academicJournals

Vol. 8(27), pp. 2631-2638, 2 July, 2014
DOI: 10.5897/AJMR2014.6793
Article Number: 08271E945864
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Detection and biovar discrimination of *Ureaplasma urealyticum* colonization in preterm neonates under ventilation and correlation with bronchopulmonary dysplasia

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Received 24 March, 2014; Accepted 2 June, 2014

Despite numerous studies, controversy regarding the association between *Ureaplasma urealyticum* colonization of premature neonates and bronchopulmonary dysplasia (BPD) still exists. The aim of the present study was to determine the colonization rate of preterm ventilated neonates with the two *U. urealyticum* biovars, parvo and T960, and to assess the correlation between colonization and the development of BPD. A prospective study was done from February 2013 to January 2014 in neonatal intensive care unit (NICU) in Mansoura University Children Hospital (MUCH) and NICU of different specialized medical centers, with 100 ventilated premature neonates (< 34 weeks) in the first 24 h of life, where tracheal secretions were aspirated, transported and cultured on 10B broth and A7 agar media. Culture was positive in 10 out of 85 samples (11.8%) as compared to 15/85 (17.6%) samples obtained by PCR technique. PCR assay demonstrated that Parva biovar (biovar 1) was the predominant, found in 9 (60%) out of 15 Ureaplasma isolates, as compared to T960 biovar (biovar 2) in 6 (40%) isolates. None of the neonates were co-colonized with both biovars. There was a statistically significant difference in the mean gestational age and mean birth weight between neonates with positive Ureaplasma colonization and neonates without colonization. BPD was significantly higher among colonized neonates than non-colonized one, 12 (80%) of 15 colonized neonates developed BPD as compared to 21 (30%) of 70 non colonized neonates. BPD was found to be correlated to decreasing gestational age (r = -0.341, p=0.001), low birth weight (r = -0.328, p= 0.002), and Ureaplasma colonization (r = 0.391, p < 0.001). Logistic regression analysis revealed that Ureaplasma colonization was a more important predictor for development of BPD than decreasing gestational age. BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1) (83.3% vs. 77.7%) with statistical significant difference (p = 0.001). colonization of respiratory tract by *U. urealyticum*, particularly biovar 2 (T960 biovar) in premature ventilated neonates was related to the development of BPD. PCR is a sensitive and specific technique for detecting Ureaplasma and for distinguishing its biovars (Parvum and T960) directly from clinical samples.

Key words: *Ureaplasma urealyticum*, bronchopulmonary dysplasia, chronic lung disease, prematurity.

INTRODUCTION

Ureaplasma spp. are members of class *Mollicutes* that colonize human mucosal surfaces of the respiratory and urogenital tracts (Waites et al., 2005). Data from 16srRNA sequencing led to classification of *Ureaplasma* serovars into two biovars; (*Ureaplasma parvum*, biovar 1, parvo) and (*Ureaplasma urealyticum*, biovar 2, T960) including a total of 14 serovars, biovar 1 included serotypes 1, 3, 6 and 14, and the remaining 10 serovars belonged to biovar

2 (Robertson et al., 2002). Characteristics of all serovars include lack of cell walls, limited biosynthetic abilities, small genome size, and mucosal association in the human host (Viscardi, 2010). The unique characteristic of *Ureaplasma* is their ability to hydrolyze urea to generate metabolic energy (Waites et al., 2005, 2009). Some debates still occur regarding whether there is a difference in pathogenicity among these 14 serovars and 2 biovars (Sung, 2010).

Although *U. urealyticum* is a common commensal microorganism in the genital tract of sexually mature women (Abele-Horn et al., 1997a; Cunliffe et al., 1996), it is strongly associated with genitourinary syndromes and pregnancy complications such as chorioamnionitis, stillbirth, preterm delivery, neonatal morbidity and perinatal death (Abele-Horn et al., 2000; Yoon et al., 2001; Waites et al., 2005; Viscardi, 2010). They are the most common perinatally acquired pathogens in preterm infants (Viscardi, 2010). It appears that in-utero infection of the fetus is common, but neonates may also be colonized initially at the time of delivery (Waites et al., 2009).

A number of studies attempted to relate *U. urealyticum* colonization to the development of respiratory diseases in premature newborns and mostly with bronchopulmonary dysplasia (BPD).

Bronchopulmonary dysplasia (BPD) or previously known as chronic lung disease (CLD) of the newborn is a serious problem among very low-birth weight infants and has become even more so due to the increased survival of more immature infants (Bancallari et al., 2003; Jobe and Bancalari, 2001). It has been suggested that development of BPD (CLD) is related to pulmonary immaturity, oxidant injury due to high levels of inspired oxygen, and volutrauma associated with mechanical ventilation. However, recent research has focused on the roles of perinatal infection and the inflammatory response as critical factors influencing chronic lung injury (De Dooy et al., 2001; Lyon, 2000). Particular attention has been given to the role of *Ureaplasma* species, found in the lower genital tracts of 40 to 80% of asymptomatic women (Cassell et al., 2001).

Since BPD etiology is multifactorial and complex, the relationship of Ureaplasma respiratory tract colonization with the development of BPD has been debated (Kallapur et al., 2013). The distinction of *U. urealyticum* and *U. parvum* species could also open new perspectives of study.

U. urealyticum is among the less frequently diagnosed respiratory pathogens in a clinical environment, mainly because of the lack of standardized and specific diagnostic tests (Blanchard et al., 1993). Routine bacterial cultures are not sufficient to recover *Ureaplasma* species. It is difficult, expensive, needs special culture media and growth conditions. Furthermore, it is time consuming, and requires repeated observations with light microscopy (Mallard et al., 2005; Petrikkos et al., 2007). In newborns, an additional problem is the necessity of antibiotic administration before the diagnostic procedures are started. Antibiotics inhibit the growth of ureaplasmas, thus limiting the reliability of the culture method (Biernat-Sudolska et al., 2006).

Currently, polymerase chain reaction (PCR) appears to be the most promising method that have replaced culture for the detection of mycoplasmas from clinical specimens (Biernat-Sudolska et al., 2006; Petrikkos et al., 2007). Several PCR methodologies for the detection of *Ureaplasma*, targeting 16S rRNA (Robertson et al., 1993), urease (Scheurlen et al., 1992, Willoughby et al., 1991), and multiple-banded antigen (MBA) (Teng et al., 1994a) gene sequences, have been described. Primers directed against MBA gene has many advantages including: (i) detection of all 14 serovars of Ureaplasma, (ii) lack of detection of product from 17 other mycoplasma species including phylogenetically closely related species such as *Mycoplasma pneumoniae*, and (iii) distinction of amplicons from biovar 1 strains (serovars 1, 3, 6, and 14) from amplicons from biovar 2 strains (serovars 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13) (403 versus 448 bp, respectively) (Teng et al., 1994a; Nelson et al., 1998).

The aim of the present study was to determine the colonization rate of preterm ventilated neonates with the two *U. urealyticum* biovars, parvo and T960, and to assess the correlation between colonization and the development of BPD in a prospective study.

MATERIALS AND METHODS

Patients

One hundred premature newborns with gestational age less than 34 weeks, without congenital anomalies who were admitted within 24 h after birth for assisted ventilation to the neonatal intensive care unit (NICU) in Mansoura University Children Hospital (MUCH) and other specialized medical centers were prospectively enrolled into the study from February 2013 till January 2014.

Data on perinatal events were obtained from hospital records; mode of delivery, gestational age, birth weight, Apgar score, respiratory distress syndrome (RDS), maternal hypertension, premature rupture of membranes (PROM), chorioamnionitis and antenatal steroids administration. Gestational age was established by the last normal menstrual period and ultrasound examination before 20 weeks of gestation.

Bronchopulmonary dysplasia (BPD) (CLD) was considered to be present if the child needed supplemental oxygen at 28 days of age (Northway et al., 1967; Hannaford et al., 1999) or at 36 weeks gestational age accompanied by changes in chest radiographs (Bancalari et al., 2003).

Written informed consent was obtained from parents of the included infants. The protocol was accepted by the local ethical committee

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Sample collection

Tracheal aspirates were collected immediately after birth (within the first 24 h after intubation) to exclude nosocomial transmission of infectious agents. Blood cultures were obtained when septic infection was suspected.

Endotracheal aspirates (ETA) were collected aseptically; Sterile, preservative- free saline (0.5 ml) was instilled into the endotracheal tube or nostril, the infant was ventilated for 10 breaths. Using an appropriately sized catheter, the trachea was suctioned at a point 0.5 cm beyond the tip of the endotracheal tube; after another 10 ventilator breaths suctioning was repeated with a new catheter.

The suction catheter was flushed with 2 ml 10B broth, and the samples were transported on ice until processed. Aliquots were removed and frozen at -70°C for later analysis by PCR to ensure that PCR and culture would be performed on the same sample mixture.

Culture

ETA specimens were vortexed and 0.2 mL of each specimen was added to 1.8 mL of 10B broth. Tubes were incubated aerobically at 37°C. If color change occurred, 0.2 ml of inoculum was plated onto A7 agar plates, incubated at 37°C in 5% CO_2.

Liquid media were incubated for 72 h at 37°C, solid media for 5-7 days. The growth on liquid media was observed as a change of color of the medium (hydrolysis of urea with the release of ammonia, signaled by a colour change of a pH indicator), while on solid media by the presence of characteristic golden-brown colonies of ureaplasmas (magnification 125x).

Tube cultures and plates were examined daily for one week for color change (from yellow to pink) and typical colonies of Ureaplasma as described previously. A positive culture was defined as a positive broth (color change) confirmed by typical colony morphology on A7 agar.

Negative broths and plates were subcultured after 48 h to a new broth and plate. All broths were read twice daily, and the total incubation time for the cultures was 10 days.

PCR

DNA was extracted directly from samples using QIAamp DNA Blood Mini kits (QIAgen, Hilden, Germany) according to the manufacturer's protocol. Ureaplasma were detected and biotyped by PCR targeting MBA gene UMS-125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C) (Teng et al., 1994b).

The amplification reaction mixtures contained 50 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 µl of each deoxynucleotide triphosphate (dATP, dCTP, dTTP and dGTP (Fermentas, Germany), 2.5 U Taq DNA polymerase (Fermentas, Germany), and 30 pmol of each primer UMS 125 (GTA TTT GCA ATC TTT ATA TGT TTT CG) and UMA 226 (CAG CTG ATG TAA GTG CAG CAT TAA ATT C) synthesized by Bio Basic Inc., Canada, and 10 µl of the sample DNA. The reaction mixtures were covered with mineral oil and subjected to an initial denaturation at 95°C for 4 min; then 40 cycles at 95°C for 45 s, 60°C for 45 s, and 72°C for 45 s; and a final elongation at 72°C for 3 min.

Detection involved visualization after gel electrophoresis, with benefits of speciation by amplicon size of 403 bp for *U. parvum* (biovar 1) and amplicon size of 448 bp for T960 biovar (biovar 2).

Statistical analysis

Statistical analysis of the data was done by using Statistical Package for Social Science (SPSS) version 15.0. Data are expressed as mean value ± SD for quantitative data and as frequency (number/percent) for qualitative data. Comparisons between two different groups were carried out by unpaired t-test. Pearson's Chi-square or fisher exact test was used for comparisons of categorical data. Spearman's rank correlation was used to assess relations between variables. Some investigated parameters were entered into a logistic regression model to determine which factor is considered as a significant risk factor and identified its odds ratio. The sensitivity and specificity of culture was detected using ROC curve analysis. Differences were considered statistically significant when $P < 0.05$.

RESULTS

From 100 premature neonates who were enrolled in this study, only 85 met the eligibility criteria. 7 neonates had insufficient clinical data or insufficient sample collected, 8 neonates died before 28 days of age. We restricted analysis to neonates who survived beyond 28 days of age.

From 85 studied premature ventilated neonates who met the eligibility criteria, 15 (17.6%) were colonized with *U. urealyticum* as detected by culture and/or PCR. A sample was considered positive if 10B broth had color change and confirmed morphology on A7 agar and/or positive PCR.

Culture was positive in 10 out of 85 samples (11.8%), as compared to 15/85 (17.6%) samples obtained by PCR technique (P=0.001) (Table 1).

When PCR was used as a reference standard, the overall agreement between culture and PCR was 83.3% with 66.7% sensitivity and 100% specificity of culture as compared to PCR (Figure 1).

Mean ± SD of gestational age of neonates with Ureaplasma colonization were 29.7 ± 1.5 and neonates without colonization 30.94 ± 1.4 that showed a statistically significant difference (P= 0.004).

There was also a statistically significant difference in the mean birth weight between neonates with and without colonization (1279.33 ± 260.9 vs. 1573.99 ± 389.6).

Apart from spontaneous vaginal delivery (SVD), there was no statistically significant difference between colonized and non colonized neonates regarding other parameters including gender, antenatal steroids and maternal PROM (Table 2). 12 (80%) of 15 colonized neonates developed BPD as compared to 21 (30%) of 70 non colonized neonates. BPD was significantly higher among colonized neonates than non colonized ones ($P < 0.001$). Among the study 85 neonates, BPD developed in 33 (38.8%) cases. BPD was found to be correlated to decreasing gestational age ($r = -0.341$, $p=0.001$), low birth weight ($r = -0.328$, $p= 0.002$) and Ureaplasma colonization ($r = 0.391$, $p < 0.001$).

Logistic regression analysis revealed that Ureaplasma colonization was a more important predictor for development of BPD than decreasing gestational age (Table 3). PCR assay found that Parvo biovar (biovar 1) was predominant in 9 (60%) out of 15 Ureaplasma

Table 1. Comparison of Ureaplasma PCR results with Ureaplasma culture results.

PCR		Urea plasma culture		Total
		Positive	Negative	
Ureaplasma PCR	PCR positive	10	5	15
	PCR negative	0	70	70
Total		10	75	85

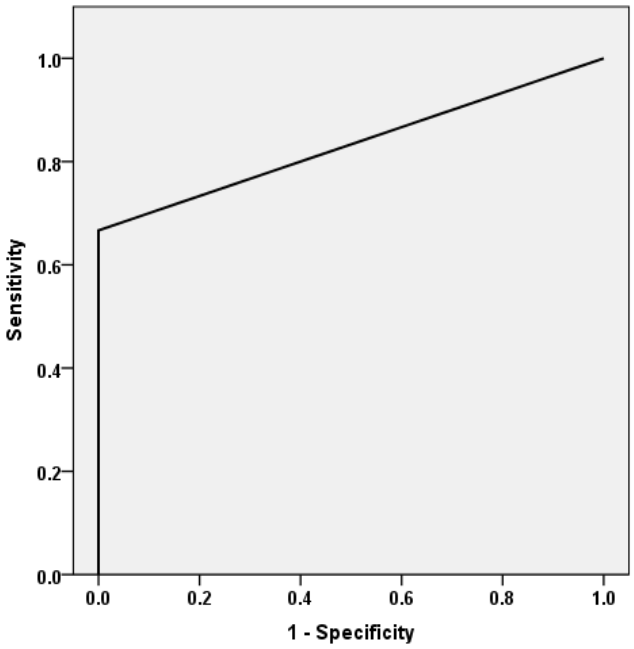

Figure 1. Roc curve.

isolates, as compared to T960 biovar (biovar 2) in 6 (40%) isolates. None of the neonates were co-colonized with both biovars (Table 4). Although, Parva biovar was more predominant than T960 biovar, BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1) (83.3 vs. 77.7%) with statistical significant difference in frequencies of BPD between neonates colonized by biovar 1 or biovar 2 (p = 0.001) (Figure 2).

DISCUSSION

The multifactorial etiology of BPD of the newborn has been reported over the last three decades (Bancalari et al., 2003; Jobe and Bancalari, 2001; Kinsella et al., 2006; Speer, 2003; Speer, 2006) With the increased survival of a greater number of more immature infants, the contribution of antenatal infection to BPD has been argued (Jobe, 2003; Lyon, 2000; Miralles et al., 2002). *Ureaplasma* spp. are associated with increased risk for preterm labor and morbidity in the preterm neonate. However, there are some controversies regarding the importance of *Ureaplasma* in the pathogenesis of BPD (Kallapur et al., 2013). We aimed to clarify the contribution of Ureaplasma biovars colonization to the development of BPD.

In the present study, 17.6% of 85 premature ventilated neonates were colonized with *U. urealyticum* as detected by culture and/or PCR. This is in agreement with the percentage of colonization of *U. urealyticum* in neonatal respiratory specimens reported in other studies from 3 to 23% (Blanchard et al., 1993; Cassell, 1993; van Waarde et al., 1997; Payne et al., 1993; Jonsson et al., 1994; Mohagheghi et al., 2013). Higher rates were reported by Nelson et al. (1998) (36%), Ollikainen et al. (2001) (33%), Pacifico et al. (1997) (40%), Ollikainen (2000) (55%) that could be explained by larger sample size, or testing multiple specimens from enrolled patients.

In the present study, 5 samples were PCR positive but culture negative for *U. urealyticum*. The sensitivity and specificity of culture when compared with PCR were 66.7 and 100%, respectively. Our results are consistent with other author's observations that culture is less sensitive (Teng et al., 1994a; Luki et al., 1998; Biernat-Sudolska et al., 2006).

The higher sensitivity of PCR may be due to the generally recognized difficulties of culturing and isolating Ureaplasma. The results of PCR amplification are less prone to being influenced by methods of sample collection, and handling. Moreover, PCR is much quicker, results can be obtained in 1-2 days, whereas it takes 5-7 days in the case of cultivation.

The ability to differentiate the Ureaplasma biovars in clinical samples has long been a challenge for investigators. Our study confirms previous observations (Abele-Horn et al., 1997a; Katz et al., 2005) that biovar 1 (parvo biovar) is the predominant species colonizing the respiratory tract of preterm infants. On the other hand, Kotecha and co-workers (2004) have described the colonization of their study patients with either biovar 1 or biovar 2 without a different distribution inside patient groups.

The detected frequency of BPD (38.8%) agrees with 17 to 60% frequencies in the previous studies (Sanchez and Regan, 1988; Illes et al., 1996). BPD rate was higher among neonates colonized with T960 biovar (biovar 2) than those colonized with parvo biovar (biovar 1). In agreement with our study, Abele-Horne et al. (1997b)

Table 2. Clinical features of 85 study neonates and Ureaplasma colonization.

Parameter		Urea plasma colonization		P
		Positive	Negative	
Gestional age (weeks) (Mean±SD)		29.7±1.5	30.94±1.4	0.004
Birth weight (g) (Mean±SD)		1279.33±260.99	1573.99±389.6	0.007
Gender	Male	7 (46.7%)	30(42.9%)	0.78
	Female	8(53.3%)	40(57.1%)	
Antenatal steroid	Yes	11(73.3%)	47(67.1%)	0.64
	No	4 (26.7%)	23(32.9%)	
Maternal PROM	Positive	7(46.7%)	23(32.9%)	0.31
	Negative	8(53.3%)	47(67.1%)	
Delivery mode	SVD	12(80%)	31(44.3%)	0.01
	CS	3(20%)	39(55.7%)	
Final outcome	Non-BPD	3(20%)	49(70%)	<0.001
	BPD	12(80%)	21(30%)	

SD: Standard deviation P: Probability CS: cesarean section SVD: spontaneous vaginal delivery; BPD: chronic lung disease.

Table 3. Logistic regression analysis with respect to BPD.

Factor	P-value	OR	95% CI
Gestational age	0.025	0.668	0.46-0.95
Ureaplasma colonization	0.008	6.748	1.64-28.0

P: Probability OR: Odds ratio CI: confidence interval

Table 4. Clinical features in relation to Ureaplasma (T960 and parvo) biovars.

Parameter		Ureaplasma biovars		P
		T 960 biovar	Parvo biovar	
Gestional age (weeks) (Mean±SD)		28.5±0.54	30.56±1.4	0.002
Birth weight (g) (Mean±SD)		1025±175.3	1448.9±138.4	0.001
gender	Male	0(0%)	7(77.8%)	0.01
	Female	6(100%)	2(22.2%)	
antenatal steroid	Yes	3(50%)	8(88.9%)	0.02
	No	3(50%)	1(11.1%)	
maternal PROM	Positive	0(0%)	7(77.8%)	0.1
	Negative	6(100%)	2(22.2%)	
Delivery mode	SVD	3(50%)	9(100%)	0.9
	CS	3(50%)	0(0%)	
final outcome	Non-BPD	1(16.7%)	2(22.2%)	0.001
	BPD	5(83.3%)	7(77.7%)	

SD: Standard deviation P: probability.

reported that the BPD rate was 2-fold higher for *U. urealyticum* (biovar 2)-colonized infants than the rate for *U. parvum* (biovar 1)-colonized infants. Heggie and coworkers (2001) found no greater risk of developing BPD among 66 Ureaplasma-colonized infants, and also found no differences between infants harboring parvo biovar as compared to those with T960 biovar. Katz et al. (2005) found no difference in BPD rates between infants colonized with either species, but a higher rate of BPD in infants positive for both species. The infants colonized

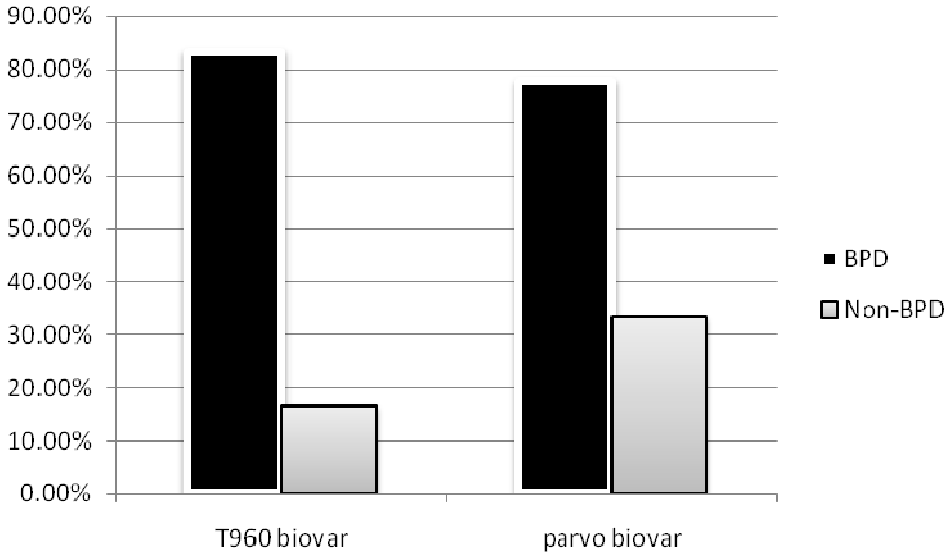

Figure 2. Frequency of BPD among neonates colonized with T960 biovar and parvo biovar.

with T960 biovar were significantly less mature and had lower birth weight than parvo biovar-colonized infants. This may explain, in part, the biovar difference in BPD rates in this study.

It was noted that neonates with positive Ureaplasma colonization had a significantly lower gestational age than non-colonized ones. This is in accordance with Pandey et al. (2007) and Mohagheghi et al. (2013). Our results are in agreement with Yada et al. (2010) and Theilen et al. (2004), Ureaplasma colonization was significantly associated with gestational age, birth weight, SVD and development of BPD.

The association between presence of ureaplasma and the development of BPD remains controversial and hotly debated. Several studies attempted to relate the possibility of an association between *U. urealyticum* respiratory colonization with the development of BPD in preterm neonates (Cassell et al., 1988; Iles et al., 1996; Garland and Bowman, 1996; Kafetzis et al., 2004; Kotecha et al., 2004). On the other hand, there have been studies that failed to detect the association (Saxen et al., 1993; Cordero et al., 1996; Van Waarde et al., 1997; Heggie et al., 2001; Ollikainen et al., 2001; Pandey et al., 2007). The great variations in the sample selection, processing, methods of identification assays applied might explain the different results observed in these studies.

Several studies declared that the most significant factor in the development of BPD was the decreasing gestational age (Smyth et al., 1993; Payne et al., 1993; Jonsson et al., 1994). The present study revealed that in addition to decreasing gestational age, Ureaplasma colonization was a more important predictor for development of BPD (OR: 6.784 and CI95%: 1.64–28.0). In 1995, a meta-analysis by Wang et al. (1995) included 1479 babies from 17 studies, reporting a significant association between BPD diagnosed at 28 days of life and ureaplasma colonization, with an overall relative risk of 1.72 (CI95%: 1.5–1.96). In a cohort of 126 preterm deliveries, Kafetzis et al. (2004) found a significant increase in BPD among ureaplasma colonized infants. Van Waarde et al. (1997) found that Ureaplasma was significantly associated with both BPD and lower gestational age, but logistic regression analysis failed to show a correlation between ureaplasma colonization and BPD. Schelonka et al. (2005) found an odds ratio (OR) of 2.83 (CI95%: 2.29–3.51) for the relationship between the presence of ureaplasma and BPD in a meta-analysis of 23 studies, and Goldenberg et al. (2008) confirmed a probable association between infection and BPD as well.

Limitations of our study

Although the BPD etiology is multifactorial and complex, our study has focused on the role of Ureaplasma colonization in development of BPD, ignoring the postnatal factors for development of BPD as oxygen toxicity, barotraumas, volutrauma, mechanical ventilation, patent ductus arteriosus (PDA) and also the role of proinflammatory cytokines.

Conclusion and future prospectives

Our finding support the evidence that Ureaplasma respiratory tract colonization in preterm ventilated neonates particularly T960 biovar (biovar 2) has been associated with the development of BPD. PCR could be a highly sensitive and specific technique for detecting

Ureaplasma and for distinguishing its biovars (Parvum and T960) directly from clinical samples. Moreover, it may be a useful tool in clinical trials studying the efficacy of early antibiotic intervention or to fully assess the benefits and risks of therapy in colonized high-risk neonates.

Furthermore, a study on large number of neonates analyzing antimicrobial therapy (macrolide) of Ureaplasma -colonized infants should be conducted in a large, multicenter, randomized clinical trial to confirm relationship between Ureaplasma colonization and BPD, and to determine whether these antibiotics are effective in reducing BPD rate and improving neonatal outcomes.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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