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Appropriate antibiotic therapy improves *Ureaplasma* sepsis outcome in the neonatal mouse

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BACKGROUND: *Ureaplasma* causes sepsis in human neonates. Although erythromycin has been the standard treatment, it is not always effective. No published reports have evaluated *Ureaplasma* sepsis in a neonatal model. We hypothesized that appropriate antibiotic treatment improves *Ureaplasma* sepsis in a neonatal mouse model.

METHODS: Two ATCC strains and two clinical strains of *Ureaplasma* were evaluated *in vitro* for antibiotic minimum inhibitory concentration (MIC). In addition, FVB albino mice pups infected with *Ureaplasma* were randomly assigned to saline, erythromycin, or azithromycin therapy and survival, quantitative blood culture, and growth were evaluated.

RESULTS: MICs ranged from 0.125 to 62.5 µg/ml and 0.25 to 1.0 µg/ml for erythromycin and azithromycin, respectively. The infecting strain and antibiotic selected for treatment appeared to affect survival and bacteremia, but only the infecting strain affected growth. Azithromycin improved survival and bacteremia against each strain, whereas erythromycin was effective against only one of four strains.

CONCLUSION: We have established a neonatal model of *Ureaplasma* sepsis and observed that treatment outcome is related to infecting strain and antibiotic treatment. We speculate that appropriate antibiotic selection and dosing are required for effective treatment of *Ureaplasma* sepsis in neonates, and this model could be used to further evaluate these relationships.

Ureaplasma is the single most common microorganism isolated from the lower respiratory tract of newborn infants (1–3) and causes neonatal sepsis (4–11) and meningitis (10,12–14). The sepsis events have been associated with pulmonary hypertension (7,11), pneumonia (9), increased systemic inflammatory response syndrome (6,10), chronic lung disease (6), and intraventricular hemorrhage (10).

Erythromycin has been the drug of choice to prevent or treat *Ureaplasma* infection in the neonate (15–17). However, it failed to prevent infection in 17 studies (18) and failed as treatment in five studies (19). More recently, several studies have reported on the variable sensitivity of *Ureaplasma* to antibiotics (20,21). A recent Cochrane review suggested that controlled trials are required to determine whether antibiotic therapy of *Ureaplasma* reduces infection or death in preterm infants (18). It would appear logical that such an evaluation should initially begin in animals so that clinical studies could be optimized. However, no one has developed an animal model of *Ureaplasma* sepsis or evaluated antibiotic prevention or treatment in such a model.

It was our objective to determine whether appropriate antibiotic treatment improves *Ureaplasma* sepsis in the neonatal mouse. Specifically, we sought to (i) determine the antibiotic sensitivity of several *Ureaplasma* strains *in vitro*; (ii) develop a *Ureaplasma* suckling mouse model of sepsis; (iii) determine the efficacy of common antibiotics (i.e., erythromycin and azithromycin) in treating *Ureaplasma* sepsis due to various strains using this model; and (iv) determine the relationship between infecting strain and/or antibiotic and outcome of sepsis in this model.

RESULTS

A description of the four selected organisms, including name, source, species, serotype, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC), is given in **Table 1**. MICs ranged from 0.125 to 62.50 and 0.25 to 1.00 µg/ml for erythromycin and azithromycin, respectively. MBCs ranged from 0.25 to 125.00 and 1.00 to 3.90 µg/ml for erythromycin and azithromycin, respectively. For the organisms evaluated, the erythromycin MIC and MBC ranged widely, whereas the range for azithromycin was much narrower.

Figure 1 illustrates the survival percentage of suckling mice after *Ureaplasma* infection by various strains and treatments. The pup survival was significantly dependent on the infecting strain ($P < 0.001$), treatment ($P < 0.001$), and interaction of the infecting strain with treatment ($P = 0.037$). For strains 33697, B140, and B079, the survival rate was significantly increased with azithromycin treatment as compared with erythromycin or saline, whereas erythromycin was not significantly different from saline. For strain 33698, the survival rate was significantly increased with azithromycin or erythromycin as compared with saline, whereas azithromycin was not significantly different from erythromycin.

Figure 2 illustrates the quantitative bacteremia of suckling mice after *Ureaplasma* infection by various strains and treatments. The quantitative bacteremia was significantly dependent on the infecting strain ($P < 0.001$) and treatment

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Table 1. Description of the organisms evaluated

Name	Source	Species	Serotype	Erythromycin (μg/ml)		Azithromycin (μg/ml)	
				MIC	MBC	MIC	MBC
ATCC 33697	Pregnant woman	*Ureaplamsa parvum*	14	0.5	0.5	1.0	2.0
ATCC 33698	Pregnant woman	*Ureplasma urealyticum*	13	0.125	0.25	1.0	3.9
Clinical B140	23-wk placenta	*U. parvum*	6	7.8	15.6	0.25	1.0
Clinical B079	25-wk placenta	*U. parvum*	14	62.5	125	0.25	1.0

MBC, minimum bactericidal concentration; MIC, minimum inhibitory concentration.

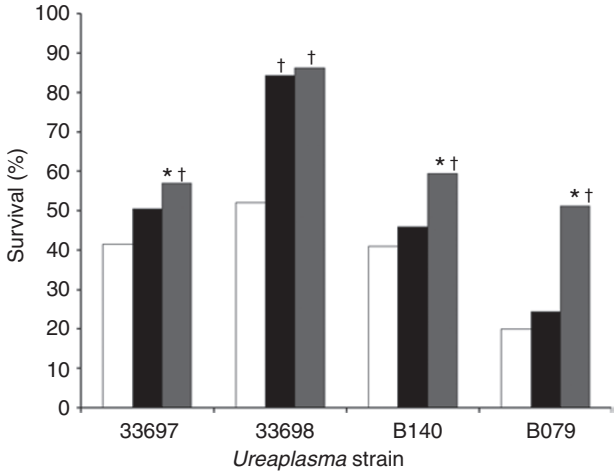

Figure 1. Percentage survival of suckling mice 8 d after *Ureaplasma* infection by various strains. Treatment groups include saline (white), erythromycin (black), and azithromycin (dark gray). For strains 33697, B140, and B079, the survival rate was significantly increased ($P \leq 0.05$) with azithromycin (*) treatment as compared with erythromycin, or azithromycin (†) vs. saline, whereas erythromycin was not significantly different from saline. For strain 33698, the survival rate was significantly increased ($P \leq 0.05$) with azithromycin (†) or erythromycin (†) vs. saline, whereas azithromycin was not significantly different from erythromycin. Each group began with 70 pups. A P value ≤0.05 was considered significant.

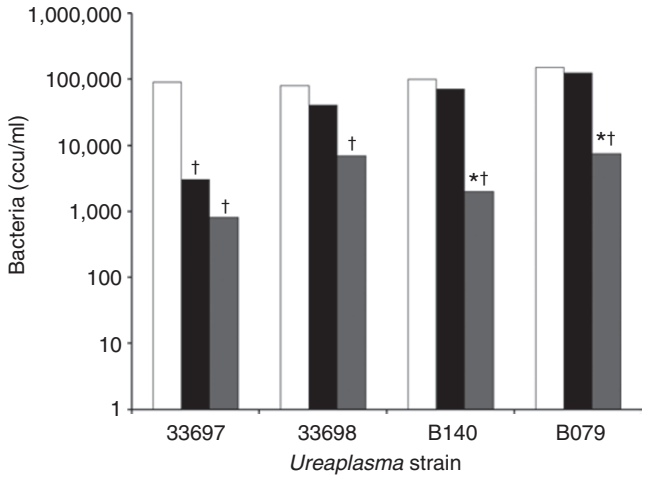

Figure 2. Mean number of bacteria (colony-changing units (ccu)/ml) in the blood of suckling mice 30 h after *Ureaplasma* infection by various strains. Treatment groups include saline (white), erythromycin (black), and azithromycin (dark gray). For strain 33697, the bacterial concentration was significantly decreased ($P \leq 0.05$) with azithromycin (†) or erythromycin (†) vs. saline, whereas azithromycin was not significantly different from erythromycin. For strain 33698, the bacterial concentration was significantly decreased ($P \leq 0.05$) with azithromycin (†) treatment as compared with saline but not erythromycin, and erythromycin was not significantly different from saline. For strains B140 and B079, the bacterial concentrations were significantly decreased ($P \leq 0.05$) with azithromycin (*) treatment as compared with erythromycin, or azithromycin (†) vs. saline, whereas erythromycin was not significantly different from saline. Each group contained 18 pups. A P value ≤0.05 was considered significant.

($P < 0.001$), and suggested an interaction of the infecting strain with treatment ($P = 0.003$). For strain 33697, the bacterial concentration was significantly decreased with azithromycin or erythromycin vs. saline, whereas azithromycin was not significantly different from erythromycin. For strain 33698, the bacterial concentration was significantly decreased with azithromycin treatment as compared with saline but not erythromycin, and erythromycin was not significantly different from saline. For strains B140 and B079, the bacterial concentrations were significantly decreased with azithromycin treatment as compared with erythromycin or azithromycin as compared with saline, whereas erythromycin was not significantly different from saline.

Figure 3 illustrates the rate of growth of suckling mice after *Ureaplasma* infection by various strains and treatments. The pup growth rate after infection did not appear to vary with the infecting strain, but did vary with the treatment group ($P < 0.05$). For strains 33697, 33698, and B 140, the rate of growth was significantly increased with azithromycin treatment as compared with saline but not with erythromycin treatment, and erythromycin was not significantly different from saline. For strain B079, the pup rate of growth was significantly increased with azithromycin treatment as compared with erythromycin or saline, whereas erythromycin was not significantly different from saline.

DISCUSSION

Specialized, but not routine, blood culture methods are required to identify *Ureaplasma* from blood specimens (22). Several studies have identified *Ureaplasma* in neonatal blood cultures as part of sepsis evaluations using specialized techniques, including: two case reports of neonatal sepsis with pulmonary hypertension (7,11); one case report of neonatal sepsis with pneumonia (9); 17.4% of cord blood cultures from 351 preterm infants (6); 18.7% of cord blood from 246 preterm infants (10); 12.6% of neonatal blood cultures from 106 preterm infants (8); and 6% of 221 preterm infants with clinical sepsis (4). Despite the potential for *Ureaplasma* sepsis, blood cultures of the neonate generally do not include investigation

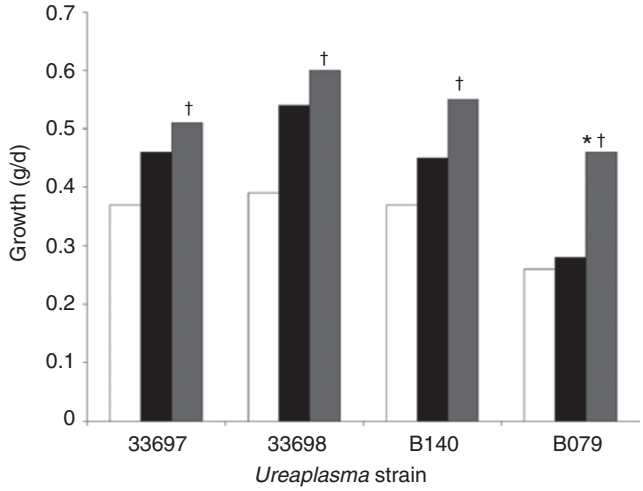

Figure 3. Growth. Mean growth (g/d) of suckling mice over 8 d after *Ureaplasma* infection by various strains. Treatment groups include saline (white), erythromycin (black), and azithromycin (dark gray). For strains 33697, 33698, and B140, the pup rate of growth was significantly increased ($P \leq 0.05$) with azithromycin (†) treatment as compared with saline but not erythromycin, and erythromycin was not significantly different from saline. For strain B079, the pup rate of growth was significantly increased ($P \leq 0.05$) with azithromycin (*) treatment as compared with erythromycin or azithromycin (†) vs. saline, whereas erythromycin was not significantly different from saline. Each group began with 70 pups. A P value ≤0.05 was considered significant.

for *Ureaplasma* as a source of infection. In part, the lack of *Ureaplasma* identification may be due to limited information about this pathogen and/or the significant additional cost of the process for identification of this organism (e.g., specialized culture media, training, staffing, additional equipment, and supplies needed for confirmation). Most laboratories send their few requests for detection of *Ureaplasma* in blood culture to central specialized laboratories, with frequent loss of the organism in transport (22).

Antibiotic treatment for suspected or confirmed *Ureaplasma* infection has generally consisted of erythromycin (15–17); however, this drug has failed to prevent (18) or treat infection (19). Recently, several studies have reported the variable sensitivity of *Ureaplasma* to antibiotics (20,21), and some small studies have found azithromycin (23) or clarithromycin (24) to be effective for treatment of chronic lung disease. A Cochrane review suggests that controlled trials are required to determine whether (and which) antibiotic therapy of *Ureaplasma* reduces infection or death in preterm infants (18).

This is the first study to report an animal model for *Ureaplasma* sepsis. Although there are several animal models for *Ureaplasma*-associated lung disease, only one has been evaluated for bacteremia (21). In that model, blood culture and PCR, following a much smaller infecting dose and several weeks after treatment, were also evaluated. Thus, the organism may have been cleared, the infecting dose may have been too small (50% of the dose used in this study), or the organism undetectable because of its mucosal and intracellular association (21). Our model is less expensive and could be used to evaluate future treatments for this infection.

This study also reveals that *in vitro* and *in vivo Ureaplasma* sensitivity to antibiotics varies with the infecting strain and antibiotic used. As a result, to optimize treatment, clinicians should be aware of the sensitivity of their infecting strain or of those in their community. Initial antibiotic therapy should begin based on local data of *Ureaplasma* antibiotic sensitivities and may need to be adjusted once susceptibility of the specific isolate is available (25). It is now possible to detect antibiotic resistance in *Ureaplasma* within 48 h of a positive culture identifying them for further molecular analysis (26).

The pharmacokinetics of similar doses of azithromycin and erythromycin therapy in mice has been previously reported (21). Whereas serum levels of erythromycin appeared to reflect clinical activity, those of azithromycin did not. It may be that azithromycin efficacy is better reflected with tissue levels due to its bacterial killing and other effects (27) such as downregulation of inflammation, increased mucous clearance, prevention of bacterial biofilm formation, reduced activation of the immune system (cytokines, chemokines, and neutrophils), decreased production of reactive oxygen species, and blocking activation of nuclear transcription factors (28). Macrolide effects are also time- and dose-dependent, although the mechanisms underlying this are not clear. Antibiotic serum levels may not be helpful in the routine treatment of this infection; however, the sensitivity of the organism *in vitro* does appear to be related to outcome in this mouse sepsis model.

We conclude that *Ureaplasma in vitro* sensitivity via MIC and MBC ranged widely for erythromycin and more narrowly for azithromycin. In our neonatal mouse model of *Ureaplasma* sepsis, the infecting strain and antibiotic selected for treatment appeared to affect survival and bacteremia, but only the infecting strain affected growth. We speculate that development of effective strategies to treat *Ureaplasma* infection in the neonate will improve clinical outcomes, and those strategies should include early identification of infected neonates, determination of organism sensitivity *in vitro*, and selection of appropriate antibiotic therapy and dosing.

METHODS

Organisms

We selected four strains of *Ureaplasma*: an ATCC (Manassas, VA) strain 33698 with serotype 13 (*Ureaplasma urealyticum*); an ATCC strain 33697 with serotype 14 (*Ureaplasma parvum*); a clinical strain B140 from a 23-wk placenta with serotype 6 (*U. parvum*); and a clinical strain B079 from a 25-wk placenta with serotype 14 (*U. parvum*). The species and serotypes selected reflected the predominant species and serotypes in the clinical strains from placentas and amniotic fluid of preterm births as reported by others (29–32) and observed by us (unpublished data: Weisman LE, *et al.* E-PAS 2009:3877.412). *Ureaplasma* species was identified utilizing PCR as described previously (33).

Organism Preparation

Strains were obtained from a frozen stock solution (5×10^6 color-changing units (ccu)/ml) diluted 1/100 with 10B broth (Remel, Lenexa, KS), grown 16 h at 37 °C, and spun down at 3,000 rpm for 30 min. Supernatant was poured off and precipitate resuspended in 900 µl of 10B. This suspension was used for intramuscular injection at a final concentration of 5×10^6 ccu/ml. The concentration was confirmed by quantitative culturing of the injectate.

Antibiotics

Erythromycin (USP, Rockville, MD) was selected based on historical data and practice (1,18,34) and administered intraperitoneally at 20 mg/kg/dose twice a day for 3 d (21). Azithromycin (Baxter, Deerfield, IL) was selected based on current and local data (17,21) and administered intraperitoneally at 12 mg/kg/dose once a day for 3 d (21).

MIC and MBC

MIC and MBC were determined using a microdilution method as previously reported (35). In brief, each antibiotic was serially twofold diluted in 10B broth to achieve final well concentrations ranging from 0.03 to 500 μg/ml. The *Ureaplasma* strain was added to achieve a final well concentration of 10^5 ccu/ml. Plates were sealed, incubated aerobically at 37 °C, and examined frequently until there was color change in the antibiotic-free control wells (usually <24 h). MIC was the lowest concentration of drug with no medium color change. From each well of the MIC microtiter plate that did not change color, 30 μl was added to 2.97 ml of 10B broth (1:100 dilution) and incubated at 37 °C for 7 d. The MBC was the lowest concentration of antibiotic in which no color change in the medium was observed. All wells had culture confirmation performed on an A7 agar plate.

Animals

FVB albino mice (Charles River Laboratories, Wilmington, MA) were time impregnated and fed antibiotic-free water and food *ad libitum*, and delivered about 10 pups per dam at 18–20 d gestation. Pups were kept with their delivering dam throughout each experiment. Animals were placed under barrier conditions before infection. Surviving pups were euthanized with injection of 2.25–4.5 ml/kg of 20% Rodent Comboanesthetic III (ketamine 37.6 mg/ml, xylazine 1.92 mg/ml, and acepromazine 0.38 mg/ml). The Baylor College of Medicine Institutional Animal Care and Use Committee approved these experiments.

Sepsis Model

Two-day-old mice pups were infected with a single strain of *Ureaplasma* twice daily for 3 d via intramuscular injection (0.1 ml day 2, 0.2 ml day 3, and 0.3 ml day 4). One hour after initial infection, littermates were randomly assigned to treatment with normal saline, erythromycin, or azithromycin intraperitoneally (refer to Antibiotics section). Pups were evaluated daily for survival and growth for 8 d.

Ureaplasma Blood Cultures

Blood of 100 μl was obtained via cardiac puncture from each pup of selected litters 30 h after treatment was initiated. The culture samples were placed in 900 μl of 10B broth, serially diluted, and incubated at 37 °C. Samples were examined for 2 wks for color change and cultures confirmed on A7 agar (36) and by PCR (21).

Statistical Analysis

Sample size was estimated to be 70 pups per group on the basis of the assumptions that the normal saline-treated survival would be 40% and antibiotic-treated survival would be 65%, with a P value < 0.05 and a power of 0.80. Survival was evaluated by χ^2 or Fisher's exact test as appropriate. Blood culture data were analyzed by Kruskal–Wallis test. Growth curves were constructed for individual pups (even those who died in the study period), slopes calculated, and means of slopes compared for each group by one-way ANOVA. A P value < 0.05 was considered significant. Multiple logistic regression or multiple linear regression was used to determine interaction of variables with outcomes. IBM SPSS Release 19 (International Business Machines, Armonk, NY) and GraphPad Prism Release 5 (GraphPad Software, La Jolla, CA) were used for analysis.

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