ENDOTRACHEAL PCR DETECTION OF MICROORGANISMS AND CHRONIC LUNG DISEASE OF PREMATURITY: A PROSPECTIVE COHORT STUDY

by:

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ABSTRACT:

Purpose: Infectious microorganisms are hypothesized to contribute to the pathogenesis of chronic lung disease of prematurity (CLD) in very low birthweight (VLBW) infants. This hypothesis remains controversial. We hypothesized that colonization of the respiratory tree with *Ureaplasma urealyticum (Uu)*, Adenovirus, or *Chlamydia sp.* increases the risk of CLD. Both *Ureaplasma urealyticum* and Adenovirus have been associated with CLD in previous studies. We analyzed endotracheal aspirate samples using PCR, a highly specific and sensitive method.

Methods: Intubated, VLBW (<1500 g) infants admitted to a university-based Level III NICU at <72hrs of age between 1/01/99 and 12/31/02 were studied. *Ureaplasma urealyticum,* Adenovirus, and *Chlamydia sp.* were detected by PCR from endotracheal aspirate samples. Outcome measures included CLD (O2 supplementation at 36 weeks corrected gestational age (CGA), and a combined outcome of death from lung disease or CLD at 36 weeks CGA. The frequency of detection of *Uu,* Adenovirus, and *Chlamydia sp.* was compared between patients with and without CLD using chi-squared and Fisher's exact tests. Logistic regression analysis was used to control for covariates.

Results: 139 patients were enrolled of which 33 (25%) screened positive for *Uu.* Of 136 patients screened for Adenovirus, 22 (16%) were positive; of 133 patients screened for *Chlamydia*, 8 (6%), were positive. At 36 weeks CGA, 18 patients had died or been transferred, and 68 (57%) were O2 dependent. Detection of *Uu* was associated with CLD at 36 wks CGA ($p < 0.001$), Adenovirus ($p=0.52$) and *Chlamydia* ($p= 0.33$) were not. The frequency of CLD at 36 wks CGA was 77% in *Uu* positive, versus 12% in *Uu* negative infants. Controlling for birthweight, gestational age, sepsis, patent ductus arteriosus, days of mechanical ventilation, and the interaction of mechanical ventilation

with gestational age, OR for *Uu* and CLD at 36 weeks CGA was 3.08 (95% CI, 0.78, 11.6). 59% of subjects met the criteria for the outcome of CLD at 36 weeks or death prior to this point due to lung disease. *Ureaplasma urealyticum* was significantly associated with this outcome ($p < .0001$); Adenovirus and Chlamydia were not. Using the same model as that for CLD at 36 weeks, OR for *Uu* and CLD at 36 weeks or death due to lung disease was 4.7(95% CI,1.03, 21.2).

Conclusions: In our population, detection of *Ureaplasma urealyticum* in the respiratory tree of VLBW infants is associated with CLD at 36 weeks CGA or death due to lung disease. Adenovirus was commonly detected in our population, but was not associated with need for supplemental O2. *Chlamydia sp.* were rarely detected, and were not associated with need for supplemental O2.

INTRODUCTION:

Overview:

Bronchopulmonary dysplasia, (BPD), represents an ongoing challenge in neonatal care. BPD is a mixed obstructive/restrictive lung disease that occurs in preterm infants, with infants in the very low birth weight (VLBW, <1500 g) category being at highest risk (1). It significantly increases morbidity and mortality in this vulnerable population (2). Historically, BPD has been attributed to the effects of treatment of infant respiratory distress syndrome (iRDS), particularly oxygen supplementation and mechanical ventilation (3). However, despite routine use of surfactant, gentle ventilation techniques, and judicious use of oxygen therapy, BPD remains a major morbidity for very low birthweight infants. Infants with only minimal exposure to mechanical ventilation are still affected by BPD (4, 5). This suggests other factors are involved in the pathogenesis of BPD. A common pathway may link many of these factors: inflammation, resulting in alveolar developmental abnormalities (2). *Ureaplasma urealyticum* and other infectious agents are currently under investigation as potential contributors to the pathogenesis of BPD.

CENTRAL HYPOTHESIS

Ureaplasma urealyticum is a pathogen of the premature neonate. Colonization of the lower respiratory tract with this organism contributes to the pathogenesis of bronchopulmonary dysplasia, independently of other risk factors.

Maternal colonization with *Ureaplasma urealyticum* is associated with premature birth and chorioamnionitis (6). This organism is transmitted from the mother to the fetus, with premature infants in the very low birthweight (VLBW) category (birthweight

<1500 g) at the highest risk of colonization or infection (7). These infants are also at highest risk for BPD (1).

Many investigators have found an association between neonatal pulmonary colonization with *Ureaplasma urealyticum* and development of BPD (8-17). This association remains controversial, as some studies show no association, and there are design limitations in many studies (18-25). Frequent limitations include: small sample sizes, failure to take degree of prematurity or birthweight into account during statistical analysis, failure to consider other possible pathogens, and varying definitions of BPD.

In addition, polymerase chain reaction (PCR) is a more sensitive method for detection of *Ureaplasma*, with a lower false-negative rate than the clinical cultures used in most of these studies (26-28). PCR has unique features make it an ideal assay for fastidious organisms that can be difficult to culture under routine clinical laboratory conditions, including *Ureaplasma urealyticum*.

Several of the studies that demonstrate an association were also performed before the routine use of surfactant for treatment of iRDS, a treatment now almost universal in this population, and known to decrease the risk of BPD. There is also evidence in the literature that other organisms other than *Ureaplasma*, including Adenovirus and *Chlamydia sp.*, may be associated with BPD. Many previous studies of the association between *Ureaplasma* and BPD did not include screening for these pathogens, raising concerns of confounding.

Ureaplasma urealyticum has been shown to be associated with a systemic and specific pulmonary inflammatory response (29-36), thus suggesting a mechanism for contributing to BPD.

Clarification of the association of *Ureaplasma* and BPD, with a larger sample size, taking all previously studied confounders into account, testing for other pathogens,

and using the most clinically useful definition of BPD will aid in the overall understanding of BPD. Ideally, this study can contribute greatly to the resolution of the controversy surrounding *Ureaplasma urealyticum* and BPD. This will stimulate further investigation, particularly development of long-needed clinical trials of treatment of *Ureaplasma urealyticum* and its effects on incidence of BPD.

***Ureaplasma urealyticum* and BPD: Literature review**

Ureaplasma urealyticum is a common colonizing organism of the female genital tract, with 40-80% of adult women affected (37). Colonization in pregnant women is associated with increased incidence of amnionitis, preterm delivery, and premature rupture of membranes (6). *Ureaplasma* is vertically transmitted to the fetus, with the highest rates of transmission in the smallest, most immature infants (7). These infants are also the population at highest risk of BPD.

Ureaplasma urealyticum has been linked with the development of BPD, which has been defined as typical radiographic changes on chest radiograph, accompanied by persistent oxygen requirement at either 28 days of age or 36 weeks (wks) corrected gestational age (CGA) (8-10, 12-17, 38, 39). *Ureaplasma urealyticum* has also been linked to pneumonia and early death in neonates (40). Autopsy findings show increased interstitial fibrosis in Ureaplasma-infected infants when compared with infants who died of similar causes but were Ureaplasma-negative by PCR (41). A long-term follow-up study of affected infants showed that *Ureaplasma urealyticum* colonization in the neonatal period increases the need for hospital admission in the first year of life, as compared to gestational-age matched controls with a similar incidence of BPD (42).

Despite this evidence, the association remains controversial, as there is also a body of similar studies that did not support an association (18-25). See Appendix A.

Using a Medline search of all publications between 1980 and 2004, using the search terms "ureaplasma", "bronchopulmonary dysplasia", "chronic lung disease of prematurity", "premature infant", and "very low birthweight infant", 23 primary publications of studies which screened patients for *Ureaplasma* colonization and followed them for development of BPD were identified. 22 of these studies were conducted as prospective cohort studies, one was a case-control study. Twelve of these publications demonstrated statistically significant relationships between BPD and *Ureaplasma* colonization, either by univariate analysis in five, with adjustment for potential confounders by multivariate logistic regression analysis in seven. Eleven investigators reported no association between *Ureaplasma* colonization and BPD. The first 13 fully-published publications investigating the relationship of BPD and *Uu* were reviewed in a meta-analysis by Wang in 1995.

Meta-Analysis of studies of *Ureaplasma* and BPD between 1988-1994

A meta-analysis of 17 publications between 1988-1994 supports the association of *Ureaplasma urealyticum* and BPD (39). Wang evaluated the 13 then-existing fully published studies investigating *Uu* and BPD (12 prospective cohort studies, one case-control), and 4 studies presented in abstract form. These 13 fully published studies are included in the 23 studies identified by this investigator as well.

All of these studies defined BPD as a requirement for oxygen at 28 to 30 days of age. All studies used culture methods for the detection of *Ureaplasma*, which were the state of the art at the time. Several of the included studies did use logistic regression to control for covariates (10, 18, 20, 21, 43), but the meta-analysis dealt strictly with the univariate relationship between BPD and *Ureaplasma*.

Wang discussed the population completeness in the included studies. Population completeness was judged to be "good" in 7 studies, defined as inclusion of the majority

of eligible patients. Population completeness was judged "fair" in 2, if the proportion of eligible patients included was not discussed, but the design included an explicit specimen procurement procedure. Two studies were judged to have "poor" completeness, one of which was a case-control study, and the other which depended on the attending physician to select patients from whom samples were obtained. The remaining two studies did not describe population selection at all.

1479 patients were included in these 17 studies. They varied in gestational age between 23 weeks and 36 weeks. The estimate of RR for each individual study was greater than one in all studies, indicating an increased risk of BPD in colonized infants. In seven of the studies, however, the lower limit of the 95% confidence interval was less than one, indicating statistically insignificant results. The pooled estimate of the relative risk for BPD in *Ureaplasma*-colonized infants was 1.75 for all subjects (95% CI 1.53, 1.99). The Breslow-Day test was performed, with no significant heterogeneity of study results demonstrated ($p = 0.09$).

Studies of BPD and *Ureaplasma*, 1995-2004

Many of the studies of *Ureaplasma* and BPD were performed during a time period when the common definition of BPD was an oxygen requirement at 28 days of age, and used this definition as the primary outcome measure (15 of 23 studies). This includes the 13 studies included by Wang in her metanalysis, and two subsequent papers (15, 24). It is now recognized that in the smallest preterm infants, this outcome is not predictive of long-term morbidity, and BPD is now commonly defined as an oxygen requirement at 36 weeks corrected gestational age. The remaining eight studies use this more clinically useful definition of BPD.

Of those eight studies, five used logistic regression to correct for potential confounding by known risk factors for BPD. In several of these studies, the model was

not published, and in one, *Ureaplasma* colonization was not included in the model. All investigators corrected for the effect of gestational age, by far the most sensitive predictor of BPD. Other common confounders considered were birthweight (1 study), patent ductus arteriosus (2 studies), post-natal sepsis (2 studies). It is possible that many more potential confounders were considered in these studies, but the manuscripts mention only these. Three of these five studies find a significant association while using logistic regression to control for confounding, the remaining two show no association. These eight studies, which used the 36 week CGA time-point in the definition of BPD will be discussed in detail.

Studies of BPD defined at 36 weeks CGA and *Ureaplasma*: Positives

Iles and colleagues, in 1996, studied a population of infants born at less than 31 weeks gestation (GA), with median GA of 27 weeks, and mean birthweight of 842 g. They therefore represented a high-risk population for both BPD and *Uu* colonization. 47 infants had endotracheal aspirate samples obtained prior to surfactant administration, and weekly thereafter, until the child was extubated. The selection criteria for these infants, or the size of the underlying population of similar infants they were drawn from was not specified. *Ureaplasma* colonization was determined by standard mycoplasmal culture methods. Thirty-three percent of the patients were positive for *Uu*. There were no differences between the colonized and uncolonized infants with respect to gestational age, birthweight, surfactant use, or days of mechanical ventilation. The univariate OR for *Uu* and BPD at 36 weeks CGA was 8.9, (95% CI of 1.7, 49). The investigators used multivariate logistic regression to adjust for the effects of gestational age and infection, and state that the p value for the association of *Uu* with BPD is 0.02, but no OR was published (13). This study used appropriate culture methods (state of the art at the time), and did compare important risk factors for BPD between the

colonized and uncolonized infants. The use of logistic regression to adjust for gestational age and infection was helpful, but it is unknown if they used any other variables in the process of building their model. The subject selection also seems to have been by convenience, and is not described at all. The sample may be affected by selection bias.

Pacifico and colleagues, in 1997, studied a well-described population of infants weighing less than 1500 g at birth, mean GA was 28 weeks, and mean birthweight was 1000 g. 94 of a possible 110 patients were included. There were specific exclusion criteria stated, and all exclusions fell within these guidelines. Subjects were screened by standard culture methods, and fifty percent were found to be colonized with *Uu*. These babies are slightly older and larger than the highest risk population for BPD, but the high population prevalence of *Uu* increased the study's power. A subset of these patients, who were less than 1000 g at birth, were analyzed to determine if *Uu* colonization was associated with BPD at 36 weeks. They determined that colonized infants were 11 times more likely to develop BPD. Using multivariate logistic regression, they determined that the p value for the association of *Uu* and BPD was 0.0001, after adjustment for at least gestational age and patent ductus arteriosus (14). The specific model was not published, and may have included only these three variables. This study was well-planned and appropriate statistical analysis was chosen. It is unclear, however, why the association of BPD at 36 weeks and *Uu* was modeled for only a subset of the entire study population. This may represent selection bias, with only the highest risk patients chosen for the subset analysis, which would tend to inflate the effect size, although it also increases the study's power to detect a difference in BPD prevalence.

Hannaford and colleagues studied a population of infants born at less than 28 weeks gestation. The infants were small (mean BW 827 g), and of early GA (mean GA 25.8 weeks), representing a high-risk population for both BPD and *Uu* colonization. There were specified inclusion and exclusion criteria, and patients were enrolled consecutively during a specified time period. *Uu* colonization was determined by culture methods, and 27% of the subjects were colonized. Using a multivariate logistic regression model, which included at least gestational age, pre-natal antibiotic use, and *Uu* colonization, the OR for the relationship between *Uu* was 3.0, (95% CI 1.0, 9.1)(16). This study was appropriately carried out and analyzed, although once again, the exact risk factors considered in the logistic regression analysis are unclear. The most important risk factor, gestational age, was included, however.

The three studies, by Hannaford, Pacifico, and Iles, represent 273 patients who are members of a high-risk population for BPD and *Uu*. Their high-risk status increases the studies' power to detect an association between BPD and *Uu,* but does risk inflating the effect size due to selection bias. All three used appropriate statistical measures to adjust the OR for the relationship between BPD and Ureaplasma, although the risk factors adjusted for differ between studies. Population completeness was fair in all three studies, but incomplete discussion of the selection criteria in two of the studies raises additional concern for selection bias. Overall, these three studies make a good case for a true association between BPD at 36 weeks, and Ureaplasma colonization, using culture detection methods. Due to the selection criteria of the studies, this association should only be generalized to infants born at less than 28 weeks, however. The methodology is consistent across the studies, as are the results, and the effect size for all three studies.

Studies of BPD defined at 36 weeks CGA and *Ureaplasma*: Negatives

Da Silva and colleagues studied a cohort of VLBW infants, screened for *Ureaplasma* colonization by polymerase chain reaction (PCR) testing. This study was the first to use PCR methods to investigate the relationship between *Uu* and BPD. The population was well-described, and 108 of 135 eligible patients were enrolled. Mean GA and birthweights were 27.5 weeks, and 1040 g, respectively. The prevalence of *Uu* colonization as detected by PCR was 45%, with contemporaneous culture methods yielding a prevalence of 37%. This finding demonstrates the superior sensitivity of PCR techniques for screening. There were no differences in birthweight, GA, days of ventilation, or days of oxygen use between the colonized and uncolonized infants. The OR for BPD and *Uu* was 1.2 (95% CI 0.8, 1.8)(23). A logistic model was used for risk factors for BPD, but *Uu* was not considered in the analysis, as it was not associated in univariate analysis. This study was well-designed, with appropriate care to avoid selection bias.

Perzigian and colleagues studied 105 VLBW infants in 1998. The selection criteria are clearly defined, although the number of potentially eligible patients admitted to the study NICU during the enrollment period was not reported. Culture methods were used to detect *Uu.* The study was powered to detect a 30% difference in BPD at 28 days, and the OR for *Uu* and this outcome was 1.89 (95% CI, 1.26, 2.83). The OR for BPD at 36 weeks and *Uu* was 1.26 (95% CI 0.57, 2.79)(17). The analysis of this secondary outcome was not planned for in the study design, and the authors state that they have insufficient power to detect a difference in BPD at 36 weeks. This study did not use any statistical correction method to examine risk factors for BPD at either 28 days or 36 weeks CGA.

Courocli et al studied the relationship between *Uu* and BPD using PCR methods in 2000. The study took place over 11 mo, and the population included infants born at

less than 30 weeks gestation. 98 patients were enrolled, but the number of eligible patients admitted during the study time frame were not reported. There is no demographic information reported about these patients, such as mean gestational age or birthweight, or other neonatal outcomes. The authors do state that there were no differences between the BPD and non-BPD groups for GA or birthweight. The OR generated for BPD at 36 weeks and *Uu* was 1.38 (95% CI 0.46, 4.14) in univariate analysis (44). A logistic model was created that included *Uu*, but the OR for BPD and *Uu* in that model was not reported, and is presumably non-significant. This study investigated multiple organisms and BPD, and was the first to report an association between Adenovirus and BPD. Although there is not much reported about the study population, the prevalence of *Uu* colonization (23%) and the prevalence of BPD (53%) are very similar to other studies. If *Uu* increases the risk for BPD preferentially in smaller and younger patients, this cohort may not have represented the correct population. If the mean GA and birthweights of these infants were significantly higher than those reported in other studies, the underlying population may not be the same. These data are unknown, however, so any such statements are speculation.

Ollikainen and colleagues, in 2001, reported a study of a cohort of Finnish infants born at less than 34 weeks gestation. The inclusion criteria are specific, and the proportion of eligible subjects enrolled is clearly stated. 145 infants were studied, and *Uu* was detected using culture methods. The cohort was larger (no infants smaller than 1140 g) and more gestationally mature (no infants less than 28 weeks) than US cohorts. For this reason, this study describes a significantly different population than most of the US studies. The OR for *Uu* and BPD at 36 weeks was 1.23 (95% CI 0.57, 2.64), in univariate analysis (25). No statistical methods were used to examine multiple risk factors for BPD, or to adjust for confounding.

Heggie et al studied a cohort of 175 VLBW infants. The population is well-described, with 43% of eligible patients enrolled. Those not enrolled tended to be intubated for brief periods (representing low-risk patients for BPD and similar to our study), or to have died shortly after birth. The included cohort had mean BW of 914 g, and gestational age of 27.3wks, similar to those in other such cohorts. This cohort is in the high-risk categories for BPD and *Uu* colonization. *Ureaplasma* colonization was determined by culture, and the prevalence was similar to other studies (39%). The prevalence of BPD at 36 weeks was also similar to other published cohorts (59%). These investigators used multivariate logistic regression to adjust for the effects of birthweight, gestational age, sepsis, patent ductus arteriosus and abnormal head ultrasound. In this model, *Uu* was not a significant risk factor for BPD (22). This study was well-designed and executed, and the statistical adjustment methods were appropriate.

_Uu_ and BPD literature review: conclusions

The body of evidence above clearly demonstrates that colonization with *Ureaplasma urealyticum* is associated with an increased prevalence of BPD as defined at 28 days of age. However, more extremely preterm infants are now surviving than when many of these studies were executed, and in these patients, an oxygen requirement at 28 days is the norm. Only about half of those with O2 requirement at 28 days continue to need supplemental O2 at 36 weeks, and it is these babies who suffer long-term morbidity from BPD.

The data regarding *Uu* and BPD at 36 weeks CGA is more heterogeneous. Most of the studies have some question as to population completeness, as the total eligible populations are rarely enumerated. This issue of population completeness affects studies with positive and negative results equally. With the exception of the Finnish

study (25), the cohorts are fairly similar. The proportion of *Uu* colonized infants, and the prevalence of BPD is reasonably similar across the 7 remaining studies. The infants included in the negative studies may be less gestationally mature and smaller at birth than those in the positive studies, but there is considerable overlap in the standard deviations for these measurements across studies. The gestational age and birthweight data for the cohort studied by Couroucli is also unknown, making it difficult to determine if this cohort is a part of the same underlying population as the other investigators' cohorts. Essentially, half of the studies show an association, and half do not. The study methods are similar in both the positive and negative studies, and there is no clear superiority of methodology in either group. There are some potential explanations for these differences, however, which will be explored below.

Factors potentially contributing to the disagreement in the literature:

***Ureaplasma urealyticum* and PCR**

There may be several factors contributing to the disagreement in the literature. The character of *Ureaplasma urealyticum* itself may play a part. *Ureaplasma urealyticum* is one of the smallest known microorganisms, and is difficult to culture in clinical situations. *Ureaplasma urealyticum* is a member of the Mycoplasmacetae family, which comprises some of the smallest, simplest microorganisms. With their small genomes and lack of a cell wall, these bacteria rely on the host organism for vital nutrients and have limited viability outside of the host cell (Gilroy and Taylor-Robinson) making them somewhat challenging to grow in culture. Due to the small colony size, plates must be examined under a microscope to identify the organism. Studies have shown that even with the gold standard of liquid and solid media cultivation, detection rates can be as low as 80% in typical laboratory conditions (45). Studies relying on a

single clinical culture specimen for identification may not reliably detect its presence (13, 28).

Studies involving various clinical specimens, including neonatal endotracheal aspirates, have shown PCR to have superior sensitivity when compared to conventional culture methods (46), (27), (26). A study comparing detection rates of *Ureaplasma urealyticum* in simulataneously obtained endotracheal aspirate samples from VLBW infants was undertaken in our laboratory in 1996. This served as part of the foundation for the current study. We studied a cohort of 26 infants with birthweights <1250gm who had endotracheal aspirate samples obtained and tested for the presence of *Ureaplasma urealyticum* by both PCR and culture. 50% of patients were positive for *Ureaplasma urealyticum* by PCR, and forty percent of PCR positives had negative culture results. The PCR positive patients had a significantly higher incidence of death due to lung disease and supplemental oxygen requirement at 36 weeks corrected gestational age, a clinically useful definition of BPD. The incidence of BPD or death was 85% in PCR positive patients versus 38% in PCR negative patients. If culture methods alone had been used, 40% of the positive patients would have been misclassified as negative, thereby masking a true association (28). Prior investigations using culture methods may have also been affected by misclassification in this fashion, which may have contributed to confusion about the pathogenicity of *Ureaplasma urealyticum* in the premature infant. Additional advantages of PCR analysis over culture techniques exist. Unlike culture techniques, fastidious handling of specimens is not necessary, as organisms do not have to be viable to be detected. PCR can also detect much lower organism counts than culture techniques. These differences could be expected to result in fewer false negatives when the PCR method is employed, and likely explain the superior sensitivity of PCR demonstrated by several investigators.

Ureaplasma population prevalence

In addition, prevalence of colonization within a population can affect study results. Maternal colonization prevalence reported in the literature ranges from 30% to 90% and infant colonization varies from 13% to 50% (37). If the population prevalence is low, a larger sample size is needed to prevent a type II error. Some of the previously published studies with sample sizes limited to 80-100 total patients in populations with relatively low prevalence of colonization (<25%) may be affected by this problem.

Other Microorganisms and BPD

Many studies also fail to take into account other possible pathogens implicated in the development of BPD. In 2000, Couroucli and colleagues published the first report of an association between endotracheal colonization with Adenovirus species and BPD in premature infants (44). No previous *Ureaplasma urealyticum* studies in the surfactant era included screening for viral pathogens. Subsequent investigations have failed to confirm this result. (47). Studies of inflammatory mediators in the endotracheal secretions or systemic circulation of Adenovirus colonized infants have not been published to date.

Chlamydia trachomatis is a pathogen of the female genitourinary tract that has long been investigated in conjunction with BPD. It has been serologically linked to BPD in older studies, prior to routine maternal screening and treatment (48). However, culture based studies performed in the routine maternal screening era yield low numbers of colonized infants, and no association is demonstrated (12, 23). However, as these organisms have been linked with BPD, a well-designed study should include screening for their presence as well.

Choice of sample location

Colonization of the deep pulmonary tree and the subsequent inflammatory response is the hypothesized mechanism for *Ureaplasma urealyticum's* contribution to BPD (29-36). Therefore, endotracheal specimens have been considered the optimal study material. Many previous studies used nasopharyngeal aspirates, gastric aspirates, or surface skin swabs to screen for colonization. Cassel and colleagues demonstrated poor correlation between simulataneously collected endotracheal and nasopharyngeal aspirate samples (37). As it is pulmonary injury that is the concern, recovery of the organism from the pulmonary tree is the most accurate measure of true lung colonization.

Study Rationale

We proposed to use the experience of previous investigators and address many of the proposed problems from previous studies. We undertook a prospective cohort study of patients in the highest risk group for both *Ureaplasma urealyticum* colonization and BPD. Using the experience of previous investigators, we planned to measure and control for confounding in the most rigorous manner possible, to maximize the usefulness of our results.

Delineating any association of *Ureaplasma urealyticum* and BPD with a larger study using sensitive detection methods and taking into account all previous reported risk factors will help resolve the existing controversy, opening the way for future investigations. If the association of *Ureaplasma urealyticum* and BPD is substantiated, clinical studies regarding its treatment should be undertaken.

Hypothesis

We hypothesized that detection of infectious microorganisms in the endotracheal secretions of newborn VLBW infants is associated with increased incidence of BPD, independent of other known risk factors. We prospectively studied VLBW infants

admitted to our University based Level III NICU over four years, collecting endotracheal aspirates within the first 72 hrs of life, and screening them for *Ureaplasma urealyticum*, Adenovirus, and *Chlamydia sp.* Extensive clinical data was also collected, to control for confounding factors.

METHODS:

Study Design, Inclusion/Exclusion Criteria:

This study was designed as a prospective cohort study of the contribution of endotracheal colonization with infectious microorganisms to the development of BPD in VLBW infants. The Institutional Review Board of Oregon Health & Science University approved the study protocol, and a waiver of patient consent was granted, as the study samples were deemed medical waste. This waiver of consent was sought to enhance study enrollment.

All VLBW infants, inborn or admitted by transport to OHSU hospital at less than 72 hours of age who required intubation and mechanical ventilation were eligible for study entry. Patients were excluded from study entry if they had congenital anomalies, congenital heart disease, or chromosomal anomalies. Endotracheal aspirates were obtained within the first 72 hours of life. These aspirates were obtained as a part of routine pulmonary toilet, in compliance with our IRB waiver of consent. Bedside nursing or respiratory care providers obtained the samples. Specimens were screened for *Ureaplasma urealyticum*, Adenovirus, and *Chlamydia sp.*, by PCR. Clinical data regarding known risk factors for chronic lung disease was collected, and patients were followed for the development of chronic lung disease, the outcome of interest.

Outcome Definitions

We utilized two definitions of BPD commonly reported in the existing literature: need for supplemental O2 at 28 days of age; and need for supplemental O2 at 36

weeks corrected gestational age (CGA). Infants were deemed to meet these definitions if they had a room air SpO2 of <88% at these time points. This was determined by analysis of the nursing flow chart document for the day in question (28 days of age, or CGA 36 0/7 weeks), and noting whether or not the patient was receiving oxygen supplementation on that day.

In addition, a combined outcome of death due to lung disease or BPD at 36 weeks CGA was modeled, as is increasingly common in the literature. The hospital charts of all patients who died prior to 36 weeks CGA were examined to determine cause of death, which was abstracted from the attending neonatologist's death note. Causes of death including respiratory failure, pneumothorax, pulmonary hemorrhage, iRDS, and pulmonary hypertension were included in the death due to lung disease group. These patients were added to the survivors with BPD at 36 weeks CGA to form the combined outcome group.

Clinical Data Collection

Data concerning respiratory hospital course, co-morbid conditions, and preexisting demographic risks for BPD were collected as well, for analysis as potential confounding variables.

Clinical data was abstracted from hospital charts after the patient's discharge from the hospital. Data collected included microbiologic variables (incidence of detection of *Uu, Chlamydia,* or Adenovirus sp.); as well as a variety of potential confounding variables.

Potential confounding variables included:

1. Respiratory variables: days of mechanical ventilation, use of high frequency ventilation, highest PIP used, highest FiO2 used;

2. Demographic variables: gender, gestational age (determined by first trimester ultrasound or last menstrual period), birthweight.
3. Prenatal variables: length of ruptured membranes, use of prenatal steroids for prevention of BPD, use of prenatal antibiotics, maternal diagnosis of chorioamnionitis;
4. Neonatal Morbidities: postnatal sepsis, patent ductus arteriousus (PDA) requiring treatment, respiratory distress syndrome (RDS) requiring surfactant use, necrotizing enterocolitis, retinopathy of prematurity (ROP) requiring surgical therapy, intraventricular hemorrhage, length of hospital stay.

Please see Appendix C for a complete list of variables ascertained for this study and their specific definitions.

The variables chosen represent a comprehensive list of potential contributors to BPD, and were selected based on a review of existing literature regarding the causation of BPD. One investigator (TC) recorded all data at chart review and entered these clinical data into an MS Access database.

To keep study personnel blinded to study outcome and measurement of *Ureaplasma,* Adenovirus, and *Chalmydia sp.*, samples were coded anonymously, such that it was impossible to determine patient identity during PCR analysis. Samples were stored for 2-6 months prior to processing, so that no research data could be transmitted to the clinical care team and thus impact management. Lab results were initially maintained in a notebook and MS Access database, by code. Only after all of the clinical data were collected were the laboratory results decoded and added to the clinical database.

Sample Collection

Endotracheal aspirates were obtained in sterile fashion using in-line suction sets and sterile suction traps (Busse Hospital Disposable, Hauppauge, NY). Up to one ml of sterile, non-bacteriostatic saline was instilled into the trachea, with a resultant 0.5 to 1.0 ml obtained. Bedside nursing and respiratory care providers obtained the samples. In compliance with our waiver of consent, there was no specified protocol for sample collection, hence the variation in the amount of sterile saline used. Samples were stored at -80°C until DNA was isolated. Samples were coded such that the identity of the patient would be unknown during sample processing and PCR analysis. The laboratory results were entered into the clinical MS Access database described above.

Sample Preparation

300 μl of endotracheal/nasopharyngeal aspirate sample was centrifuged at 14,000 x g for 20 min. The pellet obtained was incubated at 60°C for 60 min with 50 μl of Solution A (10 mM Tris HCl, pH 8.3, 100 mM KCl, and 2.5 mM $MgCl_2$), and 100 μl of Solution B (1% Tween 20, 10 mM Tris HCl, 1% Triton X, 2.5 mM $MgCl_2$, and 120 μg/ml proteinase K). It was then heated at 95°C for 10 min to inactivate the proteinase K and quenched on ice and stored at -20°C until PCR analysis.

Primers

Primers used for detection of *Ureaplasma, Chlamydia*, and Adenovirus were synthesized and purified by Invitrogen, Carslbad, CA. A summary of the primer sequences, source genes, and initial publication is found in **Table 1**.

Ureaplasma PCR

Primers designed and validated in neonatal endotracheal aspirate samples by Blanchard and colleagues (49) were used to amplify a 429bp fragment of the urease

gene common to all serotypes of *Ureaplasma urealyticum*. The Ampliwax (Perkin-Elmer ABI) mediated hot-start PCR technique was used: PCR was performed in a 50 μl reaction mix consisting of upper and lower suspensions. The lower suspension contains 2.5 μl of each primer (U4 and U5, 10μmol), 1μl dNTP, and ddH20 to make 20 μl volume. A 50 μl Ampliwax gem was added and the tube heated to 72°C until the gem has melted. The upper suspension was placed into the tube above the wax, and contained: 10X PCR Buffer (5 μl), 25 mM $MgCl_2$ (6 μl), 1U Taq polymerase, ddH20 to make 25 μl volume. Last, and in a different location from where the rest of the PCR reaction mixes are assembled to minimize potential contamination of reagents by PCR products, 5 μl of experimental sample was added. Appropriate positive and negative controls were used with each experiment. A GenAmp 2400 DNA thermal cycler (Perkin-Elmer-Cetus) was used with the following PCR protocol: 40 cycles of 20 s at 95°C for denaturation, 60 s at 62°C for primer annealing, and 30 s at 72°C for extension.

Pathogen Screening for Chlamydia and Adenovirus

Chlamydia trachomatis and viruses such as Adenovirus have also been associated with BPD, and both are amenable to PCR detection (44). The patient samples prepared as above were used. The Ampliwax hot-start PCR technique described above for *Ureaplasma urealyticum* was used, substituting appropriate primers, and adjusting PCR protocols as noted below.

A 330bp fragment of Adenovirus was amplified using nested PCR as described by Courourcli et al (44). The Ampliwax hot-start PCR technique was used as above, with the ADH-01 and ADH-02 primers. A GenAmp 2400 DNA thermal cycler was used (Perkin-Elmer-Cetus). The protocol for the primary reaction was: after 5 min of

incubation at 94°C, 40 cycles of 45 s at 94°C, 45 s at 64°C, and 45 s at 72°C. 2 μl of the primary PCR product was used as the sample DNA for the secondary PCR reaction, which used the ADH-11 and ADH-22 primers: The reactions were otherwise as described for the *Ureaplasma urealyticum* PCR, and the amplification protocol is identical to the primary reaction.

A 609bp fragment of *Chlamydia (trachomatis, psittaci, pneumoniae)* (16S ribosomal gene) was amplified with a single PCR reaction. The PCR protocol used was: 5 minute incubation at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 64°C, 45 s at 72°C.

Positive and Negative Controls

Known samples of *Ureaplasma urealyticum, Chlamydia trachomatis,* and Adenovirus were used as positive controls for all PCR reactions. Negative controls for all PCR reactions consisted of pH 7.5 TE buffer instead of nucleic acid in the reaction mix. Each sample was tested at least twice to ensure reliable results.

Analysis of PCR Products

Amplified products were analyzed by electrophoresis through a 2% SeaKem LE agarose gel (FMC) at 60 volts for 3 hours, stained with 0.5 μg/ml ethidium bromide (Sigma Chemical Co., St. Louis, MO), and visualized with UV light. Permanent record of the gels was made by photography using a Polaroid MP-4 camera and type 667 film.

Data Analysis

Appropriate analyses, including Chi-Squared, Fisher's Exact Test, Student's T-test, and simple linear regression were used to identify variables which were associated with the development of BPD in subjects. Variables found to be significantly associated with the development of BPD ($p < 0.25$) were used to build two logistic regression models with incidence of BPD at 36 weeks, and the combined outcome of BPD at 36

weeks CGA or death due to lung disease as the dependent variables. All initial variables of interest ($p < 0.25$) were initially entered, and removed and reassessed using methods described by Hosmer and Lemeshow (50). The models were built completely separately, although the final models were identical for the two outcomes.

After an appropriate main-effects models were thus identified, interaction terms were added using backward stepwise logistic regression, candidate interaction terms were chosen based on likelihood of clinical relevance. For the outcomes of BPD at 36 weeks CGA, and BPD at 36 weeks or death, the interaction of estimated gestational age and days of mechanical ventilation was significant. Given that the importance of days of ventilation differ for different levels of gestational age, contrast tests were performed, fixing EGA at various levels and assessing the impact of ventilator days at these points. Upon examination of the distribution of EGA within the study population, contrasts were performed for 25, 26, and 28 weeks gestation. These contrasts were performed to better understand the relationship between these variables at various gestational ages, and to aid in interpreting the models in clinically useful ways.

The outcome of BPD at 28 days of age was not modeled in the same way, as the majority (83%), of our patients had BPD at this time point. Due to the small number of patients who did not meet the definition of BPD at 28 days, estimates from logistic regression models would be mathematically unstable and not helpful in understanding contributing factors to oxygen use at 28 days. In addition, this definition of BPD is falling out of favor in the literature, as oxygen dependency at 36 weeks is far more predictive of true long-term pulmonary morbidity in the VLBW population.

Instead, differences between subpopulations of the infants who were oxygen dependent at 28 days were explored. An attempt was made to determine the differences between the infants who went on to meet the criteria for BPD at 36 weeks

CGA, and those whose oxygen requirement resolved prior to that time point. Analysis was performed with SPSS 11.0 for Macintosh, and the SAS package, version 8.0, for Windows.

RESULTS:

139 VLBW infants born between January 1, 1998 and December 31, 2002 who required intubation within 72 hrs of birth and were free of major congenital anomalies or chromosomal disorders were enrolled. This sample included 79% of all eligible patients.

The average gestational age of the subjects was 26.8 +/- 1.88 weeks, and average birth weight was 941 +/- 257 grams. The cohort was 59% male. 72% of mothers received IV antibiotics prior to delivery, and 80% received at least one dose of antenatal betamethasone. 93% of the infants received surfactant, 45% required high frequency ventilation, and the mean number of days on the ventilator was 21 +/- 23. The mean number of days on oxygen was 58 +/-37, and mean hospital stay was 83 days. 14 patients died prior to 36 weeks corrected gestational age, and 4 were transferred to other institutions prior to this time point. **(Table 2)**

126 patients were still inpatients at our institution at 28 days of life, 13 having either died (10) or been transferred to other institutions (3). At 36 weeks CGA, 121 patients were still in the cohort, 14 having died, and 4 who were transferred to other institutions prior to that time. The transferred patients included two with BPD at 28 days, who were transferred prior to 36 weeks CGA, one of whom was positive for *Ureaplasma*. The other two patients were weaned from oxygen at less than a week of age, making a diagnosis of BPD at 28 days or 36 weeks unlikely. One of the

transferred patients who were weaned from oxygen at four days of age was positive for adenovirus. The proportions of transferred infants with *Ureaplasma* (25%), and Adenovirus (25%) was similar to those retained in the cohort.

At 28 days of age, 83% of infants were oxygen dependant (104/126) thus meeting one definition of BPD. At 36 weeks corrected gestational age 57% of the population required oxygen (69/121), and thus were diagnosed with BPD. Fifty-nine percent of the patients had either been diagnosed with BPD at 36wks CGA, or had died from lung disease prior to that age (76/130).

Twenty-four percent of (34/139) patients were positive for *Ureaplasma urealyticum* by PCR, and16% (22/136) were positive for Adenovirus. These prevalence numbers are consistent with previously published values (22, 23, 44, 47). *Chlamydia sp.* was more rarely detected; 6% (8/133) of patients had positive results. **(Table 2)**

Completeness of the population

OHSU participates in the Vermont-Oxford Network (VO), a group of over 500 NICUs which collects a uniform body of data about very low birthweight infants. OHSU-specific Vermont-Oxford data are available from 1999 to the present. Using this database, for the years 1999-2002, there were 207 patients born at less than 1500 g admitted to OHSU who were mechanically ventilated. 174 had endotracheal aspirates obtained for study. 35 patients were excluded from study per the previously stated criteria. Therefore, samples were obtained in 79% (174/207) of potentially eligible patients.

BPD at 28 days:

83% of surviving infants in our cohort were oxygen dependent at 28 days of age. Infants who required oxygen supplementation at 28 days of age were less gestationally mature and smaller than those who did not ($p = <0.001$ for both variables). They were

more likely to be colonized with *Ureaplasma urealyticum*, 29% vs. 4% (p = 0.012), and to have required high-frequency ventilation 49% vs. 25%, a rescue modality in our institution (p = 0.042). They also were more likely to have suffered post-natal sepsis 41% vs. 19%, although this result is not statistically significant (p = 0.067); and to have been diagnosed with stage 3 or 4 ROP 30% vs. 5% (p = 0.023). Interestingly, Adenovirus colonization was more common among infants who did not require oxygen at 28 days 25% vs. 12%, although this result is not statistically significant (p = 0.091). Chlamydia was detected in only 6 patients, all of whom were oxygen dependent at 28 days. (Table 4)

Differences between subpopulations of the infants who were oxygen dependent at 28 days were explored, using logistic regression modeling, with BPD at 36 weeks CGA as the dependent variable. Only the patients with BPD diagnosed at 28 days were included. Comparisons were made between the infants who went on to meet the criteria for BPD at 36 weeks CGA, and those whose oxygen requirement resolved prior to that time point. In a logistic model including EGA, birthweight, days of mechanical ventilation, patent ductus arteriosus (PDA), sepsis, and *Ureaplasma* colonization status, days of ventilation and PDA emerged as significant predictors. Infants requiring oxygen at 28 days who were treated for PDA had 3-fold increase in risk for BPD at 36 weeks (OR 3.1, 95% CI 1.1, 8.8). For each day of mechanical ventilation, risk of BPD at 36 weeks increased 5% (OR 1.05, 95% CI 1.0, 1.1).

BPD at 36 weeks CGA:

Individually, several factors emerged as significant predictors of BPD at 36 weeks CGA. As expected, smaller (874g vs 1070g, p = <0.001), and less gestationally mature (26.3 vs. 27.7 wks, p = <0.001) infants were more likely to develop BPD, as were those who required longer courses of mechanical ventilation. Mean days of ventilation was 30 days in the BPD group versus 10 in the unaffected group. Each day of mechanical ventilation increased the risk of BPD 8% (OR 1.08, 95% CI 1.04, 1.12). These infants were also more likely to require high frequency ventilation (57% vs. 26%, p = 0.001), and to be treated for PDA (77% vs. 53%, p = 0.01). Sepsis was more prevalent in patients with BPD at 36 weeks, 44% of patients in the BPD group had at least one episode of sepsis, while 27% of the unaffected group did. This result shows only a trend toward statistical significance (p = 0.07). BPD affected infants also had significantly higher incidence of stage 3 or 4 ROP (36% vs. 8%, p = <0.001). *Ureaplasma* colonization was significantly associated with BPD at 36 weeks (37% vs. 8%, p = <0.001), and Adenovirus (16% vs 12%, p = 0.52) and *Chlamydia* (9% vs. 4%, p = 0.33) colonization were not. **(Table 5)**

The logistic model created for the 36 weeks CGA outcome point included EGA, PDA, sepsis, *Ureaplasma* colonization, days of ventilation, and the interaction of days of ventilation and sepsis. Significant predictors of BPD at 36 weeks CGA included the interaction of days of ventilation with estimated gestational age. The OR for these gestational age points are found in Table 6. Days of ventilation were increasingly important as risk factors for BPD as babies became more gestationally mature. The OR for each additional ventilator day at 25 weeks was 1.05, indicating that each day on the ventilator increased risk by 5%. At 26 weeks, the risk was 8% per day, and at 28 weeks, 14% per day. The Hosmer and Lemeshow test of goodness of fit demonstrated

that this model did not have significant lack of fit. Residual plots were generated that showed no pattern in the residuals. **(Table 6, appendix D)**

BPD at 36 weeks or death due to lung disease:

Individual predictors of BPD or death due to lung disease were very similar to those for BPD at 36 weeks CGA. **(Table 7)**

The model for the combined outcome of BPD at 36 weeks corrected gestational age or death from lung disease was identical to that for BPD at 36 weeks, although it was arrived it via an independent model-building process. Significant predictors of BPD using this outcome definition include the interaction of ventilation with gestational age, and *Ureaplasma urealyticum* colonization. The OR for *Ureaplasma urealyticum* colonization and BPD at 36 weeks CGA or death due to lung disease was 4.2 (95% CI 1.03, 17), indicating a four-fold increase in risk of BPD or death in *Uu* colonized infants, after statistical adjustment for the effects of PDA, sepsis, EGA, and days of ventilation. Contrasts were performed to generate OR for days of ventilation at various EGA points, in a manner identical to that described above. As seen above, ventilation became increasingly important as gestational age at birth increased. At 25 weeks, the risk was 2% elevated per day, 5% per day at 26 weeks, and 12% per day at 28 weeks. The Hosmer and Lemeshow test of goodness of fit demonstrated that this model did not have significant lack of fit. Residual plots were generated that showed no pattern in the residuals. **(Table 8)** See appendix C for logistic regression table and residuals plots.

Discussion:

In our population, endotracheal colonization with *Ureaplasma urealyticum* emerged as an important predictor for the combined outcome of BPD or death due to lung disease. *Uu* colonization was a significant predictor of the combined outcome of

BPD at 36 weeks or death from lung disease after statistical adjustment for the effects of gestational age, prevalence of PDA, prevalence of sepsis, and days of ventilation. Over 20 candidate variables were investigated as potential confounders of the association between *Ureaplasma urealyticum* during the statistical analysis. Our study is the first using PCR methods to report this result with a statistical correction for time of mechanical ventilation, the most important known risk factor for BPD other than gestational age. By using the most currently useful definition of BPD, a very sensitive detection method, and adjusting for the several most significant risk factors for BPD, this study improves upon the previous literature.

Inflammation, *Ureaplasma*, and BPD

The most likely explanation for our finding is the effect of systemic and pulmonary inflammation that is demonstrated with *Ureaplasma urealyticum* colonization/infection. Infants who develop BPD, regardless of colonization, show signs of inflammation absent in those who do not (1, 2).

Ureaplasma urealyticum causes a variety of inflammatory responses in preterm neonates. Inflammatory cytokines have been shown to be present in the endotracheal secretions of colonized infants at significantly higher levels than in uncolonized controls. Groneck and Speer demonstrated higher levels of IL-1 and Leukotriene B-4 in the endotracheal secretions of *Uu* colonized VLBW infants on day of life one, suggesting prenatal exposure (31). Ratios of IL-1β to IL-6 and TNF-α were shown to be 7-18 times higher in *Uu* colonized infants on days 1 and 7 of life, and ratios increased at the later time points.

Evidence of a systemic inflammatory response to *Uu* outside the lungs also exists. Cord blood inflammatory cytokine levels have been shown to be elevated in *Uu*

colonized infants (51). Multiple investigators have found elevated peripheral white blood cell counts in *Uu* colonized infants as compared with similar uncolonized infants (29, 30, 52). Given the evidence that *Uu* does cause both pulmonary and systemic inflammation, it follows that this inflammation can predispose infants to BPD, as does inflammation from other causes.

Chorioamnionitis has been shown to produce an inflammatory response in the neonate. *Ureaplasma urealyticum* is the most common single organism cultured in cases of chorioamnionitis in very preterm deliveries (53, 54). A fetal systemic inflammatory response has been demonstrated in VLBW infants exposed to choriomanionitis prenatally (53). Chorioamnionitis has been shown to be linked to the development of BPD in epidemiologic studies (55).

BPD and *Ureaplasma*

The relationship between BPD and *Ureaplasma urealyticum* has been a topic of investigation for over 20 years. From the data reviewed in the introduction, it seems that a connection is likely, however, epidemiologic studies of the association find conflicting results. Most of the literature published in the pre-surfactant era clearly demonstrated an association; a meta-analysis of 18 publications between 1988 and 1994 demonstrated an increased risk of BPD in colonized infants (OR 1.72) (39).

Since surfactant came into common usage, the data conflict. Four studies in infants with mean birthweights of 827 – 1000 g demonstrated OR of 2.0 to 3.8. (13, 15-17) yet in three studies in larger infants (975-1300 g) there was no association between *Uu* and BPD (22, 25, 44). This ambiguity may be related to the difference in the size of the infants, as the smaller infants are at much larger risk of BPD to begin with. A population with a lower prevalence of BPD would require a higher sample size to detect any effect of colonization. Six of these seven studies used culture methods, one used

PCR to detect *Uu*. Our study used PCR, a very sensitive detection method, to screen for organism colonization, and our population had a high prevalence BPD. This increases our potential to detect differences in BPD prevalence between colonized and uncolonized infants.

Ureaplasma detection methods

Another potential explanation for the controversy in the literature may be related to the modality used for detection of *Uu*. Culture studies rely on live organisms, and *Uu* is a fastidious organism, which can be difficult to grow. Studies have shown that relying on a single clinical culture specimen for classification misclassifies up to 40% of positive patients as negative (23, 27, 52, 56). Surfactant has also been demonstrated to inactivate *Ureaplasma urealyticum* in vitro, although this has not been studied in infants (Walsh et al; 1994) If this is true in infants as well, culture detection after surfactant administration could be impaired, but PCR methods of detection would not be affected. PCR methods are more sensitive than culture, and can detect low numbers of organisms, and dead organisms (26, 27, 46). Our study, by using PCR, avoids any potential false negatives due to surfactant inactivation of *Ureaplasma* cultures.

Ureaplasma: Colonization vs Infection

The implications of colonization vs. true infection with *Uu* are also under debate. Both PCR and culture methods screen for the presence of the organism in endotracheal secretions, but neither can determine the overall pulmonary or systemic response to the organism. Epidemiologic studies of *Uu* published to date do not include data on pulmonary or systemic inflammatory mediator levels, although, as seen above, pulmonary cytokine levels and peripheral white blood cell counts are elevated in *Uu* colonized patients.

It is likely that a subset of patients exist for whom the presence of *Uu* in their bronchial tree does not cause significant inflammatory insult, perhaps those who acquire *Uu* at birth, rather than prenatally. There is currently no way to distinguish these patients in existing epidemiologic studies, including this study, from those for whom *Uu* has caused a significant pulmonary or systemic inflammatory response, thereby increasing their risk for BPD. Patients could thus be misclassified, combining patients with incidental *Uu* colonization, and therefore at no increased risk of BPD due to *Uu* with those with true infection, who are at increased risk. If this occurred, OR would be expected to be skewed towards 1.0, and a true effect could be masked. In our study, which did demonstrate a significantly increased risk of BPD in colonized patients, this type of misclassification would act to dampen the true effect size, falsely lowering the OR. Future studies that combine cytokine measurements, white blood cell counts, and other measures of inflammatory response with clinical outcomes and microbial screening would be helpful in answering this question definitively.

***Ureaplasma* and BPD: Studies of treatment**

Treatment for infants colonized with *Ureaplasma urealyticum* is controversial as well. It seems logical that if *Uu* causes BPD, treatment with antibiotics should prevent BPD in exposed patients. Erythromycin has been suggested as the appropriate agent, but resistance exists and adverse effects have been reported. (57-59). The effects of treatment have not been thoroughly evaluated. Data from three clinical studies show no decrease in the incidence of BPD in colonized patients who are treated (60-62). However, subject numbers are small in all reported studies, treatment courses vary widely, and not all studies were randomized. Existing studies relied on culture data to diagnose colonization, and treatment was not begun until after 7 days of age. It is possible that early treatment could be more beneficial. Based on studies of

inflammation in infants exposed to choriomamnionitis in utero, it is likely that pulmonary inflammation and the alveolar developmental anomalies it causes occurs before birth, making early treatment important to halt further damage (2, 53). The time advantage of PCR detection of *Ureaplasma* over culture methods (1-2 days vs. 3-5) makes this method ideal in future studies of early treatment. Studies of newer antibiotics should be undertaken to determine the safest, most effective treatment. Also, a large randomized study of early treatment and subsequent development of BPD needs to be performed to determine the importance of *Ureaplasma urealyticum* in the pathogenesis of BPD.

Mechanical Ventilation, Inflammation, and BPD:

In our population, the most significant predictor of chronic lung disease by any outcome definition is days of mechanical ventilation. This is true in both univariate analysis, and after adjustment for other risk factors with logistic regression. Other significant predictors in univariate analyses include: PDA, sepsis, use of surfactant, use of high frequency ventilation, and endotracheal colonization with *Ureaplasma urealyticum*.

Mechanical ventilation has long been known to be a powerful predictor of BPD, as have PDA and sepsis (1, 2, 55, 63). The use of surfactant in this case acts as a marker for infants who had significant RDS, in and of itself a strong predictor for BPD. However, in our population, as seen elsewhere, infants who did not have severe RDS in the first days of life went on to develop BPD (4, 5). In our institution, high frequency ventilation is used as a rescue modality, so this variable is also acting as a marker of more severe initial lung disease, and should not be interpreted as causal in the development of BPD. Adjusting for a wide variety of other risk factors, ventilation clearly emerged as the largest contributing factor to BPD in our population, regardless of

definition. Mechanical ventilation likely represents an inflammatory insult to the preterm lung, similar to chorioamnionitis and *Ureaplasma urealyticum*.

Inflammation is emerging as an important contributing factor in the development of BPD, and a common link among many risk factors. Groneck and Speer showed that infants who went on to develop BPD had higher concentrations of IL-8 and macrophage inflammatory protein-1, and levels of the counter regulatory cytokine IL-10 were lower than in those infants who did not develop BPD (31). Levels of inflammatory mediators including IL-6, TNF-α, and IL-8 in the airways of VLBW infants who develop BPD have been shown to be higher than those in infants who do not develop BPD (64).

Inflammation due to mechanical ventilation has been shown in animal models to impair alveolar and pulmonary vascular development, leading to BPD-like changes. Seven days of mechanical ventilation and high oxygen exposure in the 140-day preterm baboon model has been shown to severely decrease the numbers of alveoli, and the same finding has been demonstrated in the 125-day preterm baboon, without exposure to high oxygen. Pulmonary inflammatory cytokine levels and white blood cells are also persistently increased in these animals (65, 66). Avoidance of mechanical ventilation in preterm infants has been shown to decrease the incidence of BPD in observational studies, but no randomized trials have been completed (67). (A large trial is underway within the Vermont-Oxford Neonatal Network).

Adenovirus and *Chlamydia* and BPD:

We set out to investigate the contributions of Adenovirus and *Chlamydia* colonization on the development of BPD as well. Although we detected Adenovirus at a similar rate as other investigators (44, 47), there was no association between Adenovirus colonization and BPD at 36 weeks, or for our combined outcome. Our

sample size and colonization prevalence was similar to that of Couroucli, who first reported this association, and therefore should have been adequate to reproduce this finding. Prosch and colleagues (2003) also screened a similar population with a similar detection rate to that of Couroucli, and did not demonstrate an association between Adenovirus and BPD. Prosch et al also demonstrated that not only is it possible to detect Adenoviral DNA by PCR in the endotracheal secretions of preterm infants, but that viable virus can be recovered by viral culture methods as well (47). Recovery of virus suggests that Adenovirus could act as a low-level pathogen in VLBW infants, but there are no studies demonstrating inflammation due to Adenovirus in the lungs of these patients. Chlamydia was uncommonly detected in our population, and not associated with BPD at any time point. As *Chlamydia* was so rarely detected in our population (6 patients), it is difficult to truly assess the effect of this organism on the development of BPD. In this respect, our experience is similar to that of other investigators.

Potential Study Limitations:

Like all epidemiologic studies, ours contained potential limitations. As delineated above, we obtained samples on only 79% of eligible infants in our population. Given known sample collection patterns, these patients were probably intubated for shorter durations, however no data were collected to support this assertion. If intubated for shorter duration, they would represent a group at lower risk for BPD, and perhaps, if included, would lead to a shift in the OR of our logistic models toward 1. This is a potential source of selection bias.

Another potential source of selection bias in our study lies in including only intubated infants. The overall rate of BPD at 36 weeks CGA in the total population of VLBW infants admitted to our NICU surviving to this time point, according to the Vermont–Oxford (VO) database, is 38%. This number is significantly lower ($p = 0.02$)

than the 57% of our study population with BPD at the same time point. The VO data includes all VLBW infants, and the denominator for this figure includes the 32% of infants that were never intubated, and thus were not potential subjects for our study. The VO dataset does not separate the outcome measurements by intubation status, so there is no way to determine the incidence of BPD in the entire intubated VO cohort. Our study cohort consisted of a subset of the total admitted VO population who were smaller, younger, and, in general, at higher risk of BPD (see table 3). It is unknown how many of the non-intubated patients developed BPD, and their exposure status for the organisms of interest is also unknown. There is therefore no definitive way to determine if selection bias occurred in the formation of our cohort.

It is known that infants that never require mechanical ventilation can still develop BPD. As mechanical ventilation is being used more and more sparingly in this population, risk factors for BPD other than mechanical ventilation become more important to understand. Given the large impact of mechanical ventilation to the risk of BPD in our population, there was no way to adequately asses the effects of some other, likely important risk factors, such as sepsis, PDA, maternal chorioamnionitis. The sample size and the large effect size for mechanical ventilation precluded this. Therefore, our study cannot be generalized to the entire VLBW population, only those who have been mechanically ventilated.

As noted above, PCR is a very sensitive method for the detection of microorganisms. It is possible to produce false positive results with this technique, due to DNA contamination of work surfaces, equipment, etc. We attempted to minimize this by preparing PCR reactions in a clean room into which no DNA samples were allowed. False positive results due to contamination would lead to differential misclassification,

the results of such misclassification would be unpredictable, but could lead to an exaggeration of the association.

In this vein, the sensitivity of PCR also makes it possible, indeed likely, that one might detect very low levels of organism colonization, which do not adversely affect the patient. To separate those patients with a true pathogenic colonization and those with incidental colonization, several methods could be used. A quantitative PCR technique could be employed, however, the threshold organism levels for pathogenicity is not known for these organisms, making interpretation of such an assay impossible with current knowledge. Simultaneous measurements of cytokine levels in the endotracheal aspirate samples would also shed light onto this problem, and these were not performed. Due to our waiver of consent, we cannot standardize the sample collection methods, making standardization of ELISA assays for cytokines difficult.

Since we are not able to differentiate pathogenic from incidental colonization in our study population, patients with incidental colonization would be misclassified into the exposed group. This type of misclassification would tend to drive the OR for organism colonization and BPD towards the null. We demonstrated a clear association between *Ureaplasma urealyticum* and BPD, so even if differential misclassification of this type occurred, it only lowered the OR from the unknown "true" OR for *Uu* and BPD. For Adenovirus or *Chlamydia*, a true effect could have been masked in this way, however.

The burden of evidence suggests that *Ureaplasma urealyticum* contributes to the development of BPD in VLBW infants. Our study, which used a high-risk population for BPD, was carefully designed to correct for confounding factors, took into account other possible pathogens, and used the most sensitive detection method simply confirms the investigations of multiple prior investigators. The aim of this study was to take the previous experience and improve on it, with a more sensitive detection method for *Uu*,

and taking into account all other known risk factors for BPD, to delineate the relationship between BPD and *Ureaplasma*.

We clearly demonstrated that after accounting for other common risk factors for BPD, *Ureaplasma* colonization predisposed VLBW infants to BPD or death due to lung disease. *Ureaplasma* is an independent risk factor for BPD. The mechanism for this increased risk is not known, but is likely inflammation, which may arise prior to birth, due to intra-uterine exposure. Future studies should be aimed at combining epidemiologic studies with contemporaneous measurement of inflammatory cytokines, to determine if *Uu* is causing an inflammatory response in neonates who are then followed for the development of BPD. Biovar ascertainment would also be helpful in such studies to pin down any varying pathogenicity of the *Ureaplasma* species. Finally, no large prospective studies of antibiotic treatment of *Ureaplasma*, starting in the first days of life, exist. Such a trial could contribute much to the understanding of the impact of this microbe in this vulnerable population. Understanding the pathogenesis of BPD is essential to intervening to stop this disease. This represents another step towards optimizing the health and long-term well-being of our tiniest patients.

Table 1. PCR Primers

Organism	Name	Sequence	Size of PCR product	Gene of Origin	Original Publication
Ureaplasma urealyticum	U5 U4	5'–CAATCTGCTCGTGAAGTATTAC–3' 5'–ACGACGTCCATAAGCAACT–3'	429 bp	urease	Blanchard et al, 1993.
Adenovirus **First step** **Second step**	 ADH1 ADH2 ADH11 ADH12	 5'–ACTACAAYATTGGCTACCAGG–3' 5'–CAAAACATAAAGAAGKGTGGGC-3' 5'-AACTTCCAGCCCATGAGCMG–3' 5'–CTCAAAAGTCATGTCBAGCGC–3'	 330 bp	hexon	Couroucli et al, 2000
Chlamydia sp	CHLAM01 CHLAM02	5'–ACACTCGCAAGGGTGAAACTC-3' 5'-CGACTTCATCCYAGTCATCAG–3'	609 bp	16S ribosome	Couroucli et al, 2000

Table 2. Study Population Demographics

	N = 139
Sex (male)	59% (83/139)
Mean estimated gestational age	26.8 wks (+/- 1.9wks)
Mean birthweight	941 g (+/- 257g)
Deaths prior to 36 weeks CGA	14
Deaths due to lung disease	8
Transfers prior to 36 weeks CGA	4
Prenatal steroids	80% (108/135)
Prenatal Antibiotics	72% (97/135)
Surfactant	93% (129/139)
Hours of ruptured membranes	41 hrs (+/- 133 hrs)
Chorioamnionitis	17% (23/137)
Patent ductus arteriosus	66% (88/133)
Days of supplemental O2	58 (+/- 37days)
Days of mechanical ventilation	21 (+/- 23 days)
Hosp Days	83 (+/- 23 days)
High Frequency Ventilation	45% (62/139)
ROP stage 3 or 4	22% (31/119)
IVH grade III or IV	14% (18/129)
NEC	7% (10/139)
Sepsis	37% (51/139)
Oxygen use at 28 days	83% (105/129)
BPD at 36 weeks CGA	57% (69/121)
Ureaplasma	24% (34/139)
Adenovirus	16% (22/136)
Chlamydia	6% (8/133)

Table 3. Study population vs Total VLBW population

	Study Population N=139	OHSU Vermont-Oxford population N = 344
Median GA category	24-26 weeks	27-29 weeks
Median BW category	751 - 1000 g	1001 – 1250 g
% Male	59%	56%
Intubated patients	100%	68%
Antenantal Steroids	80%	65%
Oxygen use at 28 days	83%	55%
Oxygen use at 36 weeks	57%	38%
Patent Ductus Arteriosus	66%	35%

Table 4. Bronchopulmonary Dysplasia at 28 days, univariate analyses

	BPD 28 days (105/129)		No BPD 28 days (24/129)		p value
Sex (male)	59%	(62/105)	54%	(13/24)	0.82
Mean EGA	26.5 wks	(+/- 1.7wks)	28.8 wks	(+/- 1.8wks)	<.001
Mean BW	914 g	(+/- 222 g)	1163 g	(+/- 228 g)	<.001
Prenatal steroids	79%	(83/105)	83%	(20/24)	0.62
Surfactant	99%	(104/105)	88%	(21/24)	0.24
Hours of ruptured membranes	48	(+/- 148hrs)	21	(+/-69 hrs)	0.39
Chorioamnionitis	19%	(20/105)	8%	(2/24)	0.37
Patent ductus arteriosus	69%	(72/105)	57%	(14/24)	0.28
Days of supplemental O2	72	(+/- 30 d)	13	(+/- 9 d)	<.001
Days of mechanical ventilation	25	(+/- 24 d)	6	(+/- 6 d)	<.001
Hospital Days	88	(+/- 24 d)	57	(+/-21 d)	<.001
High frequency ventilation	49%	(51/105)	25%	(6/24)	0.042
ROP stage 3 or 4	30%	(31/105)	4%	(1/24)	0.023
Necrotizing enterocolitis	8%	(8/105)	4%	(1/24)	0.69
Sepsis	41%	(43/105)	19%	(5/24)	0.07
Ureaplasma	29%	(30/105)	4%	(1/24)	0.012
Adenovirus	12%	(12/103)	25%	(6/24)	0.09
Chlamydia	0		8%	(8/101)	0.16

Table 5. Bronchopulmonary Dysplasia at 36 weeks CGA, univariate analyses

	BPD 36 weeks (69/121)		No BPD 36 weeks (52/121)		p value
Sex (male)	57%	(39/69)	57%	(30/52)	1
Mean EGA	26.3 wks	(+/-1.6 wks)	27.7 wks	(+/- 1.9wks)	<.001
Mean BW	872 g	(+/-219 g)	1070 g	(+/-227 g)	<.001
Prenatal steroids	82%	(57/69)	79%	(38/52)	0.60
Surfactant	99%	(68/69)	85%	(44/52)	0.004
Hours of ruptured membranes	46	(+/-117 hrs)	40	(+/-165 hrs)	0.82
Chorioamnionitis	14%	(10/69)	21%	(11/52)	0.47
Patent ductus arteriosus	77%	(52/69)	53%	(27/52)	0.01
Days of supplemental O2	86	(+/-25 d)	32	(+/-20 d)	<.001
Days of mechanical ventilation	30	(+/-28 d)	11	(+/-12 d)	<.001
Hospital Days	95	(+/-22 d)	66	(+/-22 d)	<.001
High frequency ventilation	57%	(39/69)	27%	(14/52)	0.001
ROP stage 3 or 4	36%	(25/69)	8%	(4/52)	<.001
IVH grade III or IV	16%	(11/69)	6%	(3/52)	0.43
Necrotizing enterocolitis	7%	(5/69)	9%	(5/52)	0.74
Sepsis	44%	(30/69)	27%	(13/52)	0.07
Ureaplasma	37%	(26/69)	8%	(4/52)	<.001
Adenovirus	16%	(11/69)	12%	(6/52)	0.52
Chlamydia	9%	(6/69)	4%	(2/52)	0.33

Table 6. Odds ratios for Risk Factors for BPD at 36 weeks

Factor	OR	95% CI	p value
PDA	1.77	0.66, 4.77	0.26
Sepsis	0.65	0.23, 1.88	0.43
Ureaplasma colonization	2.85	0.8, 10	0.10
At EGA 25 wks, each day of mechanical vent	1.05	0.99, 1.1	0.053
At EGA 26 wks, each day of mechanical vent	1.08	1.03, 1.13	0.003
At EGA 28 wks, each day of mechanical vent	1.14	1.06, 1.24	0.0008

Table 7. Bronchopulmonary Dysplasia at 36 weeks CGA or death due to lung disease, univariate analyses

	Death or BPD at 36weeks (76/130)		No Death or BPD at 36 weeks (54/130)		p value
Sex (male)	60%	(45/76)	56%	(30/54)	0.72
Mean EGA	26.2 wks	(+/-1.5wks)	27.6 wks	(+/-1.9wks)	<.001
Mean BW	854 g	(+/-212 g)	1055 g	(+/-240 g)	<.001
Prenatal steroids	82%	(60/74)	73%	(42/52)	0.97
Surfactant	99%	(74/75)	85%	(45/53)	0.003
Hours of ruptured membranes	42	(+/-114 d)	40	(+/- 165 d)	0.94
Choriomanionitis	15%	(11/76)	19%	(10/54)	0.63
Patent ductus arteriosus	73%	(54/74)	53%	(27/51)	0.02
Days of supplemental O2	78	(+/-30 d)	32	(+/- 20 d)	<.001
Days of mechanical ventilation	26	(+/-20 d)	11	(+/-12 d)	<.001
Hospital Days	95	(+/-22 d)	66	(+/- 22 d)	<.001
High frequency ventilation	60%	(45/75)	26%	(14/53)	<.001
ROP stage 3 or 4	36%	(27/76)	8%	(4/54)	<.001
Necrotizing enterocolitis	7%	(5/76)	9%	(5/54)	0.74
Sepsis	41%	(30/74)	27%	(13/48)	0.13
Ureaplasma	38%	(29/76)	6%	(3/53)	<.001
Adenovirus	17%	(13/75)	12%	(6/51)	0.39
Chlamydia	8%	(6/74)	4%	(2/50)	0.36

Table 8. Odds ratios for risk factors for BPD at 36 weeks or death due to lung disease

Factor	OR	95% CI	p value
PDA	1.59	0.6, 4.22	0.35
Sepsis	0.70	0.24, 2.03	0.51
Ureaplasma colonization	4.23	1.03, 17	0.04
At EGA 25 wks, each day of mechanical vent	1.02	0.97, 1.06	0.42
At EGA 26 wks, each day of mechanical vent	1.05	1.004, 1.1	0.03
At EGA 28 wks, each day of mechanical vent	1.12	1.04, 1.21	0.003

Appendix A: Previous Studies of *Ureaplasma urealyticum* and BPD

STUDY	Study Design and Population Completeness (N)	Detection Method (source site)	BPD definition	Prevalence of *Uu*	OR for *Uu* and BPD (95% CI)	Statistical correction method	Covariates Corrected for				
							EGA	BWT	PDA	Sepsis	Vent Days
Cassell et al.; 1988	Prospective Cohort Good (135)	Culture (ET, NP)	28 days	17%	1.98 (1.2, 5)	None					
Sanchez and Regan, 1998	Prospective Cohort Good (111)	Culture (surface, throat)	30 days	41%	5.32 (1.7, 15.9)	None					
Wang et al.; 1988	Prospective Cohort Good (107)	Culture (gastric, ET, NP)	28 days	33%	2.6 (1.7, 3.4)	None					
Izraeli et al.; 1991	Prospective cohort Good (99)	Culture (ET), or surface	28 days	22%	3.2 (0.5, 21)	None					
Horowitz et al.; 1992	Prospective cohort Good (114)	Culture (ET), or surface	28 days	24%	4.1 (1.2, 13.6)	None					
Dyke et al.; 1993	Case-Control Poor (112 pairs)	Culture (gastric)	28 days	11%	1.4 (0.95, 2.07)	LR (model not published)		X			
Payne et al.; 1993	Prospective cohort Good (93)	Culture (ET), or surface	28 days	18%	1.66 (1.24, 2.2) univariate, p value 0.062 with multivariate	Stepwise LR	X	X	X		
Saxen et al.; 1993	Prospective cohort Good (49)	Culture (ET)	28 days	29%	1.5 (0.67, 3.34)	None					
Smyth et al.; 1993	Prospective cohort Unknown (70)	Culture (ET)	28 days	13%	2.97 (0.57, 15)	LR (model not published)	X	X			
Valencia et al.; 1993	Prospective cohort Fair (69)	Culture (NP, CSF)	28 days	30%	7.12 (2.53, 19.9)	None					

Appendix A: Previous Studies of *Ureaplasma urealyticum* and BPD, cont.

STUDY	Study Design and Population Complete-ness (N)	Detection Method (source site)	BPD definition	Prevalence of *Uu*	OR for *Uu* and BPD (95% CI)	Statistical correction method	Covariates Corrected for				
							EGA	BWT	PDA	Sepsis	Vent Days
Heggie et al.; 1994	Prospective cohort Poor (54)	Culture (ET)	28 days and 36 weeks	17%	1.27 (1.03, 1.57) univariate, NS when corrected	Excluding babies <26 weeks, because all infants <26 weeks were colonized	X				
Jonsson et al.; 1994	Prospective cohort Fair (93)	Culture (ET or surface)	28 days	19%	0.96 (0.25, 3.6)	LR	X				
Wang et al.; 1995	Meta-analysis, 1479 patients	Culture (ET, NP, surface)	28 days	33%	1.72 (1.5, 1.96)	Not possible					
Garland and Bowman, 1996.	Prospective cohort Fair (44)	Culture (ET)	28 days	14%	9.6 (1, 91.6)	None					
Iles et al.; 1996	Prospective cohort Fair (40)	Culture (ET)	36 weeks CGA	33%	8.9 (1.68, 49.4) – univariate, for multivariate, p value reported p 0.02	LR	X			X	
Panero et al.; 1997	Prospective Cohort Good (94)	Culture (ET, NP)	36 weeks CGA	50%	Not published, p=0.0001; univariate OR 11 (1.6, 75.5)	LR	X		X		
Da Silva et al.; 1997	Prospective cohort Good (108)	PCR and Culture (ET)	36 weeks CGA	45%	1.2 (0.8, 1.83)	LR, *Ureaplasma* not in model					
van Waarde et al.; 1997	Prospective cohort Good (108)	Culture (ET)	28 days	21%	3.11 (1.17, 8.33) univariate, NS in multivariate	LR	X				
Abele-Horn et al.; 1998	Prospective, mulit-center cohort Fair (97)	Culture (ET)	28 days	36%	Varied by days of ventilation, Only sig after 28 days of ventilation 5.53 (1.27, 24.02)	LR,	X	X			X

*** for studies in which no OR was reported, it was calculated if possible from data reported, with 95% CI generated using the delta method**

****Sample sites: ET = Endotracheal; NP = Nasopharynx**

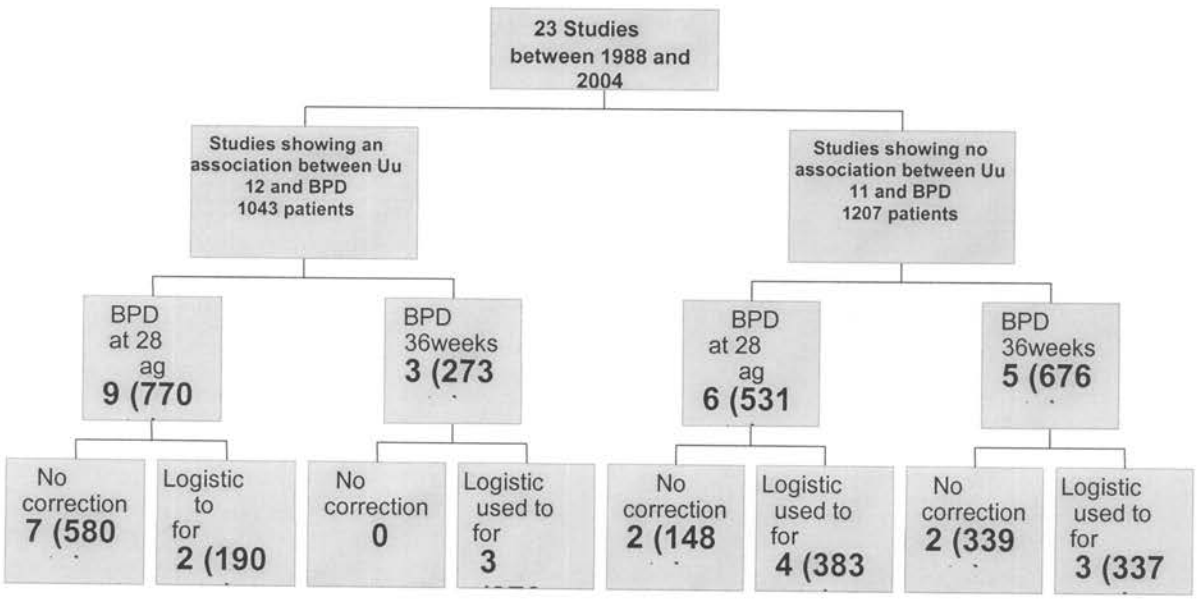
Appendix A: Previous Studies of *Ureaplasma urealyticum* and BPD, cont.

STUDY	Study Design and Population Completeness (N)	Detection Method (source site)	BPD definition	Prevalence of *Uu*	OR for *Uu* and BPD (95% CI)	Statistical correction method	Covariates Corrected For				
							EGA	BWT	PDA	Sepsis	Vent Days
Perzigian et al.; 1998	Prospective Cohort Good (105)	Culture (ET)	36 weeks CGA	27%	1.26 (0.57, 2.79)	None, univariate analysis only					
Hannaford et al.; 1999	Prospective Cohort, 2 centers Fair (139)	Culture (ET)	36 weeks	27%	3.0 (1, 9.1)	Backward LR (model not published)	X				
Couroucli et al.; 2000	Prospective cohort Fair (89)	PCR (ET)	36 weeks	23%	1.38 (0.46, 4.14)	No sig assoc					
Heggie et al.; 2001	Prospective cohort Good (175)	Culture (ET)	36 weeks	38%	Not reported, not sig	LR	X	X	X	X	
Ollikainen et al.; 2001	Prospective cohort Good (145)	Culture (ET or blood)	36 weeks	33%	1.23 (0.57, 2.64)	none					

* for studies in which no OR was reported, it was calculated if possible from data reported, with 95% CI generated using the delta method

**Sample sites: ET = Endotracheal; NP = Nasopharynx

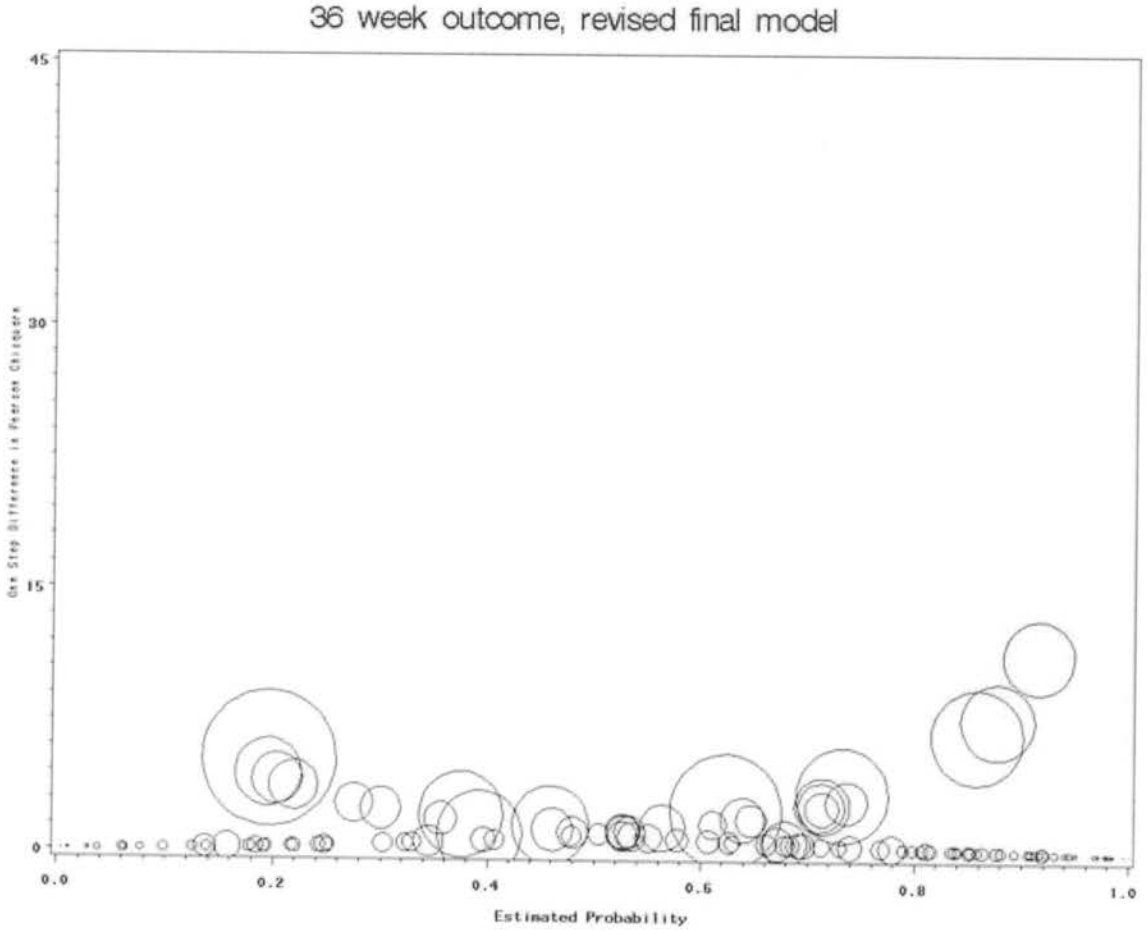
Appendix B: Flowchart of *Ureaplasma*/BPD studies 1980-2004

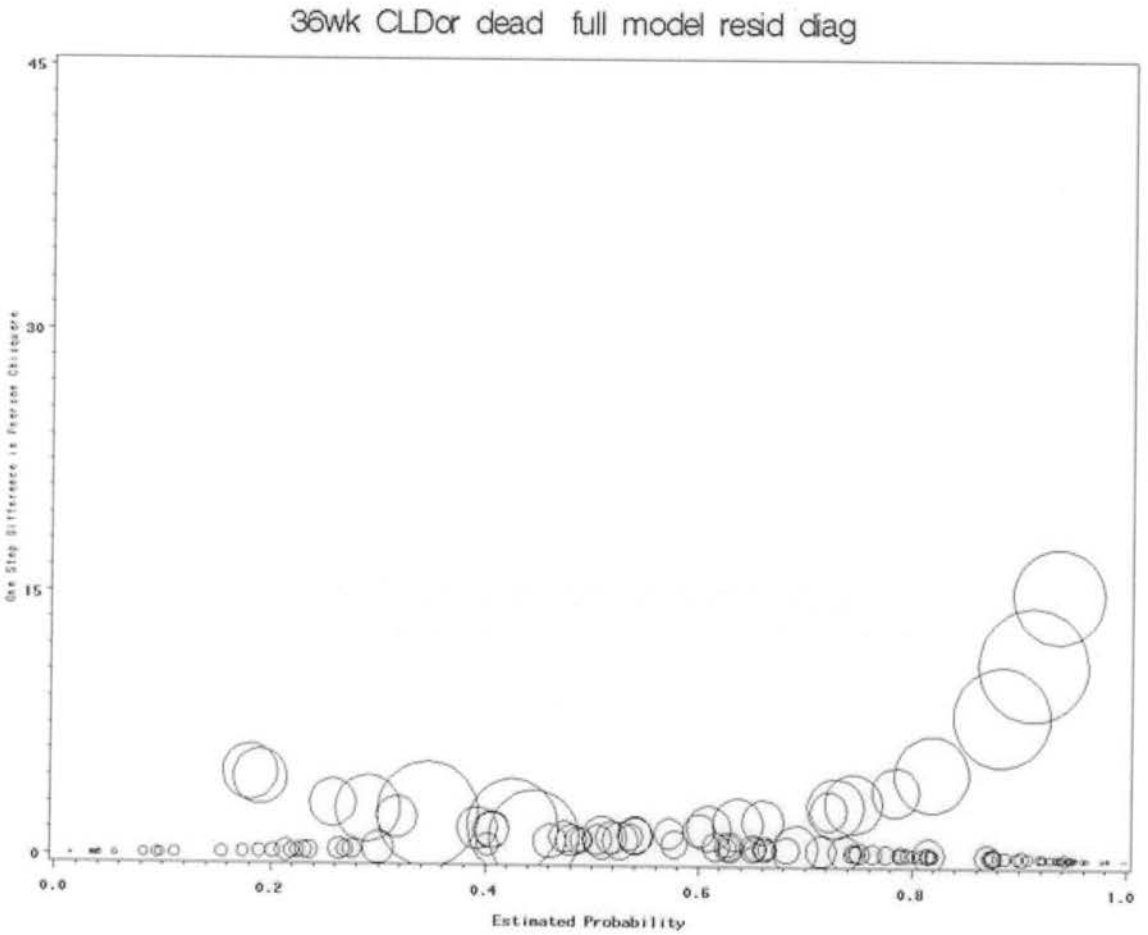


Appendix C: Study Variables

Variable Name	Definition
Estimated Gestational Age	Fractional weeks of gestation completed, according to 1st trimester ultrasound (preferred), or last menstrual period
Birthweight (grams)	Weight obtained in the delivery room
Chorioamnionitis	Clinically defined by Obstetrics provider OR Diagnosed by placental pathology
Antenatal Antibiotics	At least one dose of intravenous antibiotics received at least 4 hours prior to birth
Antenatal Steroids	At least one dose of betamethasone or dexamethasone received prior to birth
Hours of Ruptured membranes	Total hours between rupture of membranes and birth, to the nearest hour
Maximum Peak Inspiratory Pressure	Highest recorded PIP used during mechanical ventilation, per RT charting
Maximum FiO2	Highest recorded FiO2 used during hospitalization, per RT charting
High Frequency Ventilation	Use of flow interrupter (Infant Star) or Oscillating high freqeuency ventilator (Sensormedics 3000) at any time
Days of mechanical ventilation	Number of days patient received mechanical ventilation via endotracheal tube, total. (Courses of ventilation separated by time periods without ventilation were added to achieve total days)
Days of oxygen use	Total number of days that patient received oxygen therapy. (Courses of oxygen separated by time periods without oxygen were added to achieve total days)
Hospital Days	Total days from birth or admission to first hospital discharge
Surfactant	At least one dose of surfactant received during hospital
Sepsis	At least one positive blood culture for any organism during hospitalization
PDA	Patent ductus arteriosus, diagnosed by echocardiogram, requiring treatment with indomethacin and/or surgical ligation
NEC	Necrotizing enterocolitis, diagnosed by abnormal abdominal exam and radiograph, with or without bloody stools, which caused the infant to be made NPO and treated with antibiotics.
ROP	Most severe stage of ROP documented in routine screening exams during hospitalization
BPD at 28 days	Use of oxygen supplement on the 28th day of life
BPD at 36 weeks	Room air SpO2 of <88%, and use of oxygen supplement at CGA 36 0/7 weeks
***Ureaplasma* colonization**	*Ureaplasma* DNA detected by PCR in endotracheal aspirate sample
Adenovirus colonization	Adenoviral DNA detected by PCR in endotracheal aspirate sample
***Chlamyida* colonization**	*Chlamydia* DNA detected by PCR in endotracheal aspirate sample

Appendix D: Logistic Regression Residual Plots




36wk CLDor dead full model resid diag
45
30
15
0
0.0
0.2
0.4
0.6
0.8
1.0
Estimated Probability

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