 2 were also resistant as biofilm-forming cells. Two additional isolates were sensitive to erythromycin as planktonic cells, but were resistant after biofilm formation.

The MIC₅₀ and minimum biofilm inhibitory concentrations (MBIC₅₀) for *U. urealyticum* clinical isolates for azithromycin were higher than for MIC₅₀ and MBIC₅₀ for *U. parvum* isolates (Fig. 2). Both *U. parvum* planktonic and biofilm-forming cells were more susceptible to azithromycin than to erythromycin. The clinical outcomes of the patients from whom the isolates were selected included 14 no BPD, 10 mild BPD, 15 moderate-severe BPD, and 4 deaths. There were no differences in MIC₅₀ or MBIC₅₀ among isolates from BPD/death infants and non-BPD infants (Fig. 3).

DISCUSSION

Considerable effort has focused on determining whether the 10 distinct *U. urealyticum* and 4 *U. parvum* serotypes differ in pathogenic potential. *Ureaplasma parvum* serovars are the most common in clinical specimens, but some earlier antibody-based studies suggested that specific *U. urealyticum* serovars may have greater pathogenic potential.³²⁻³⁴ Utilizing recently developed serovar specific primers/probes for all 14 serovars,³⁵ we previously demonstrated that serovars 3 and 6, alone and in combination, accounted for 96% of *U. parvum* isolates from colonized preterm infants in our institution.⁸ In our population, the *U. urealyticum* isolates were commonly a mixture of 2 or more serovars. Serovar 11 was the most common *U. urealyticum* serovar, detected as a single serovar or in combination with other serovars in 56% of *U. urealyticum*-positive samples. There were no statistical differences among the *U. parvum* and *U. urealyticum* species in BPD severity, but the sample size was inadequate to detect a difference at the serovar level. We proposed that there might be virulence genes that are common across multiple serovars that could be targeted to prevent or ameliorate the adverse effects of *Ureaplasma* infection in the immature lung. Using the genotyped, banked isolates from our previous study,⁸ the current study was conducted to determine whether biofilm-forming capacity is shared among the *Ureaplasma* serovars.

The present study confirms that most clinical *Ureaplasma* isolates derived from preterm infants and *U. parvum* laboratory strains form biofilms *in vitro*. We tested isolates representing both species and multiple isolates from the same serovar. Although both non-biofilm forming clinical isolates were serovar 6 strains, other serovar 6 isolates formed biofilms *in vitro*, suggesting that biofilm-forming capacity is not serovar-specific. Four of five ATCC isolates formed biofilms, indicating that the biofilm-forming capacity is likely stable after multiple passages.

Our study extends the observations of *in vitro* biofilm-forming capacity of clinical *Ureaplasma* isolates derived from healthy men and those with urethritis and chronic prostatitis.³⁰ In the previous study, two *U. parvum* serovar 3, five *U. urealyticum* serovar 7

and 2 of four *U. urealyticum* serovar 13 isolates formed biofilms *in vitro*. Scanning electron microscopy demonstrated that biofilm-forming bacteria adhere to the prostate ductal epithelium *in vivo*, contributing to organism persistence, chronic inflammation, and antibiotic treatment failures.³⁶ Recently, Romero and co-workers reported a case of *M. hominis* *in vivo* biofilm in the intrauterine cavity detected as “amniotic fluid sludge” by transvaginal ultrasound and the complex structure confirmed by scanning electron microscopy.³⁷ *Mycoplasma pulmonis* intranasally inoculated in mice formed biofilms on the tracheal epithelium *in vivo*.²⁵ These studies suggest that ureaplasmas colonizing the preterm infant respiratory epithelium may form biofilms that protect the organisms from host defenses and antibiotic treatment. To confirm the biologic relevance of the current *in vitro* studies, biofilm-formation of clinical *Ureaplasma* isolates should be tested in *in vivo* experimental models.

Since erythromycin and azithromycin are the most common antibiotics used clinically in preterm infants to treat *Ureaplasma* infections, we tested whether biofilm formation by *Ureaplasma* isolates altered susceptibility to these antibiotics. In agreement with previous studies of *in vitro* susceptibilities to macrolides, the *Ureaplasma* isolates in the current study were more susceptible to azithromycin than erythromycin.³⁸⁻⁴⁰ In contrast to the decreased susceptibility to erythromycin of sessile cells compared to planktonic cells of *Ureaplasma* isolates from adult males,³⁰ there was no detectable difference between erythromycin or azithromycin MIC₅₀ and MBIC₅₀ for the *Ureaplasma* isolates from preterm infants. Since the activity of macrolides *in vitro* is affected by pH of the medium,^{41, 42} as well as inoculum size,⁴¹ the higher MICs observed in our study may be due, in part, to the acidic pH (6.0) of the 10B broth and variable inoculum size of the transferred sessile cells from the peg lids.

Despite *in vitro* susceptibility of *Ureaplasma* to erythromycin and azithromycin,^{14, 43} trials of erythromycin or azithromycin therapy in the first few weeks of life in *Ureaplasma* colonized preterm infants have failed to demonstrate efficacy to prevent BPD,^{18, 19, 24} or eradicate respiratory tract colonization.²¹ The failure to prevent BPD in these studies may have been due to the small sample size of each study, or to the initiation of antibiotic therapy too late to prevent the lung inflammation and injury that contribute to the pathogenesis of BPD. Recently, Walls *et al.*⁴⁴ demonstrated that azithromycin, but not erythromycin prophylaxis, improved outcomes and reduced inflammation in a murine neonatal *Ureaplasma* infection model. This suggests that azithromycin may be effective if administered immediately after birth. An initial single dose pharmacokinetic study of 10 mg/kg azithromycin in infants 24-28 weeks gestation suggested that this dose was well-tolerated, but likely insufficient to maintain azithromycin concentrations above the MIC₅₀ for *Ureaplasma*.⁴⁵ In the current study, none of the isolates as planktonic or sessile cells were resistant to azithromycin, but 10-15% were resistant to erythromycin. Additional clinical pharmacokinetic/pharmacodynamic studies are needed to determine the optimal dosing regimen for azithromycin to eradicate *Ureaplasma* from the respiratory tract and potentially prevent BPD. Biofilm formation may require higher doses and longer course of therapy for effective eradication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Comparison of antibiotic susceptibility of planktonic and sessile cultures of ATCC and clinical isolates. Conventional MIC susceptibility testing with erythromycin and azithromycin were performed for each ATCC (N=5) and clinical (N=43) isolate in quadruplicate by the broth microdilution method and the MBIC determined following biofilm-formation as previously described³⁰. Data are presented as box plots with the bottom, top, and line through the middle of the box corresponding to the 25th percentile, 75th percentile, and 50th percentile (median), respectively. The whiskers extend from the 10th percentile to the 90th percentile.

Erythro, erythromycin, AZI, azithromycin

*p=0.006 vs AZI MIC₅₀ ATCC strains by Wilcoxon sign rank test

Figure 2.

Comparison of antibiotic susceptibility of planktonic and sessile cultures of *U. parvum* and *U. urealyticum* isolates. Conventional MIC susceptibility testing with erythromycin and azithromycin were performed for each *U. parvum* (N=26) and *U. urealyticum* (N=17) isolate in quadruplicate by the broth microdilution method and the MBIC determined following biofilm-formation as previously described³⁰. MIC₅₀ and MBIC₅₀ for erythromycin (Erythro) and azithromycin (AZI). Data are presented as box plots with the bottom, top, and line through the middle of the box corresponding to the 25th percentile, 75th percentile, and 50th percentile (median), respectively. The whiskers extend from the 10th percentile to the 90th percentile.

*p<0.01 compared to *U. parvum* isolate erythromycin MIC₅₀ and MBIC₅₀ by Wilcoxon sign rank test

†p<0.001 compared to azithromycin MIC₅₀ and MBIC₅₀ *U. urealyticum* isolates by Wilcoxon sign rank test

Figure 3.

Comparison of clinical isolates MIC₅₀ and MBIC₅₀ by clinical outcomes. There were no significant differences in antibiotic susceptibility among biofilm-forming isolates from infants with and without BPD/death. Data are presented as box plots with the bottom, top, and line through the middle of the box corresponding to the 25th percentile, 75th percentile, and 50th percentile (median), respectively. The whiskers extend from the 10th percentile to the 90th percentile.