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Frequency of *Ureaplasma* Serovars in Respiratory Secretions of Preterm Infants at Risk for Bronchopulmonary Dsyplasia

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Keywords

Ureaplasma parvum; *Ureaplasma urealyticum*; Bronchopulmonary dysplasia; prematurity

INTRODUCTION

Ureaplasma spp. are members of the *Mollicutes* class that colonize human mucosal surfaces of the respiratory and urogenital tracts [1]. *Ureaplasma* has 14 known serovars and is separated into two new species; *U. parvum* (serovars 1, 3, 6, and 14) and *U. urealyticum* (serovars 2, 4, 5, and 7–13) [2]. The characteristics of all serovars include lack of cell walls, limited biosynthetic abilities, small genome size, hydrolysis of urea to generate ATP, and mucosal association in the human host [1]. *Ureaplasma* spp. can be found as commensals in the genital tract of 40–80% of sexually mature asymptomatic women and, hence, they have been considered of low virulence [1, 3]. However, the *Ureaplasma* spp. are associated with multiple pregnancy complications including chorioamnionitis, stillbirth, preterm delivery, neonatal morbidity, and perinatal death [1, 3]. They are the most common perinatally acquired pathogens in preterm infants [3, 4]. Vertical transmission from mothers to their infants occurs either *in utero* or during delivery.

Ureaplasma respiratory tract colonization has been associated with respiratory distress syndrome [5], persistent pulmonary hypertension [6], neonatal pneumonia [7], and the development of bronchopulmonary dysplasia (BPD) [1, 8, 9]. Two meta-analyses confirmed a significant association between *Ureaplasma* respiratory colonization and the development of BPD whether defined as oxygen-dependence at 28 d or at 36 weeks post-menstrual age (PMA) [8, 9]. It has been suggested that infants with a pattern of persistent respiratory colonization/infection are more likely to develop BPD than infants with transient colonization [10]. Due to recent improvements in perinatal care, BPD has become a disease limited to the most immature infants, but it remains the major respiratory morbidity of prematurity [11, 12] occurring in 30% of infants ≤ 30 weeks gestation[11]. Currently, no treatment modality has been shown to prevent or treat *Ureaplasma*-mediated neonatal lung injury.

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There is conflicting evidence concerning whether there are differences in virulence among the *Ureaplasma* serovars. It has been suggested that some *Ureaplasma* serovars have greater association with adverse pregnancy outcomes than others [13–16]. However, there has been no study to date that has investigated the relationship of specific *Ureaplasma* serovars and the development of BPD. The aim of this study was to determine the distribution of *Ureaplasma* serovars in neonatal respiratory secretions by real-time PCR using serovar-specific primers/probes and to assess whether there are predominant serovars associated with BPD in a prospective cohort of preterm infants less than 33 weeks gestation.

METHODS

Study design

The University of Maryland School of Medicine Institutional Review Board approved the study protocol and parental consent was obtained. Eligible subjects were premature infants without congenital anomalies with gestational age <33 weeks admitted to the neonatal intensive care unit <72 h age at the University of Maryland Medical Center from May 2007 to March 2010. During the first sampling period (1–3 d), both a tracheal aspirate and nasopharyngeal wash were obtained from intubated infants and a nasopharyngeal wash only was obtained from non-intubated infants for *Ureaplasma* culture. At subsequent sampling periods (7–10 d and 28–30 d), a single sample for culture was obtained from each infant as a tracheal aspirate from infants who remained intubated or a nasopharyngeal wash from infants who were not intubated. Sterile, preservative-free-saline (0.5 ml) was instilled into the endotracheal tube or nostril and then immediately aspirated back into a suction trap and the process was repeated. The suction catheter was flushed with 2 ml 10B broth [17], and the samples were transported on ice until processed.

Sample collection and PCR

Nasopharyngeal (NP) and/or endotracheal (ET) specimens were vortexed and 0.2 mL of each specimen was added to 1.8 mL of 10B broth prepared as described by Shepard [17]. Serial 10-fold dilutions were made to 10^{-4}. Tubes were incubated at 37°C. If color change occurred, 0.2 ml of inoculum was plated onto A8 agar (Northeast Laboratory, Waterville, ME) and incubated at 37°C in 5% CO_2/air. Tube cultures and plates were examined daily for 1 week for color change and typical colonies of *Ureaplasma* as described previously. A positive culture was defined as a positive broth (color change) confirmed by typical colony morphology on A8 agar. All remaining original samples and positive isolates were aliquoted and stored at − 80°C for later DNA extraction and amplification.

DNA was extracted from tracheal and nasopharyngeal aspirate samples and positive isolates using QiAmp DNA Blood Mini kits (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The DNA was first analyzed by multiplex real-time PCR to differentiate the two *Ureaplasma* species simultaneously as previously described using the Roche LightCycler 2.0 [18]. Serovars of each species were then determined by monoplex real-time PCR assays [18]. Each primer/probe set was confirmed to amplify the designated serovar but none of the other 13 serovars when the optimized PCR conditions previously described were used [18]. All primers were synthesized by Invitrogen (Carlsbad, CA) and probes were manufactured by Roche Diagnostics (Indianapolis, IN).

Clinical outcomes

BPD severity was defined according to NIH criteria [12]. Infants receiving supplemental oxygen at 28 d were assessed at 36 wk PMA 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 ≥ 30% at the assessment time point. Other clinical variables that were recorded included (1) duration of ventilator and oxygen support, (2) duration of hospitalization, (3) incidence of clinical chorioamnionitis [19], (4) occurrence of preterm labor (POL) or preterm premature rupture of membrane (PPROM), and (5) demographic variables such as birth weight, gestational age, sex, and race.

Statistical analysis

Based on BPD rates reported for infants colonized with *U. parvum* (25%) and *U. urealyticum* (50%) by Abele-Horn et al. [14], and an expected 40% respiratory colonization rate, we estimated that 360 infants <33 wks gestation would need to be enrolled to detect a 25% difference in rates of moderate-severe BPD between the species with alpha=0.05 and 80% power. This report contains an interim analysis of the first 136 infants enrolled in this prospective study.

Continuous variables were analyzed by the two-tailed Student t-test or ANOVA if normally distributed or the Mann-Whitney U test or Wilcoxon rank sum test if not normally distributed. The Z-test for 2 proportions was used to determine if differences between two groups were significant. The Chi ^{2} or Fisher exact test was used to compare categorical variables. Univariate odds ratios and 95% confidence intervals were calculated for association of *Ureaplasma* variables (species, respiratory sample source, and colonization pattern) with BPD outcome. A *P*-value of < 0.05 was considered significant. All statistical analyses were performed using Stata 7.0 (Stata Corp., College Station, TX, USA).

RESULTS

Subject and Ureaplasma characteristics

Of 324 infants <33 wks gestation who were eligible for the study, 20 were missed due to lack of parental contact, 168 declined consent and parental consent was obtained for the remaining136 infants. Four hundred twenty aspirates (114 ET (27%) and 306 NP (73%) were analyzed by culture and species-specific PCR (Table 1). There were only 96 subjects available at 28–30 d for sampling due to death (N=2) or discharge/transfer (N=38) prior to this timepoint. Five samples were positive by PCR only. Paired ET/NP aspirates were obtained from 52 subjects (38%). There was significant agreement between ET and NP paired sample culture/PCR results (88.7%, kappa 0.722, p<0.001).

Fifty-one infants (37.5%) had one or more positive *Ureaplasma* NP and/or ET aspirate cultures during the first month of life. Respiratory colonization was inversely related to gestational age (OR = 0.821, CI: 0.720–0.935). Sixty-five percent of infants <26 wk gestation compared with 31% infants ≥ 26 wk were culture/PCR positive (see figure, (Supplemental Digital Content 1, http://links.lww.com/INF/A677) which demonstrates the percentage of *Ureaplasma* culture/PCR positive infants at each gestational age). Thirty-seven culture/PCR positive infants (77%) who had ≥ 2 positive aspirates during this period were classified as persistent colonization, while 11 (23%) who had a single culture positive in the first week of life were classified as transient colonization. Two infants who were *Ureaplasma*-positive at the 28 d only and one infant who was culture-positive on day 7, but not day 1, and expired on day 16, were not assigned to a colonization pattern group.

PCR assays demonstrated that *U. parvum* was the predominant species (N=32, 63%), compared with *U. urealyticum* (N=17, 33%) regardless of sample source or colonization pattern (see figure, (Supplemental Digital Content 2, http://links.lww.com/INF/A678) which demonstrates the distribution of *Ureaplasma* species at each gestational age). Cultures from 2 infants contained mixtures of both species (4%). There were no significant differences in clinical variables between infants colonized with *U. parvum* compared with those colonized

with *U. urealyticum* (table, (Supplemental Digital Content 3, http://links.lww.com/INF/A679) which shows the comparison of clinical variables in subjects with *U. parvum* and *U. urealyticum* respiratory colonization).

Serovar genotyping was performed on at least one bacterial isolate from 45 positive subjects. As shown in Figure 1A, serovars 3 and 6, alone and in combination, accounted for 96% (27/28) of *U. parvum* isolates. Serovar 14 was detected only in an aspirate from one infant as a mixture with serovars 3 and 6. The *U. urealyticum* isolates were commonly a mixture of 2 or more serovars (Figure 1B). Serovar 11 was the most common *U. urealyticum* serovar alone or in combination with other serovars (10/18, 56%). Serovars 4 and 5 were not detected in any sample.

Relationship of Ureaplasma species and serovars to BPD

We analyzed the association of *Ureaplasma* respiratory tract colonization variables with pulmonary outcomes. We did not observe evidence of a difference in BPD rates between the *Ureaplasma* species (Table 2). However, with the current sample size, we are not able to rule out the possibility that true differences exist within the confidence interval range (Table 2). Previously, a pattern of persistent, but not transient respiratory tract colonization was associated with BPD [10]. However, in the present study, persistent colonization during the first month of life alone did not increase the risk for moderate-severe BPD (Table 2). In contrast, the source of the respiratory sample was an important factor. Infants who had been mechanically ventilated for any duration and had a positive tracheal aspirate with or without a paired positive NP sample had a 7.9-fold increased risk (OR = 7.86, CI: 1.31–47) to develop moderate-severe BPD than mechanically ventilated infants with a positive NP sample alone. Moreover, we compared the BPD severity of infants colonized with the different serovars. There were no statistical differences among the *U. parvum* and *U. urealyticum* serovars in BPD severity (Fig. 1), but the sample size was inadequate to detect a difference at the serovar level. The moderate to severe BPD rate for infants colonized with serovar mixtures was similar to the rate for infants colonized with single serovars (Table 2).

DISCUSSION

The ability to differentiate the *Ureaplasma* serovars in clinical samples has long been a challenge for investigators. Previous antibody-based phenotyping methods [20–25] and serologic testing [26] were time-consuming and results were difficult to reproduce and interpret due to multiple cross-reactions and poor ability to discriminate among multiple serovars in clinical samples. More recently, gel-based traditional [2, 27, 28] and real-time PCR [29–32] methods targeting the urease gene, the *mba* gene, or 16s rRNA gene have been developed to discriminate between the two *Ureaplasma* species. Real-time PCR assays yield quantitative results and are more rapid, specific, sensitive, and less subject to contamination than culture and traditional PCR methods. Previous attempts to distinguish all 14 serovars by genotyping have been only partially successful, particularly for discriminating among the 10 *U. urealyticum* serovars. Availability of the genomic sequences for all 14 *Ureaplasma* serovars enabled primers/probes to be designed that are specific for each serovar without any cross-reactions [18]. In this paper, we report for the first time, the frequency of the 14 serovars in respiratory secretions in a prospective cohort of preterm infants at risk for BPD.

Our study confirms previous observations [5, 33] that *U. parvum* is the predominant species colonizing the respiratory tract of preterm infants. In a study of a previous cohort from the University of Maryland, *U. parvum* was the more common species detected in samples from multiple compartments (blood, CSF, and tracheal aspirates) [4]. In contrast, Heggie et al. [34] reported a similar distribution of the two species by species-specific PCR of culture-positive broths from endotracheal aspirates. Differences in study results may be due to differences in

study populations or in selection bias of the DNA source. By performing PCR only on culture-positive broths, Heggie et al. [34] may have missed PCR-positives of culture-negative aspirates and broth medium may have supported differential growth of one or the other of the two species.

It has been debated whether one species is more pathogenic than the other in development of BPD in preterm infants. In agreement with Heggie et al. [34], we did not observe evidence of a difference in the incidence of moderate to severe BPD between infants less than 33 weeks gestation colonized with *U. parvum* than infants colonized with *U. urealyticum*. Katz et al. [33] also found no difference in BPD rates between infants colonized with either species, but a higher rate of BPD in infants positive for both species. A limitation of that study was that the study samples were endotracheal aspirates submitted for culture for clinical indications rather than samples collected prospectively using a defined protocol. Abele-Horne et al. [14] reported that the BPD rate was 2-fold higher for *U. urealyticum*-colonized infants than the rate for *U. parvum*-colonized infants. However, the infants colonized with *U. urealyticum* were significantly less mature and had lower birth weight than *U. parvum*-colonized infants. This may explain, in part, the species difference in BPD rates in their study [14].

Using antibody-based methods, previous investigators sought to ascertain differential pathogenicity of ureaplasmas for adverse pregnancy and neonatal outcomes at the serovar level. Serovar 4 was 4-fold more common in cervical samples from women with recurrent abortions compared with controls [13]. Quinn et al. [35] reported that infants of mothers with histories of pregnancy losses had increased mean antibody titers for serovars 6 and 8, while the mothers had elevated mean titers to serovar 4 and 8. Serologic responses were assessed in preterm infants with respiratory diseases [26]. Compared with infants without lung disease, infants with lung disease had elevated titers to serovars 4, 7, and 8. For serovars 4 and 8, the mean titers were higher in non-survivors than in survivors, while titers to serovar 5 were elevated in survivors. In the current study using genotyping, the most common serovars alone and in combinations with other serovars were *U. parvum* serovars 3 and 6 and *U. urealyticum* serovar 11. Interestingly, serovars 4 and 5 were not detected in any sample. There was no difference in BPD severity among the serovars. This supports the contention that *Ureaplasma* virulence is species and serovar-independent with regards to neonatal lung disease, but this will need to be confirmed in a larger sample.

In summary, using serovar-specific real-time PCR tests, we have provided a description of the distribution of the *Ureaplasma* serovars among respiratory secretions from preterm infants. Many isolates, predominantly *U. urealyticum*, contained multiple serovars. It is unknown whether these isolates represent mixtures of multiple serovars or are hybrids of a single organism expressing markers of multiple serovars as the result of horizontal gene transfer as is now known to occur in *Ureaplasma* spp. (Xiao, L., Paralanov, V., Glass, J.I., Duffy, L.B., Cassell, G.H. Waites, K.B. Extensive horizontal gene transfer in human ureaplasmas questions the utility of serotyping for diagnostic purposes. Abstract, 18th Congress of the International Organization for Mycoplasmology, Chianciano Terme, Italy, July, 2010). There were no differences in BPD rates between infants colonized with multiple serovars compared with infants colonized with a single serovar. These findings suggest that serotyping of ureaplasmas for diagnostic purposes may be of limited value. A search for specific virulence genes that may be common across multiple serovars may identify targets to prevent or ameliorate the adverse effects of *Ureaplasma* infection in the immature lung.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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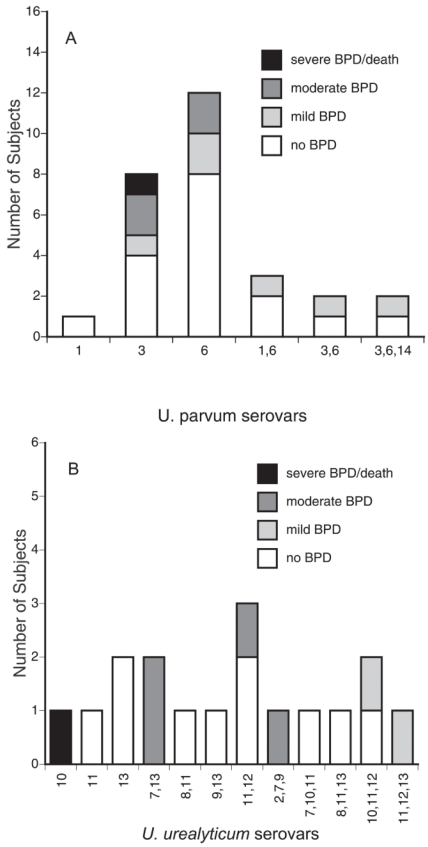

Figure 1.

BPD severity among infants colonized with *U. parvum* (A) and *U. urealyticum* serovars. Serovars were determined by monoplex real-time PCR. BPD severity was defined using NIH criteria [12].

Table 1

Ureaplasma status by respiratory source at each sampling period*

Sampling Period	Source	*Ureaplasma* positive N (%)
Day 1–3	ET (N=52)	15 (29)
	NP (N=136)	30 (22)
	Total (N=188)	45 (24)
Day 7–10	ET (N=38)	10 (26)
	NP (N=98)	28 (29)
	Total (N=136)	38 (28)
Day 28–30	ET (N=24)	5 (21)
	NP (N=72)	14 (19)
	Total (N=96)	19 (20)

* Both ET and NP sample obtained d 1–3 if subject intubated and NP only if not intubated. For other sampling periods, ET samples only from infants who remained intubated and NP samples only from non-intubated infants. Number of subjects sampled per sampling period: day 1–3, 136; day 7–10, 136; day 28–30, 96 due to death (N=2) or discharge/transfer (N=38) prior to 28 d. A sample was classified as *Ureaplasma* positive if 10B broth had color change and confirmed morphology on A8 agar and/or positive PCR.

Table 2

Ureaplasma respiratory tract colonization variables associated with BPD outcome.

Variable	None or mild BPD	Moderate to Severe BPD*	Odds Ratio	P value
Species				
Ureaplasma parvum (N=31)	23 (74)	8 (26)	1.19 (0.39–3.52)	0.760
U. urealyticum (N=17)	13 (76)	4 (24)		
Serovar mixture				
Single serovar (N=23)	19 (83)	4 (17)	1.19 (0.26–5.52)	0.827
Multiple serovar (N=20)	16 (80)	4 (20)		
Aspirate source				
NP (N=33)	30 (91)	3 (9)	7.86† (1.31–47.04)	0.024
ET (N=17)	7 (41)	10 (59)		
Persistence‡				
Transient (N=11)	10 (91)	1 (9)	3.67 (0.41–32.49)	0.243
Persistent (N=37)	26 (70)	11 (30)		

*Moderate to severe BPD was defined as receipt of supplemental oxygen and/or positive pressure support at 36 wk PMA or discharge which ever came first.

†Regression analysis restricted to subjects who were mechanically ventilated for any duration during the first month of life.

‡Respiratory tract colonization was considered persistent if *Ureaplasma* spp. were ≥ 2 positive aspirates during the first month of life.