Antenatal exposure to *Ureaplasma* species exacerbates bronchopulmonary dysplasia synergistically with subsequent prolonged mechanical ventilation in preterm infants

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INTRODUCTION: The presence of microorganisms in gastric fluid in neonates at birth is postulated to reflect antenatal infection and also to be associated with the development of bronchopulmonary dysplasia (BPD).

RESULTS: A logistic regression analysis, after controlling for other risk factors, indicated that *Ureaplasma*–positive infants were not at increased risk for moderate/severe BPD (adjusted odds ratio (OR): 2.58, 95% confidence interval (CI): 0.57–6.89, $P = 0.12$). However, the association between the presence of *Ureaplasma* species and the risk for moderate/severe BPD increased significantly in infants on mechanical ventilation (MV) ≥2 wk (adjusted OR: 4.17, 95% CI: 1.62–44.1, $P = 0.009$). An analysis using a lung injury marker indicated that *Ureaplasma*–positive infants with MV ≥2 wk, but not other infants, showed higher serum KL-6 levels in samples taken from cord blood, and that KL-6 levels increased time-dependently up to 4 wk of age.

DISCUSSION: Antenatal exposure to *Ureaplasma* species induces lung injury prior to birth and synergistically contributes to the development of BPD in infants requiring prolonged MV (≥2 wk).

METHODS: We recovered gastric fluid specimens from 122 infants with gestational age (GA) <29 wk or birth weight <1,000 g to investigate whether these microorganisms influence respiratory outcome of BPD. A PCR analysis was used to detect urease and 16S ribosomal RNA (rRNA) genes to classify neonates into *Ureaplasma*–positive or *Ureaplasma*–negative infants.

Bronchopulmonary dysplasia (BPD) is a common disorder that affects morbidity and mortality rates in premature infants. BPD has been associated with lung injury and inflammation resulting from oxygen toxicity, barotrauma/volutrauma, and infection in the premature lung (1,2). In particular, over the past 2 decades, the level of evidence has increased regarding the existence of a link between intrauterine infection and BPD (3–5).

Ureaplasma species represent one of the most prevalent microbial causes of intrauterine and neonatal respiratory infection (6,7). The presence of *Ureaplasma* species in the maternal upper genital tract or in the neonatal airway has a close relationship with adverse neonatal outcomes including preterm delivery and the development of BPD (7–11). On the other hand, exposure to antenatal infection or inflammation triggered by bacteria other than *Ureaplasma* species may also have an important pathogenic role in the development of BPD (12–14). In an experimental model, the intra-amniotic injection of lipopolysaccharide induced an inflammatory response that caused deterioration in the lung architecture of premature animals in combination with mechanical ventilation (MV) (14).

Miralles *et al.* reported that the presence of a bacterial 16S ribosomal RNA (rRNA) gene in gastric fluid at birth was correlated with antenatal infection, thereby indicating that gastric fluid samples obtained from neonates soon after birth could be used as an alternative specimen to reflect antenatal infection (15). We recently found that the presence of a bacterial 16S rRNA gene in gastric fluid in infants was associated with the development of severe BPD (16). A biochemical analysis showed that, in a group of infants who tested positive for bacteria in gastric fluid at birth, the levels of KL-6, a lung injury marker, were high. This indication of lung injury was associated with an increased severity of subsequent BPD. Because KL-6 is a mucinous glycoprotein that is expressed by alveolar type II cells and bronchiolar cells and is known to be specifically correlated with the severity of respiratory distress in infants with BPD, increased plasma KL-6 levels at birth are presumed to be due to injury in the immature lungs resulting in the subsequent development of BPD (17,18). However, we could not clarify precisely which microorganism was most likely to play a pathogenic role in the ongoing process of BPD.

In this study, we investigated whether (i) antenatal exposure to *Ureaplasma* species, bacteria other than *Ureaplasma* species, or both, have a close association with the development of BPD; (ii) whether this association, if present, is modulated by postnatal lung injury induced by MV; and, if so, (iii) to what extent these microorganisms influence the respiratory morbidity associated with BPD.

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RESULTS

During the study period, 136 infants with gestational age (GA) <29 wk or a birth weight <1,000 g were admitted to our units. Among them, seven infants did not qualify for enrollment in our study at birth (two had chromosomal anomalies, two had multiple congenital anomalies, and three had complicated congenital heart diseases). In addition, data relating to seven infants were excluded from the final analysis: five of these infants were transferred to other facilities, and gastric fluid or blood specimens were not obtained from two infants. In total, data relating to 122 infants (90% of all the eligible infants) were included in this study.

Of the total number of study infants, 35 (29%) tested positive for *Ureaplasma* species in the gastric fluid soon after birth and were assigned to the *Ureaplasma*–positive group; 87 infants (71%) were assigned to the *Ureaplasma*–negative group. Among the group of *Ureaplasma*–negative infants, 31 (25% of the study infants) were positive for the 16S rRNA gene and were therefore assigned to the bacteria–positive subgroup, whereas the other 56 infants in the group (46% of the study infants) were negative for the 16S rRNA gene and were therefore assigned to the bacteria–negative subgroup.

Clinical characteristics and outcomes were compared among all the study infants (**Table 1**). The mean GA in *Ureaplasma*–positive infants was significantly lower than the mean GA values in both bacteria–negative infants (26.4 vs. 27.9 wk, $P = 0.009$) and bacteria–positive infants (26.4 vs. 27.7 wk, $P = 0.013$). As compared with bacteria–negative infants, the incidence of chorioamnionitis was significantly higher in *Ureaplasma*-positive infants (bacteria–negative vs. *Ureaplasma*–positive: 11 vs. 79%, $P < 0.0001$) and in bacteria–positive infants (bacteria–negative vs. bacteria–positive: 11 vs. 63%, $P = 0.0001$). The proportion of grade 2 or 3 chorioamnionitis was also higher in the *Ureaplasma*–positive infants (bacteria–negative vs. *Ureaplasma*–positive: 3.8 vs. 64%, $P < 0.0001$) and in bacteria–positive infants (bacteria–negative vs. bacteria–positive; 3.8 vs. 23%, $P = 0.012$).

Table 1 Comparison of the clinical characteristics and outcomes among *Ureaplasma*–negative infants (bacteria–negative infants, bacteria–positive infants) and *Ureaplasma*–positive infants

	Ureaplasma–negative ($n = 87$)		*Ureaplasma*–positive ($n = 35$)	
	Bacteria–negative ($n = 56$)	Bacteria–positive ($n = 31$)		*P* value
Male:female	29:27	14:17	18:17	0.76
GA (wk)	27.9 (23.7 to 31.1)	27.7 (24.4 to 31.9)	26.4 (23.0 to 29.7)[a,b]	0.0001
BW (g)	930 (376 to 1,626)	926 (460 to 1,420)	862 (470 to 1,410)	0.089
Z score	0 (−2.7 to 2.0)	0.20 (−2.9 to 1.8)	−0.10 (−1.6 to 1.5)	0.39
Apgar 1 min	5 (1 to 9)	5 (1 to 9)	4 (1 to 8)[a,b]	0.042
Apgar 5 min	8 (2 to 10)	7 (2 to 10)	7 (3 to 9)	0.27
CS, *n* (%)	49 (88%)	24 (77%)	19 (54%)[a]	0.001
Antenatal steroid, *n* (%)	38 (68%)	16 (55%)	24 (69%)	0.36
Chorioamnionitis, *n*/*N* (%)[c]	6/53 (11%)	19/30 (63%)[a]	26/33 (79%)[a]	<0.0001
Chorioamnionitis ≥ grade 2, *n*/*N* (%)[c]	2/53 (3.8%)	7/30 (23%)[a]	21/33 (64%)[a]	<0.0001
RDS, *n* (%)	40 (69%)	20 (65%)	22 (63%)	0.74
PDA, *n* (%)	28 (48%)	11 (35%)	16 (46%)	0.33
Sepsis, *n* (%)	1 (1.7%)	1 (3.2%)	3 (8.6%)	0.48
Pneumonia, *n* (%)	4 (6.9%)	2 (6.5%)	3 (8.6%)	0.92
Death due to lung disease	2 (3.4%)	0 (0%)	1 (2.8%)	0.97
Death at PMA 36 wk	2 (3.4%)	0 (0%)	2 (4.5%)	0.93
Supplemental oxygen (d)	38 (0 to 146)	38 (2 to 219)	58 (0 to 200)	0.27
Mechanical ventilation, *n* (%)	43 (77%)	24 (77%)	30 (86%)	0.89
Mechanical ventilation (d)	5 (0 to 146)	8 (0 to 67)	16 (0 to 129)[a]	0.046
Postnatal steroid, *n* (%)	1 (1.8%)	2 (6.5%)	4 (11.4%)	0.098
BPD, *n* (%)	33 (57%)	22 (71%)	28 (80%)[a]	0.019
Mild BPD, *n* (%)	24 (41%)	16 (52%)	14 (40%)	0.45
Moderate BPD, *n* (%)	10 (18%)	3 (9.7%)	8 (23%)	0.75
Severe BPD, *n* (%)	1 (1.7%)	3 (9.7%)	6 (17%)[a]	0.010
Moderate/severe BPD, *n* (%)	11 (16%)	6 (19%)	14 (40%)[a]	0.005

Values are expressed as the median (total range), or occurrences (%). The *P* value indicates the differences among three groups as analyzed by the Kruskal–Wallis or χ^2 tests.

BPD, bronchopulmonary dysplasia; BW, birth weight; CS, cesarean section; GA, gestational age; PDA, patent ductus arteriosus; PMA, postmenstrual age; RDS, respiratory distress syndrome.

[a]$P < 0.05$ vs. bacteria–negative infants. [b]$P < 0.05$ vs. bacteria–positive infants. [c]Histologic examination of placentas obtained from 53 of 56 bacteria–negative infants, 33 of 35 *Ureaplasma*–positive infants, and 30 of 31 bacteria–positive infants.

The occurrence of BPD in *Ureaplasma*–positive infants was significantly greater than in the bacteria–negative infants (80 vs. 57%, $P = 0.014$). The analysis of BPD severity indicated that, in comparing *Ureaplasma*–positive infants with bacteria–negative ones, the difference in occurrence was more prominent with respect to moderate/severe BPD (40 vs. 16%, respectively, $P = 0.002$) and severe BPD (17 vs. 1.7%, respectively, $P = 0.020$).

Predictor variables for the development of moderate/severe BPD were analyzed using univariate analysis (**Table 2**). Several factors emerged as significant predictors of moderate/severe BPD. Younger GA, lower birth weight, lower *Z* score, the occurrence of respiratory distress syndrome (RDS), and the presence of MV ≥2 wk were all factors indicating a higher likelihood of developing moderate/severe BPD. The presence of *Ureaplasma* species was also a risk factor for moderate/severe BPD (odds ratio (OR): 2.75, 95% confidence interval (CI): 1.72–9.80, $P = 0.006$), but not the presence of bacteria other than *Ureaplasma* species. Therefore, further analyses were restricted to the comparison of clinical data from *Ureaplasma*–positive infants and *Ureaplasma*–negative infants.

Logistic-regression models were created to assess significant predictors of the development of moderate/severe BPD in the study infants. As shown in **Table 3**, a lower GA, smaller *Z* score, and MV ≥2 wk were independent risk factors for moderate/severe BPD. The adjusted OR (aOR) of *Ureaplasma* species for the development of moderate/severe BPD was slightly higher, and tended to be positively correlated with moderate/severe BPD, although the correlation was not significant (aOR: 2.58, 95% CI: 0.57–6.89, $P = 0.12$). To examine whether antenatal exposure to *Ureaplasma* species had an interaction with MV, the risk of developing moderate/severe BPD was compared among the *Ureaplasma*–negative group × MV <2 wk ($n = 59$), the *Ureaplasma*–positive group × MV <2 wk ($n = 18$), the *Ureaplasma*–negative group × MV ≥2 wk ($n = 28$), and the *Ureaplasma*–positive group × MV ≥2 wk ($n = 17$), after controlling for other risk factors. This analysis showed that the risk for the *Ureaplasma*–positive group × MV ≥2 wk was significantly higher than those for the *Ureaplasma*–negative group × MV <2 wk (aOR: 8.95, 95% CI: 2.12–73.5, $P = 0.001$) and the *Ureaplasma*–negative group × MV ≥2 wk (aOR: 4.17, 95% CI: 1.62–44.1, $P = 0.009$) (**Table 4**).

The time-dependent changes in KL-6 levels were compared among *Ureaplasma*–positive infants ($n = 35$), bacteria–positive infants ($n = 31$), and bacteria–negative infants ($n = 56$) of <4 wk of age (**Figure 1**). This analysis was performed on data within the respective groups for MV ≥2 wk and <2 wk. Overall, 17 of 35 (49%) *Ureaplasma*–positive infants, 10 of 31 (32%) bacteria–positive infants, and 18 of 56 (32%) bacteria–negative infants were in the group with MV ≥2 wks (**Figure 1a**), and the other infants were in the group with MV <2 wk (**Figure 1b**). In both these groups, the median KL-6 levels in the *Ureaplasma*–positive infants increased time-dependently after birth and, at 2 wk of age, these levels were significantly higher than in the bacteria–negative infants (**Figure 1a,b**). Furthermore, in the analysis of the group with MV ≥ 2 wk, the median KL-6 level at 4 wk of age was significantly higher in the *Ureaplasma*–positive infants than in the bacteria–negative infants (**Figure 1a**). On the other hand, in the analysis of the group with MV <2 wk, the median KL-6 levels at 4 wk of age were not significantly different among the *Ureaplasma*–positive, bacteria–negative, and bacteria–positive infants (**Figure 1b**).

DISCUSSION

In this study, we investigated whether the morbidity related to BPD may be affected by antenatal exposure to *Ureaplasma*

Table 2 The results of a univariate analysis between predictor variables and the development of moderate/severe BPD among all study infants

	Total	Moderate/severe BPD			
Factor	*N*	*n* (%)	OR	95% CI	*P* value
	122	31 (25%)			
GA (wk)			0.53	(0.36–0.75)	<0.0001**
BW (100 g)			0.54	(0.41–0.69)	<0.0001**
Z score			0.70	(0.45–0.97)	0.045*
Male gender					
No	61	15 (25%)	Reference	—	—
Yes	61	16 (26%)	1.09	(0.48–2.50)	0.83
CS					
No	30	10 (33%)	Reference	—	—
Yes	92	21 (23%)	0.59	(0.24–1.49)	0.26
PDA					
No	67	13 (19%)	Reference	—	—
Yes	55	18 (33%)	2.02	(0.89–4.56)	0.092
RDS					
No	40	4 (10%)	Reference	—	—
Yes	82	27 (33%)	4.42	(1.75–17.7)	0.004**
Chorioamnionitis[a]					
No	65	17 (26%)	Reference	—	—
Grade 1	21	2 (9.5%)	0.30	(0.07–1.89)	0.30
≥ Grade 2	30	12 (40%)	1.88	(0.76–5.02)	0.16
Ureaplasma					
No	87	17 (20%)	Reference	—	—
Yes	35	14 (40%)	2.75	(1.72–9.80)	0.006*
Other bacteria					
No	91	25 (27%)	Reference	—	—
Yes	31	6 (19%)	0.63	(0.22–1.74)	0.42
MV					
<1 wk	54	4 (7.4%)	Reference	—	—
≥1 wk, <2 wk	23	3 (13%)	1.89	(0.65–33.7)	0.25
≥2 wk	45	24 (53%)	14.3	(3.10–45.6)	<0.0001**

BPD, bronchopulmonary dysplasia; BW, birth weight; CI, confidence interval; CS, cesarean section; GA, gestational age; MV, mechanical ventilation; *N*, number of infants analyzed; *n* (%), number (proportion) of infants with moderate/severe BPD among study infants; OR, odds ratio; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

[a]The number of infants investigated was 116. *$P < 0.05$; **$P < 0.005$.

Table 3 The results of a multivariate logistic regression analysis between predictor variables and the development of moderate/severe BPD among all study infants

Factor	aOR	95% CI	*P* value
GA (wk)	0.53	(0.33–0.81)	0.003**
Z score	0.56	(0.35–0.89)	0.041*
Ureaplasma	2.58	(0.57–6.89)	0.12
MV ≥2 wk	4.60	(1.52–6.10)	0.005*
RDS	2.91	(0.43–15.2)	0.33
PDA	1.10	(0.34–3.70)	0.87

aOR, adjusted OR; CI, confidence interval; GA, gestational age; MV, mechanical ventilation; OR, odds ratio; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

*$P < 0.05$; **$P < 0.005$.

Table 4 The results of an interaction analysis between *Ureaplasma* species and prolonged mechanical ventilation for the development of moderate/severe BPD in a multivariate logistic-regression model

Factor	aOR	95% CI	*P* value
GA	0.51	(0.35–0.78)	0.001**
Z score	0.57	(0.28–0.92)	0.025*
RDS	2.95	(0.61–11.2)	0.17
PDA	1.37	(0.41–4.76)	0.61
Model 1			
Ureaplasma–negative × MV <2 wk (*n* = 59)	Reference	—	—
Ureaplasma–positive × MV <2 wk (*n* = 18)	0.38	(0.010–1.58)	0.14
Ureaplasma–negative × MV ≥2 wk (*n* = 28)	2.15	(1.20–6.46)	0.011*
Ureaplasma–positive × MV ≥2 wk (*n* = 17)	8.95	(2.12–73.5)	0.001**
Model 2			
Ureaplasma–negative × MV ≥2 wk (*n* = 28)	Reference	—	—
Ureaplasma–positive × MV ≥2 wk (*n* = 17)	4.17	(1.62–44.1)	0.009**

The interaction between the duration of MV and antenatal exposure to *Ureaplasma* species was analyzed by a logistic-regression analysis after controlling for GA, *Z* score, RDS, and PDA. Each aOR for the risk of moderate/severe BPD was estimated in the respective four groups of infants (with MV ≥2 wk or <2 wk ± *Ureaplasma* species), after being adjusted with the OR for *Ureaplasma*–negative × MV <2 wk (model 1) or *Ureaplasma*–negative × MV ≥2 wk (model 2), respectively.

aOR, adjusted OR; CI, confidence interval; GA, gestational age; MV, mechanical ventilation; OR, odds ratio; PDA, patent ductus arteriosus; RDS, respiratory distress syndrome.

*$P < 0.05$; **$P < 0.005$.

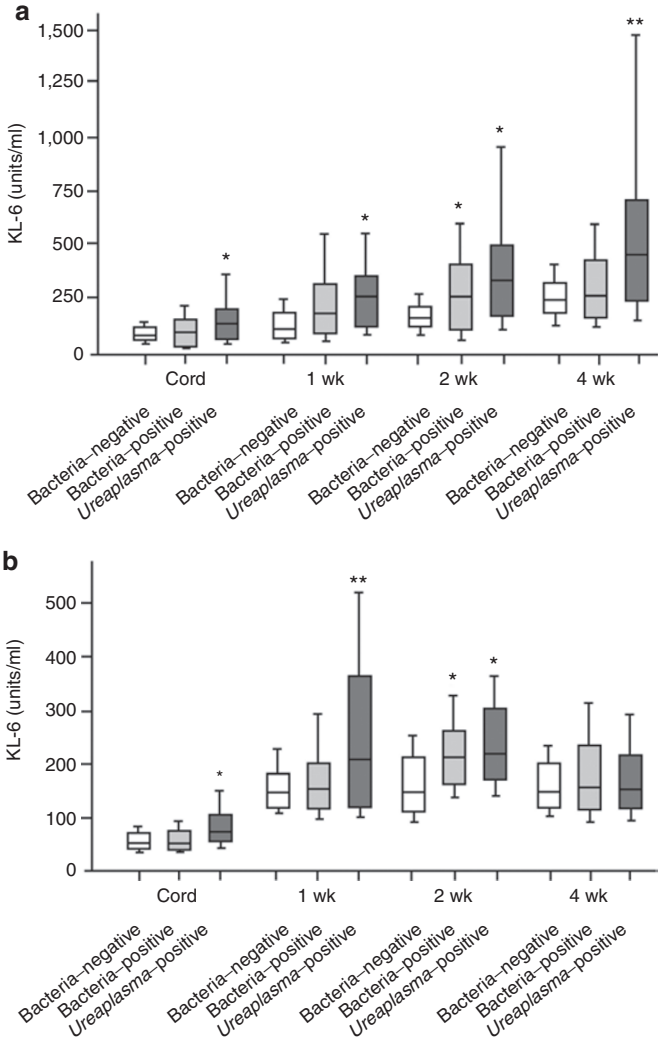

Figure 1. Comparison of median KL-6 levels within 2 wk of age. (**a**) A comparison among infants with MV ≥ 2 wk. The numbers of infants investigated (10th–90th percentiles) were 18 (15), 10 (8), and 17 (13) in the bacteria–negative subgroup, the bacteria–positive subgroup, and the *Ureaplasma*–positive group, respectively. (**b**) Comparison among infants with MV <2 wk. The numbers of infants investigated (10th–90th percentiles) were 38 (30), 21 (16), and 18 (14) in the bacteria–negative subgroup, the bacteria–positive subgroup, and the *Ureaplasma*–positive group, respectively. *$P < 0.05$, **$P < 0.005$ vs. the bacteria–negative subgroup at each time point, analyzed using the Mann–Whitney U-test. The differences in the KL-6 levels between groups were analyzed by two-way repeated-measures ANOVA ((**a**): bacteria–negative vs. bacteria–positive, $P = 0.041$; bacteria–negative vs. *Ureaplasma*–positive, $P = 0.009$; (**b**): bacteria–negative vs. bacteria–positive, $P = 0.010$; bacteria–negative vs. *Ureaplasma*–positive, $P = 0.009$). Box plots show the median values with interquartile range for KL-6, and whisker plots show the 10th and 90th percentiles of plasma KL-6. MV, mechanical ventilation.

species or other bacteria and whether the exposure to such bacteria would modulate the respiratory outcome of BPD in conjunction with subsequent MV. Our findings indicate that immature, preterm infants who are antenatally exposed to *Ureaplasma* species (but not infants exposed to other bacteria) have an increased risk of adverse respiratory outcome of BPD, in synergy with prolonged MV of ≥2 wk after birth.

The relationship between antenatal infection and subsequent development of BPD has been debated for more than two decades (3–5,19). Although the underlying mechanism is yet to be determined, recent experimental studies have provided clues. In animal studies, the intrauterine injection of endotoxin or *Ureaplasma parvum* resulted in enhanced inflammation and morphologic change in the premature lung, leading to subsequent abnormalities in lung development (5,20). A study in a baboon model clarified that intrauterine infection with *Ureaplasma* species contributes to early lung fibrosis in conjunction with MV for 14 d (21). An experimental study using

alveolar type II cells also showed that exposure to chorioamnionitis inhibits alveolar epithelial repair after lung injury (22). Taken together, these findings indicate that antenatal infection with either *Ureaplasma* species or other bacteria may inhibit alveolar epithelial repair after lung injury and remodel lung architecture, leading to developmental abnormalities. A recent clinical study reported that the placental and/or fetal inflammatory response has a protective effect against the development of BPD, presumably because of the reduced incidence of RDS and associated reduced need for MV (23). On the other hand, Marter *et al.* reported that the risk of BPD was higher in the presence of chorioamnionitis in conjunction with MV of >7 d or postnatal sepsis (24). It has therefore been postulated that chorioamnionitis in itself is not a risk factor, and it may even have a protective effect against BPD, whereas subsequent prolonged MV or postnatal infection/inflammation may increase the risk for adverse lung development, thereby leading to BPD. In the case of antenatal infection, signs of fetal inflammation, reflecting a more serious inflammatory state in the fetus, are thought to provoke an impaired surfactant response that is a characteristic of ongoing BPD (25,26). Therefore, a priming microorganism or its related signaling during antenatal infection, together with MV soon after birth may induce lung inflammation, alter the architecture of the immature lung, and compromise postnatal lung development.

We recently found that preterm newborns who tested positive for bacterial 16S rRNA in gastric fluid specimens obtained soon after birth had a higher risk of developing severe BPD (16). However, we could not clarify which microorganism (*Ureaplasma* species or other bacteria) was most frequently associated with severe BPD, and how such a microorganism induced lung injury leading to BPD. In this study, we intended to identify whether antenatal exposure to *Ureaplasma* species or other bacteria provokes lung injury leading to BPD; we also sought to investigate whether this process, if present, was induced prior to birth and thereafter enhanced merely by the exposure itself, or whether there was an interaction between prior exposure to bacteria and MV initiated soon after birth.

A univariate analysis indicated that the presence of *Ureaplasma* species (but not the presence of other bacterial species) in gastric fluid was a significant risk factor along with other potential risk factors. However, the logistic regression analysis showed that the presence of *Ureaplasma* species was no longer significant (aOR: 2.58, 95% CI: 0.89–16.3, $P = 0.12$) after controlling for other potential risk factors including GA, *Z* score, MV ≥2 wk, RDS, and patent ductus arteriosus (PDA) (**Table 3**). In another logistic regression analysis, we verified whether antenatal exposure to *Ureaplasma* species interacts with prolonged MV, and found that these two factors do combine to affect the respiratory outcome of BPD (**Table 4**). In the analysis of the group with MV ≥2 wk, the presence of *Ureaplasma* species was found to enhance the risk of moderate/severe BPD significantly (aOR: 4.17, 95% CI:1.62–44.1, $P = 0.009$) after adjusting for the risk relating to *Ureaplasma*–negative infants. This finding suggests that antenatal exposure to *Ureaplasma* species and prolonged MV may be synergistically involved in the ongoing process of BPD. Of note, in the analysis of the groups with MV <2 wk, the risk of moderate/severe BPD was much lower in the *Ureaplasma*–positive infants after adjusting for the risk relating to *Ureaplasma*–negative infants, although it was not significant (aOR: 0.38, 95% CI: 0.010–1.58, $P = 0.14$). This result may partly correlate with the finding that antenatal infection *per se* has a protective effect against BPD (23).

To clarify the effect of antenatal exposure to *Ureaplasma* species or other bacteria on premature lung tissue, we used KL-6 as a lung injury marker (because serum levels of KL-6 are known to increase after lung injury) correlating with the severity of respiratory distress in infants with BPD (18). Among the infants with MV ≥2 wk, the median KL-6 level in the cord blood of *Ureaplasma*–positive infants, was higher than in that of the bacteria–negative infants, and this difference in KL-6 levels increased time-dependently after birth (**Figure 1a**). On the other hand, the median KL-6 levels reached plateaus at 1 or 2 wk in all three groups of infants with MV <2 wk, and was subsequently lower at 4 wk (**Figure 1b**). The findings from this analysis suggest that antenatal exposure to *Ureaplasma* species induces harmful effects in the immature lung prior to birth, and exacerbates lung injury after birth in conjunction with MV ≥2 wk. Given the fact that an increase in the level of KL-6 is associated with an adverse outcome in BPD, these analysis results support our clinical findings.

Several other researchers have investigated the association between antenatal/postnatal exposure to microorganisms, including *Ureaplasma* species, and the development of BPD. Previously, van Waarde *et al.* reported that the presence of *Ureaplasma* species in tracheal aspirates was significantly associated with the development of BPD, but they found no such correlation after correction for GA (27). In their study, infants on MV with a GA <42 wk were enrolled, and BPD was defined as an oxygen requirement for 28 d after birth. Because their study design allowed them to enroll more infants with a low risk for BPD, it is likely that it underestimated the impact of *Ureaplasma* species in increasing the risk of developing BPD. Miralles *et al.* reported in their preliminary study that there was no association between the presence of microbes in neonatal and/or placental specimens and the development of BPD (15). This study has some similarities with ours, but differed in that it was designed to investigate the significance of microbes and not *Ureaplasma* species. In contrast to the findings of our study, Payne *et al.* reported that the presence of *Ureaplasma* species in gastric fluid was not related to the development of BPD, even on the basis of a univariate analysis (28). We cannot pinpoint the reasons for the opposite outcomes in the two studies; however, it would be necessary to verify several institutional factors in neonatal/maternal management and patient characteristics that affect the prognosis of BPD. For example, as compared with our study, the prevalence of maternal chorioamnionitis was much lower in the study by Payne *et al.* than in ours (20 vs. 44%). Although the prevalence of chorioamnionitis of higher grades was not indicated in their study, it is expected to have been lower than in our study (grade 2 or 3; 25%) given the overall lower rate of the condition. Because the severity of fetal inflammation/infection and

the interaction with prolonged MV would affect the prognosis of BPD, the differences between the two samples of study subjects with respect to clinical characteristics may have resulted in the differences in outcome. Colaizy *et al.* also detected *Ureaplasma* species in tracheal aspirates of infants and found an association with BPD and/or death (29). Our study was similar to theirs, but our aim was different. We intended to clarify the pathogenetic role of microorganisms, including *Ureaplasma* species, on the immature fetal lung in antenatal infection; we also intended to identify a reliable candidate for the treatment of developing BPD as early as possible after birth. An analysis using tracheal aspirate specimens makes it difficult to examine such a causal relationship, and often needs a careful interpretation. This is because the analysis of tracheal aspirate specimens may underestimate the rate of *Ureaplasma* species within 1 wk of life, as we have reported previously (16). Gastric aspirate specimens can be easily obtained soon after birth, even from critically ill infants from whom tracheal aspirate specimens often cannot be obtained. Taken together, our method of using gastric fluid specimens instead of tracheal aspirate specimens is therefore more beneficial in identifying treatment approaches for BPD in the future.

Recently, Ballard *et al.* performed a double-blind, placebo-controlled, randomized trial of azithromycin in preterm infants who were at risk of BPD and reported an improvement of the BPD rates in the *Ureaplasma* subgroup after treatment with azithromycin (30). However, routine use of azithromycin therapy for the prevention of BPD was not recommended because of the lack of a large-scale trial and an optimized treatment regimen. Our findings will contribute to identifying preterm infants at risk of BPD who could benefit the most from the prophylactic administration of azithromycin. These findings could therefore be applicable in future randomized controlled trials.

METHODS

The study protocol was approved by the ethics committee of Osaka Medical College, and informed consent was obtained from the parents of all the subjects.

Subjects

Preterm infants with GA <29 wk or birth weight <1,000 g, admitted to the neonatal intensive care unit of Osaka Medical College Hospital between January 2007 and March 2010, were eligible for this study. The exclusion criteria were: (i) complicated congenital heart diseases; (ii) multiple malformations; or (iii) documented chromosomal anomalies. Infants who died of nonrespiratory causes, those transferred to another facility before reaching a postmenstrual age of 36 wk, and those from whom gastric fluid or blood specimens were not available were also excluded. According to the results of gastric fluid tests carried out to determine the presence or absence of the urease gene soon after birth, the enrolled infants were allocated to a *Ureaplasma*–positive group or a *Ureaplasma*–negative group. The *Ureaplasma*–negative infants were further classified into two subgroups, bacteria–positive and bacteria–negative, according to the presence or absence of 16S rRNA genes.

Synchronous intermittent MV was initiated in infants who had respiratory distress and, if this failed to maintain the study criteria, high-frequency oscillation ventilation was started. Synchronous intermittent MV was delivered by volume-guaranteed, assist control mode (Babylog 8000; Dräger, Lübeck, Germany), with an initial setting of the tidal volume at 3–5 ml/kg, inspiratory time of 0.3–0.4 s, rate of 40–60/min, maximum peak inflating pressure of 25 cm H_2O, and positive end expiratory pressure of 5 cm H_2O. High-frequency oscillation ventilation was delivered using the Babylog 8000 or Calliope α (Metran, Kawaguchi, Japan), with an initial mean airway pressure of 12–14 cm H_2O. Ventilator settings and oxygen therapy were adjusted to maintain study criteria of pH ≥7.20, arterial oxygen saturation as measured by pulse oximetry (SpO_2) between 88 and 95%, and partial pressure of carbon dioxide (pCO_2) between 40 and 60 mmHg.

BPD was defined in accordance with the National Institutes of Health consensus definition for infants with GA <32 wk (fraction of inspired oxygen (FiO_2) >0.21 for at least 28 d). At a postmenstrual age of 36 wk, the infants were classified into the following three subgroups: mild BPD (FiO_2 = 0.21), moderate BPD (0.21< FiO_2 <0.30), and severe BPD (FiO_2 ≥0.30 and/or positive pressure assistance) (31). Intubated infants who needed FiO_2 ≥0.4 to maintain SpO_2 ≥90% and had typical radiographic features of RDS were diagnosed as having RDS. All infants on MV who required FiO_2 >0.60 to maintain SpO_2 >88% because of respiratory failure after the first week of life received low-dose dexamethasone (0.89 mg/kg over 10 d), as reported previously (32). If these ventilated infants required FiO_2 ≥0.60 to maintain SpO_2 >88% despite the initial low-dose dexamethasone treatment, further dexamethasone treatment was administered over 7 d (0.50 mg/kg/d for 3 d, 0.25 mg/kg/d for 3 d, and 0.10 mg/kg/d for 1 d). Chorioamnionitis was diagnosed histologically if polymorphonuclear leukocytes were seen in the fetal membranes, and its severity was graded according to Blanc (33). Infants who were treated with indomethacin or underwent ligation were diagnosed as having PDA.

Detection of *Ureaplasma* species and other bacteria

We inserted nasogastric tubes into all the infants to collect gastric fluids within the first hour after birth and used them for the extraction of DNA, as reported previously (16). The bacterial 16S rRNA gene was detected using the methods described previously (15,16). Briefly, the extracted DNA was amplified by PCR using the FD1 (AGA GTT TGA TCC TGG CTC AG) and rP1 (ACG G(T/A/C)T ACC TTG TTA CGA CTT) primers. After PCR amplification, 1 μl of the reaction product was re-amplified using another pair of primers, FD1 and rD2 (G(T/A)A TTA CCG CGG C(G/T)G CTG). For detection of *Ureaplasma* species, the extracted DNA was amplified by PCR with the primer pair U5 (CAA TCT GCT CGT GAA GTA TTA C) and U4 (ACG ACG TCC ATA AGC AAC T) to detect the urease gene, as reported previously (34).

Measurement of KL–6

Blood samples were collected from cord blood at birth and also by venipuncture at 1, 2, and 4 wk of age, after parental consent was obtained. The blood samples were immediately centrifuged at 3,000*g* for 10 min at 4 °C to obtain plasma, which was stored at −80 °C until analysis. The serum KL-6 levels were measured using a chemiluminescent enzyme immunoassay kit (Eidia, Tokyo, Japan).

Statistical Analysis

Differences between any two groups were assessed using the Mann–Whitney U-test for continuous variables or Fisher's exact test for categorical data. Differences among three groups were assessed using the Kruskal–Wallis test for continuous variables or the χ^2 test for categorical data. The variables associated with moderate/severe BPD in the univariate analysis ($P < 0.10$) were included in a logistic-regression analysis. Birth weight was not included because of its close correlation with GA ($\chi^2 = 0.79$). The first logistic-regression model included GA, *Z* score, *Ureaplasma* species, MV ≥2 wk, RDS, and PDA (**Table 3**). The second logistic-regression model was constructed to examine the interaction between *Ureaplasma* species and MV. This model included the GA and *Z* score as continuous variables, PDA and RDS as categorical variables, and MV (<2 wk or ≥2 wk) ± *Ureaplasma* species as a discrete variable (**Table 4**). For the comparison of KL-6 levels between groups, two-way repeated-measures ANOVA (time-dependent change) and the Mann–Whitney U-test (comparison at each time point) were used for the analysis. Data were analyzed using the JMP 9.0 statistical software package (SAS Institute, Cary, NC). In all analyses, $P < 0.05$ was considered to be significant.

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