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Role of Pulmonary Infection in the Development of Chronic Lung Disease of Prematurity

Michael L. Beeton*, Nicola C. Maxwell*, Philip L. Davies, Diane Nuttall, Eamon McGreal, Mallinath Chakraborty, O. Brad Spiller and Sailesh Kotecha.

Department of Child Health, School of Medicine, Cardiff University, Cardiff, United Kingdom

Corresponding Author:

Professor Sailesh Kotecha

Department of Child Health

School of Medicine

Cardiff University

Cardiff

CF14 4XN

United Kingdom

Tel: +44 (0)29 20 74 4187

Fax: +44 (0)29 20 74 4283

kotechas@cardiff.ac.uk

* These two authors contributed equally.

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Abstract

Aim: We studied the role of ante- and postnatal infection in the development of chronic lung disease of prematurity (CLD).

Methods: 192 newborn infants (61 term, 131 preterm of <34 weeks gestation: 88 with respiratory distress syndrome, 35 developed CLD and 8 died) were recruited. 16S rRNA genes were identified by PCR of DNA isolated from 840 gastric and lung fluid samples. *Ureaplasma* was also cultured.

Results: Presence of 16S rRNA genes (Odds Ratio ± 95%CI 1.6, 1.2 – 2.2) and *Ureaplasma* (OR 3.6, 1.7 – 7.7) was significantly associated with the development of CLD. This association remained if the 16S rRNA genes and *Ureaplasma* were first identified within the first three days of life (OR 2.4, 1.4 – 4.1 and 3.8, 1.4 – 10.0, respectively) or if first identified after three days of age (OR 1.7, 1.1 – 2.8 and 5.1, 1.3 – 19.8 respectively). Peak lung fluid interleukin-6 and interleukin-8 were significantly associated with presence of microbes ($p<0.0001$ and $p=0.0001$ respectively) and development of CLD ($p=0.003$ and 0.001 respectively).

Conclusion: Both early and late microbial presence in neonatal lung fluid samples was significantly associated with the development of CLD suggesting that both ante- and post-natal infection play a role in the development of CLD.

Word count for Abstract: 200 words (after deletion)

Keywords: bronchopulmonary dysplasia, chronic lung disease of prematurity, *Ureaplasma*, 16S rRNA genes, inflammation, infection.

Introduction

Chronic lung disease of prematurity (CLD) is a significant cause of morbidity and mortality in preterm infants. Although its risk factors have been defined, its exact aetiology remains unknown. Recently antenatal infection has received much attention (1,2) in the development of CLD; however, it is uncertain if *pulmonary* postnatal infection especially nosocomial infection also has a role to play in the development of CLD. *Ureaplasma*, which is thought to be acquired antenatally, appears to be associated with the development of CLD (3,4). In her case-control study of 193 infants with birth weights of <1500g, van Marter and colleagues (5) reported an association between *systemic* infection acquired postnatally and development of CLD; findings later confirmed by Hernandez-Ronquillo and colleagues in a smaller case-control study (6). Few studies have reported postnatal microbiological findings of infection and their role in the development of CLD (7,8). Identification of microbes is hindered by current microbiological methods and by frequent use of antibiotics in this high risk population thus making identification of organisms difficult by standard culture methods. However, the identification of microbial presence by polymerase chain reaction (PCR) amplification of microbe-associated 16S ribosomal RNA genes using universal bacterial primers shows promise as reported by our group and others (8,9). We hypothesised that postnatal infection plays a role in the development of CLD. In this study, (a) we aimed to demonstrate if there was an association between microbial presence identified by amplification of 16S rRNA; (b) for completeness we also searched for *Ureaplasma* by culture (9,10) in gastric and lung fluid samples. (c) We also compared early i.e. microbial presence within three days of birth and late, i.e. microbial presence after three days of birth, to the development of CLD. (d) We also examined if microbial presence in these samples was associated with increased pro-inflammatory cytokines namely interleukin-6 (IL-6) and interleukin-8 (IL-8) which we have previously shown to be increased in the presence of 16S rRNA genes (9).

Methods

Patient groups

192 newborn infants were recruited from the Regional Neonatal Unit at University Hospital of Wales, Cardiff including 61 term (≥37 weeks gestation) and 131 preterm (≤34 weeks gestation). In the preterm group, 88 infants developed and recovered from respiratory distress syndrome (RDS); 35 developed CLD (defined pragmatically as oxygen dependence at 36 post-menstrual age) and 8 infants died before 28 days of age. Gentamicin and penicillin was used as first line therapy in all infant who were suspected of respiratory illness on admission to the neonatal unit and antibiotic therapy was modified according to the clinical status of the infant by the attending clinicians.

Sample collection

Gastric fluid was obtained wherever possible. Infants admitted to the neonatal unit routinely have a naso- or oro-gastric tube inserted for feeding and for deflating the stomach. Gastric fluid was obtained within 2 hours of birth from infants who had not been fed previously and used to culture for *Ureaplasma* and for isolation of DNA for identification of 16S rRNA genes and for *Ureaplasma* urease gene (7,11). Infants who received invasive mechanical ventilation for respiratory disease underwent non-bronchoscopic bronchoalveolar lavage as described in the on line supplement (12-15) or tracheal aspirates (16,17) at the time of routine suctioning as previously described. For those who received continuous positive airways pressure (CPAP) ventilation or were self-breathing in air or increased ambient oxygen, nasopharyngeal aspirates (NPA) were obtained. Wherever possible, the lung fluid samples were obtained at the time of routine suctioning especially in infants receiving invasive mechanical ventilation. Samples were obtained daily for the first week then twice weekly until 28 days of age or until discharge whichever occurred earlier. Samples were placed at 4^{o}C

until processing within 12 hours of collection. The study was approved by the local research ethics committee and written informed consent was obtained from the parents.

Detection of *Ureaplasma* urease gene and polymerase chain reaction (PCR) and sequencing of 16S rRNA genes

DNA was extracted from samples using a commercially available Qiagen DNA/RNA extraction kit (Crawley, UK). Sample integrity of extracted DNA was determined using PCR with primers designed against human mitochondrial cytochrome oxidase (HMCO) gene as previously described (18). At least one satisfactory sample for DNA isolation to identify 16S rRNA genes was available from 164 infants. 16S rRNA genes positive samples were identified using the primer set 27f (AGA GTT TGA TC(AC) TGG CTC AG) and 1492r (TAC GG(CT) TAC CTT GTT ACG ACT T) to give the 1465 bp product and sequenced as previously described (19). *Ureaplasma* spp. was determined by amplification of a 430 bp DNA product using Blanchard's U4 (ACG ACG TCC ATA AGC AAC T) and U5 (CAA TCT GCT CGT GAA GTA TTA C) primers as previously described (7, 20).

Culture based detection of *Ureaplasma* spp.

25 µl of the sample was inoculated into 2 ml of *Ureaplasma* selective media (Mycoplasma experience, Surry, UK), incubated at 37^{o}C and checked for an increase in pH for a period of 5 days (10). Positive samples were confirmed by the *Ureaplasma* specific PCR as described above.

IL-6 and IL-8 ELISA

IL-6 and IL-8 were measured in duplicate in BAL fluid samples (9), from 63 infants including 11 term infants, 21 from the RDS group, 24 who developed CLD and 7 (6 for IL-8) who died,

by ELISA. Further details are given in the on line supplement.

Statistical analyses

Non-parametric tests including Mann-Whitney and Kruskall-Wallis were used to compare two and multiple groups respectively and Fishers exact test was used to compare tabular data (using SPSS version 15.0). A p-value of <0.05 was considered significant.

Results

Patient characteristics

Patient characteristics are shown in Table 1. Further details of admissions and causes of deaths are given on the on-line supplement. A total of 840 samples were obtained from 192 babies who were enrolled to the study including 61 who were born at term (gestation ≥37 weeks) and 131 preterm infants of ≤34 weeks gestation from which 88 developed and recovered from RDS, 35 developed CLD (defined pragmatically as oxygen dependence at 36 post-menstrual age) and 8 died. Of the 840 samples collected, 88 were from the term group, 408 from infants with RDS, 309 from the CLD group and 35 from babies who died.

Association between postnatal microbial genes and development of CLD

When the association of presence of pulmonary microbial genes and development of CLD samples was examined, 77% of the infants in the CLD group were positive compared with 48% of the RDS infants and 29% of term infants (Odds Ratio ± 95% confidence intervals: 1.6, 1.2 – 2.2, p=0.004) (Table 2). An association was noted between the presence of pulmonary *Ureaplasma* and development of CLD as shown in Table 2. Of the 35 babies in the CLD group, 37% were positive for *Ureaplasma* compared to 10% of RDS babies and 15% of term infants. When the preterm groups who developed either RDS or CLD were compared, the association with development of CLD and presence of *Ureaplasma* was highly statistically significant (OR ± 95% CI, 3.6, 1.7 – 7.7, p=0.0004).

In order to determine if the microbes were acquired antenatally or nosocomially, we divided the samples into those which were <u>first</u> noted to be positive on or before three days of age or after three days of age. The association between day of detection of colonisation with *Ureaplasma* and other microbes relative to development of CLD is shown in Table 3a and 3b,

respectively. There was a significant association between 16S rRNA genes and *Ureaplasma* and development of CLD if first identified within the first three days of life (OR ± 95% CI, 2.4, 1.4 – 4.1, p=0.003 and 3.8, 1.4 – 10.0, p=0.005, respectively) and also if first identified after 3 days of age (OR ± 95% CI, 1.7, 1.1 – 2.8, p=0.036 and 5.1, 1.3 – 19.8, 0.01 respectively).

Relationship between IL-6 and IL-8 with microbial genes and clinical outcome

The peak results for each individual infant were correlated with the presence or absence of either 16S rRNA genes or *Ureaplasma* as shown in Figures 1a and 1b. Samples positive for either microbial genes or *Ureaplasma* had median BAL fluid IL-6 and IL-8 values of 12604 pg/ml and 97358 pg/ml, respectively which were 6-fold and 4-fold greater than the median values in the microbial negative samples (2074 pg/ml and 21970 pg/ml, respectively, both $p<0.0001$). When only samples from the first three days of age were examined a significant relationship was also noted between IL-6 ($p<0.05$) and IL-8 concentrations ($p<0.05$) and presence of microbes within the first three days of life (figure 1c and 1d).

The peak values for the IL-6 and IL-8 for each diagnostic group, together with presence of absence of microbial presence or absence, are shown in Figure 2. BAL fluid samples from the term group had the lowest IL-6 and IL-8 median values of 980 and 12107 pg/ml, respectively. RDS infants had median IL-6 and IL-8 levels of 5170 and 17502 pg/ml, respectively whereas CLD babies had median levels of 15594 and 122550 pg/ml, ($p<0.005$ and $p<0.0001$, respectively comparing RDS vs. CLD groups).

Microbes associated with peaks in IL-6 and IL-8

Amplicons from 16S rRNA gene positive samples which coincided with peaks in IL-6 and IL-

8 were sequenced to determine the predominant microbial species (Table 4). *Staphylococcus epidermidis* was the most prevalent organism which was isolated from infants who went on to develop RDS with one peak of IL-6/IL-8 being associated with *Fusobacterium nucleatum*. Gram-negative organisms were more frequently associated with IL-6/IL-8 peaks in CLD infants and included *Escherichia coli*, *Haemophilus influenzae*, *Enterobacter* spp. and *Pseudomonas. aeruginosa*, although Gram-positives including *S. epidermidis* and *Staphylococcus aureus* were also identified. Twice as many *U. parvum* (2 RDS and 4 CLD) were isolated relative to *U. urealyticum* (1 RDS and 2 CLD) in both RDS and CLD babies.

Discussion

In this study, we examined over 800 lung and gastric fluid samples from 192 newborn infants and identified microbial genes by using universal bacterial primers to assess the role of postnatal infection in the development of CLD. We also investigated if the proinflammatory cytokines IL-6 and IL-8 were increased to suggest whether the microbial gene presence was indeed likely to be an infective process rather than a colonisation one. The results show that the presence of 16S rRNA genes was increased in babies developing CLD when compared to those who did not; the association was greatest when the microbial genes were detected within the first three days of life but remained significant even when 16S rRNA genes were first detected beyond three days of age. The microbial presence is likely to be due an infective process as both IL-6 and IL-8 were markedly increased in lung fluid samples from infants colonised with microbial genes. Finally, we confirm previous findings implicating *Ureaplasma* in the development of CLD (3,4).

Postnatal infection is strongly suspected in the development of CLD. Van Marter and colleagues in a case control study identified postnatal *systemic* infection, as assessed by systemic features, as a risk factor for the development of CLD (5). Similar findings have been reported by Hernandez-Ronquillo and more recently Lahra (6,21). However, these studies used epidemiological methods to suspect postnatal *systemic* infection in the development of CLD. Few studies have prospectively investigated this association of postnatal *pulmonary* infection and CLD by identification of microbial genes as we have in this study with a large number of prospectively collected samples from 192 newborn infants. Our findings suggest that 16S rRNA genes if first identified within three days of birth, suggesting antenatal acquisition, were strongly associated with the development of CLD. The association remained if microbes were first detected beyond three days of age, suggesting postnatal acquisition.

The presence of pulmonary *Ureaplasma* was similarly associated with the development of CLD; again the association remaining strongest for samples collected within three days of birth. *Ureaplasma* spps. were detected in 13 out of 35 (37%) babies who developed CLD compared to 9 out of 88 (10%) of RDS babies and 9 out of 61 (15%) term infants. The association between the presence of pulmonary *Ureaplasma* and development of CLD remained if it was first acquired after three days of age (Table 3a). Current evidence, however, suggests that *Ureaplasma,* even if first identified at few days of age, is likely to be acquired antenatally. These data support the conclusions of the meta-analyses by both Wang and Schelonka that there is an association between the development of CLD and presence of pulmonary colonisation of this organism (3,4). Nevertheless, controversy continues regarding the relevance of pulmonary colonisation by *Ureaplasma* in preterm infants as increased vaginal colonisation is observed in mothers who deliver preterm when compared to those who deliver at term (22). Its true significance in the development of CLD can only be confirmed by an adequately powered randomised control trial to determine if its eradication can decrease the rates of CLD (23,24). Our data also suggest that late manifestation of *Ureaplasma*, whether acquired antenatally or not, will need to be considered in the causation of CLD and be accounted for in any future interventional trials investigating if eradication of pulmonary *Ureaplasma* decreases the rates of CLD.

An interesting observation was that 41% of infants born at 25 weeks were colonised with *Ureaplasma*, all of whom developed CLD, whereas 42% of those born at 26 weeks were colonised but only 18% developed CLD. This implies that the most immature infants are at risk of acquiring early microbes – whether this is due to immaturity of the infant's immune system is speculative. Furthermore, the vast majority of the microbes were acquired in the

first three days of age thus also suggesting that the vast majority of infections are likely to be acquired *in utero* although in this study no differences were noted for preterm prolonged rupture of membranes. Unfortunately comprehensive data on placental histology was not available to further investigate this early association between the development of CLD and chorioamnionitis although our previous study did support this association (9).

The presence of microbial genes may simply reflect pulmonary colonisation especially in those infants who have an endotracheal tube *in situ*. Thus, although it has limitations, we measured IL-6 and IL-8 in BALF from those infants who underwent non-bronchoscopic - bronchoalveolar lavage (13-15) to minimise contamination. The increased BALF IL-6 and IL-8 imply that pulmonary inflammation is increased in babies who were positive for microbial genes thus suggesting an infective process rather than microbial colonisation. We have previously reported this association between IL-6 and IL-8 with 16S rRNA gene positivity and development of CLD by examining intrauterine tissue samples and BAL fluid from a cohort of 41 preterm infants (9). Furthermore, we have previously shown that concentrations of IL-6 and IL-8 were significantly higher on the first day of life in *Ureaplasma* infected babies compared to those who were negative (7). The significance of high IL-8 levels in BAL samples could result in the recruitment of neutrophils, the predominant inflammatory cell in CLD lungs (13). The viability of the isolated *Ureaplasma* was confirmed by culture but the presence of 16S rRNA genes may simply reflect non-viable organisms as these infants on the whole were on regular antibiotic treatment especially preterm ones during the first week of life.

It was interesting to note that the CLD infants were colonised more frequently with more virulent organisms including gram negative ones such as *E. coli, H. influenzae, Entrobacter*

Spp and *P. aeruginosa* than infants with RDS. The pro-inflammatory nature of the Gram-negative endotoxin lipopolysaccharide (LPS) has been shown in a preterm lamb model to contribute to lung injury (25). Ventilated lambs, born to pregnant ewes who had received intra-amniotic LPS, had increased inflammatory markers and fewer alveoli compared to ventilated controls. *S. epidermidis* was prevalent in a number of both RDS and CLD infants. Although it is often thought to be a commensal and contaminant, Lahra *et al* reported that postnatal systemic infection with *S. epidermidis* had similar effect on the development of CLD as more virulent organisms (21).

This study has added further evidence for the role of postnatal infection in development of CLD. It highlights the possible significance of antenatal acquisition of infection by determining the presence of organisms within three days of life in correlation with high levels of inflammatory mediators IL-6 and IL-8. It also suggests that nosocomially acquired infection is also likely to contribute to the development of CLD.

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	CLD	RDS	Term	Died
Number of patients	35	88	61	8
Number of samples	309	408	88	35
GF:BAL:NPA:ET	10:260:9:30	69:73:236:30	51:37:0:0	4:22:3:6
Gestational age (weeks ± range)**	26^{+5} (23^{+4} – 31^{+6})	30^{+5} (26 – 34)	38^{+5} (37 – 42^{+1})	26^{+6} (25^{+6} – 30^{+3})
Birth weight (Kg ± range)**	1.03 (0.53 – 2.73)	1.50 (0.61 - 3.41)	2.92 (0.91 – 4.35)	0.87 (0.55 - 1.49)
Male:Female	18:17	41:47	41:20	6:2
Vaginal: Caesarean Delivery	19:16	24:64	33: 28	3:5
Antenatal steroids >24 hours†	22/35 (63%)	71/88 (81%)	2/61 (3.3%)	7/8 (88%)
Surfactant†	35/35 (100%)	43/88 (49%)	1/61 (2%)	8/8 (100%)
pPROM	6/35 (17%)	15/88 (17%)	6/61 (10%)	1/8 (13%)
Ventilation (IPPV:CPAP:SV)	35:0:0	47:28:13	19:13:29	8:0:0

Table 1. Patient characteristics. Values are median or proportion of total patients within each group. **Abbreviations**: GF: gastric fluid; BAL: bronchoalveolar lavage; NPA: nasopharyngeal aspirate; ET**:** entotracheal aspirate; pPROM: prolonged preterm rupture of membranes. IPPV: intermittent positive pressure ventilation; CPAP: continuous positive airway pressure; SV: self ventilating. ** $p<0.001$ when groups compared and †$p<0.001$ when term infants compared to the CLD, RDS and died groups.

A.

	16S rRNA genes detected	**16S rRNA genes not detected**	**TOTAL**
Term	14 (29%)	34 (71%)	**48**
RDS	35 (48%)	38 (52%)	**73**
CLD	27 (77%)	8 (23%)	**35**
Died	2 (25%)	6 (75%)	**8**
Total	**78**	**86**	**164**

B.

	***Ureaplasma* detected**	***Ureaplasma* not detected**	**TOTAL**
Term	9 (15%)	52 (85%)	**61**
RDS	9 (10%)	79 (90%)	**88**
CLD	13 (37%)	22 (63%)	**35**
Died	3 (38%)	5 (62%)	**8**
Total	**34**	**158**	**192**

Table 2 shows the relationship between the presence of (A) 16S rRNA genes and (B) *Ureaplasma* spp. with clinical outcome. For comparison for positive and negative 16S rRNA genes for the RDS and CLD groups, p=0.004; and for *Ureaplasma* positive and negative for the RDS and CLD groups, p=0.0004. NB: Sufficient DNA could only be extracted from 48/61 of Term infants and 73/88 RDS infants due to limited cell numbers.

A.

	16S rRNA genes positive ≤ 3 days	16S rRNA genes positive >3 days	16S rRNA genes negative	Total
RDS	14 (19%)	21 (29%)	38 (52%)	**73**
CLD	14 (40%)	13 (37%)	8 (23%)	**35**
Total	**28**	**34**	**46**	**108**

B.

	Ureaplasma positive age ≤ 3 days	*Ureaplasma* positive age >3 days	*Ureaplasma* negative	Total
RDS	6 (7%)	3 (3%)	79 (90%)	**88**
CLD	8 (23%)	5 (14%)	22 (63%)	**35**
Total	**14**	**8**	**101**	**123**

Table 3. **Comparison of (A) 16S rRNA genes and (B) *Ureaplasma* with *first* positive identification at ≤ 3days of age or >3 days of age with negative samples. Comparisons were made between the early and late groups with the 16s rRNA gene negative group. Results are discussed in the text.**

CLD		RDS	
Gram-positive	Incidence	Gram-positive	Incidence
Staphylococcus epidermidis	4	*Staphylococcus epidermidis*	3
Streptococcus haemolyticus	1		
Gram-negative		Gram-negative	
Escherichia coli	2	*Fusobacterium nucleatum*	1
Haemophilus influenzae	1		
Enterobacter spp.	1		
Pseudomonas aeruginosa	1		
Other		Other	
U. parvum	4	*U. parvum*	2
U. urealyticum	2	*U. urealyticum*	1
Mixed	2		

Table 4. **Microbes associated with peaks concentration of IL-6 and IL-8 from BALF samples.**

Peak levels of IL-6 and IL-8 from BAL fluids from preterm infants were correlated with the presence of infection within a day of the peak value. 16S rRNA gene positive samples were then sequences to identify the infecting organism. The number of samples containing each organism is indicated.

Figure 1. Association between peak (A) IL-6 and (B) IL-8 concentrations and presence of microbial genes in BAL fluid samples. (C) Peak IL-6 and (D) IL-8 within three days of age. IL-6 and IL-8 were $\log_{10}$ transformed.

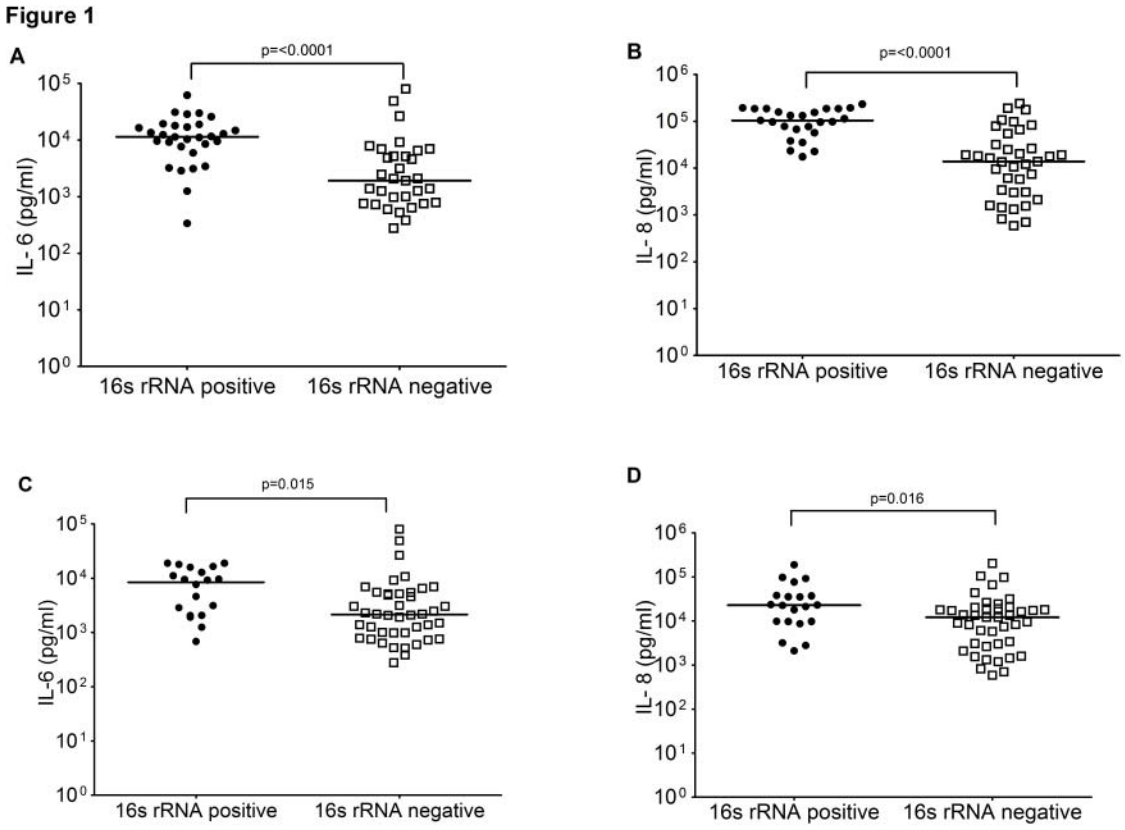
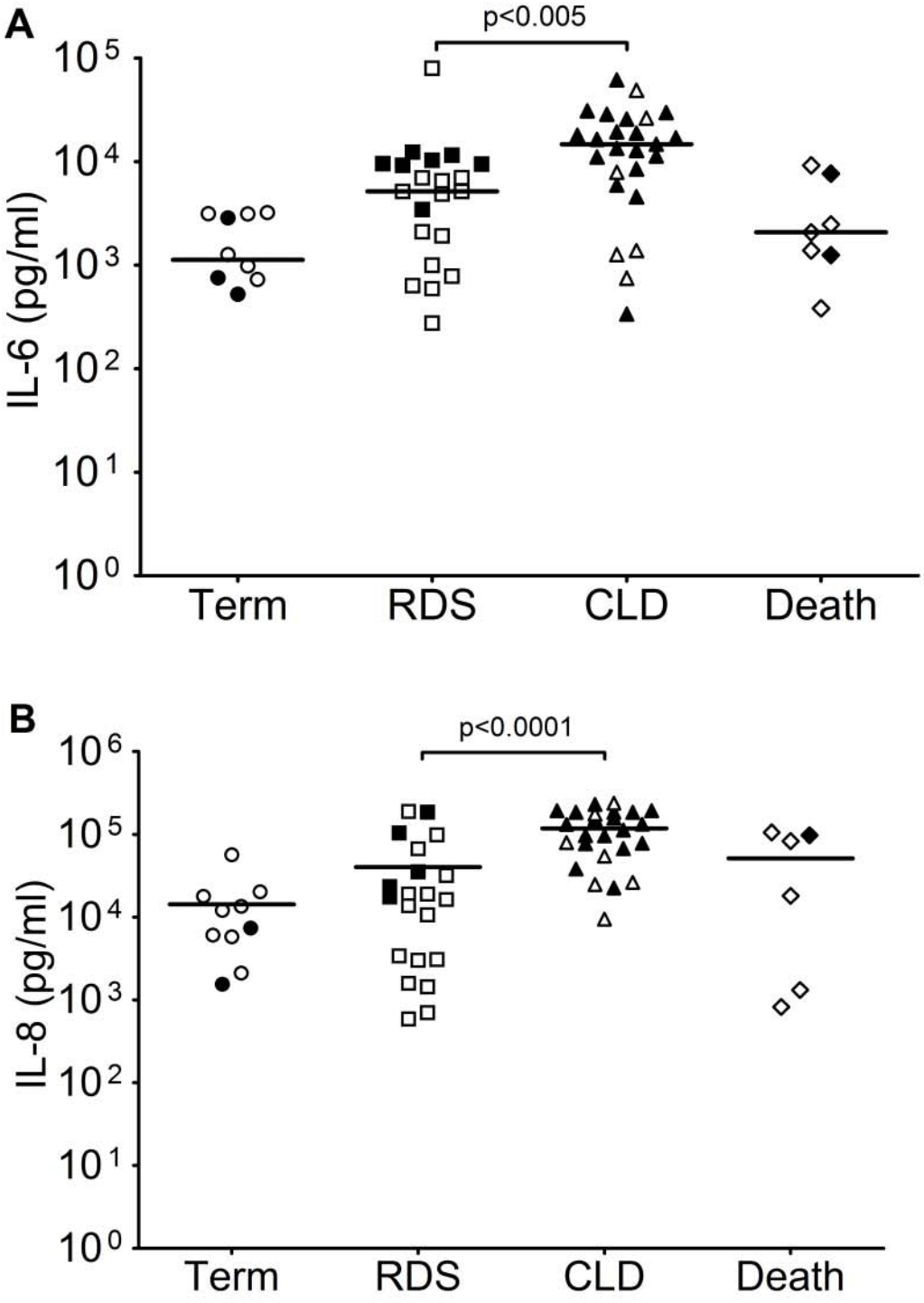


Figure 2. Peak levels of IL-6 (A) and IL-8 (B) from BAL samples for term, RDS, CLD and infants who died. Open shapes show infants negative for 16S rRNA genes and closed shapes represent infants' positive for 16S rRNA genes. IL-6 and IL-8 were $\log_{10}$ transformed.

Figure 2


A
p<0.005
IL-6 (pg/ml)
10^5
10^4
10^3
10^2
10^1
10^0
Term
RDS
CLD
Death
B
p<0.0001
IL-8 (pg/ml)
10^6
10^5
10^4
10^3
10^2
10^1
10^0
Term
RDS
CLD
Death