DETECTION OF UREAPLASMA SPECIES IN ENDOTRACHEAL ASPIRATES FROM PRETERM INFANTS WITH RESPIRATORY DISTRESS SYNDROME

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ABSTRACT

Introduction: Respiratory distress remains the most common cause of perinatal morbidity and mortality in preterm infants. Ureaplasma urealyticum and U. parvum have been associated with respiratory diseases in premature newborns, but their role in the pathogenesis of the respiratory distress syndrome (RDS) is unclear.

Aim of the Work: To investigate the colonization of the respiratory tract of preterm newborns by U. urealyticum U. parvum and Mycoplasma hominis by using Mycoplasma Duo kit and PCR to prove a possible association between the colonization of these microorganisms and the development of RDS with associated risk factors. Also to compare the performance of the Mycoplasma Duo kit with PCR for the detection of Ureaplasma species.

Subjects and Methods: Sixty paired endotracheal aspirates were obtained from premature infants ventilated due to hyaline membrane disease were analysed for detection of Ureaplasma spp. by Mycoplasma Duo assay and PCR.

Results: This study revealed that the prevalence of Ureaplasma in neonatal endotracheal aspirates with respiratory diseases was 33.3%, 35% as detected by Mycoplasma Duo kit and PCR tests respectively and U. parvum was found more than U. urealyticum in the study group. There was very good overall agreement between the Mycoplasma Duo assay and PCR (0.96). As regarding to the average gestational age and birth weight there were a statistical significant difference between positive and negative case.

Conclusion: Colonization of the lower respiratory tract by Ureaplasma spp. and particularly by U.parvum in preterm newborns was related to RDS. The Mycoplasma Duo assay is a commercially available kit that is rapid and simple to use and has a sensitivity comparable to PCR for the detection of Ureaplasma spp. in neonatal endotracheal aspirates. Also PCR could be a highly sensitive and specific technique for detecting Ureaplasma and for distinguishing U. urealyticum from U. parvum directly in clinical specimens.

Keywords: Ureaplasma urealyticum, ureaplasma parvum, RDS, Mycoplasma Duo kit, Endotracheal aspirates.

INTRODUCTION

Respiratory distress syndrome (RDS) of newborn, previously called hyaline membrane disease, is a syndrome in premature infants caused by developmental insufficiency of surfactant production and structural immaturity in the lungs. It can also result from a genetic problem with the production of surfactant associated proteins (Pramanik, 2012).

Perinatal bacterial colonization of urogenital tract of pregnant females has an implication in pathogenesis of both preterm labour and neonatal morbidity and mortality. Most common organisms involved are Ureaplasma and Mycoplasma species (Kafetzis et al. 2004).

Ureaplasma spp. are the most common microbes isolated from infected amniotic fluid, placentas and the respiratory tracts of preterm infants and their ability to induce inflammation in these sites is undeniable (Yoon et al. 1997).

Ureaplasma spp. cause lung injury through a number of mechanisms including the inhibition of pulmonary surfactant by phospholipase A2 produced by Ureaplasma and the production of soluble intercellular adhesion molecules (ICAM-1) that mediates neutrophil activation and transendothelial migration of leukocytes to sites of inflammation (Viscardi et al. 2002).
Knowledge of the biology of ureaplasmas and their behavior in the respiratory tract of preterm neonates suggest that lung disease associated with these organisms is not only due to direct damage from the bacteria themselves, but also because of their potent stimulation of proinflammatory cytokines (TNF-α, IL-1β and IL-8), nitric oxide production or perhaps blockage of counter regulatory cytokines (IL-6 and IL-10) (Cassell et al. 2001).

The prevalence of clinical disease associated with U. urealyticum is probably underestimated due to the limitations of laboratory diagnosis. U. urealyticum and Mycoplasma are fastidious organisms requiring vigorously quality-controlled medium for cultivation and several days of incubation. These procedures are costly and laborious (Nelson et al. 1998).

The development of a commercially available diagnostic kit (Mycoplasma Duo kit) offers a simpler and rapid alternative method for detection of ureaplasma species in urogenital and neonatal respiratory samples. With this kit identification of ureaplasma species is based on the hydrolysis of urea with the release of ammonia, signaled by a colour change of a pH indicator (phenol red) and results are read within 24-48 hours (Fook-Choe et al. 2005).

PCR techniques have proved useful in detecting Ureaplasma spp in clinical specimens due to time saving and the possibility of directly identifying the mycoplasma species (Fook-Choe et al. 2005).

AIM OF THE WORK

The aim of this study was to investigate the colonization of the respiratory tract of preterm newborns by U. urealyticum and U. parvum and Mycoplasma hominis using Mycoplasma Duo kit and PCR to prove a possible association between the colonization of these microorganisms and the development of RDS with associated risk factors. Also to compare the performance of the Mycoplasma Duo kit with PCR for the detection of Ureaplasma spp.

SUBJECTS AND METHODS

Study Approval: The study was approved by the Ethical Committee of our institution. Neonate's parents gave informed written consent for participation in this study.

Subjects: This study was conducted on 60 premature infants ventilated due to respiratory distress in neonatal intensive care unit in Benha University Hospital (37 premature infant) and Children Special Hospital (23 premature infant) from June to December 2014 on Sixty paired endotracheal aspirates.

Gestational age was determined by the last normal menstrual period and ultrasound examination. Infants with RDS characteristically are seen either immediately after delivery or within several hours of birth with combination of respiratory manifestations.

Selection of cases was done as follows:
- History taking from parents including: Sex, gestational age and birth weight of studied premature neonates.
- Diagnosis of respiratory distress syndrome was done according to criteria explained by (Clarence & Gowen, 2011).

1- Clinical signs of respiratory distress including expiratory grunting, tachypnea, subcostal and intercostal retraction, nasal flaring and cyanosis. This clinical presentation usually manifest in the first few hours and almost always before 8 hours of age. On auscultation, air movement is diminished despite vigorous respiratory effort. Extremely immature neonates may develop apnea and/or hypothermia.
- Requirement for supplemental oxygen of more than 40%, to maintain arterial oxygen tension above 60 mm Hg, in ventilated infants.
- Laboratory investigations: Chest X-rays exhibit bilateral diffuse reticular granular appearance, ultrasonography, echocardiography, blood gases measurement, electrolytes, hemoglobin or hematocrit measurement, sepsis work-up, tracheal aspirate for assessment of Surfactant protein A.

Method: For the Mycoplasma Duo assay, endotracheal secretions were removed under aseptic conditions by repetitive and gentle flushing of the suction catheters with 2 ml of the suspension medium provided in the kit. The specimens were then processed according to the manufacturer's recommendations: Results were first read after incubation for 24 hours; this provided the definitive result in high-titre specimens (≥10⁴ CCU/ml). A second reading performed after 48 hours incubation was done to:
- Confirm negative results.
- Detect strains present in low titres (≤10³ CCU/ml).
- Detect strains present in high titres (≥10⁴ CCU/ml) but characterized by a slow metabolic rate.
For PCR analysis, endotracheal secretions in suction catheters were flushed with 1 ml of phosphate-buffered saline and then centrifuged at a relative centrifugal force of 9,200 xg for 2 min and the supernatants were stored at -80°C (Fook-Choe et al. 2005). Genomic DNA was extracted from 200 μl of these aliquots by using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, EU Lithuania) according to manufacturer's instructions. Purified DNA samples were stored at -20°C till used in the amplification step.

PCR with species-specific primers for identification of ureaplasmas was done according to Kong et al. 2000, using Thermal Cycler (Biometra) and Maxima Hot Start PCR Master Mix (Thermo Scientific, EU Lithuania). Containing Maxima Hot Start Taq DNA polymerase, Hot Start PCR buffer, 400 μM each dNTP and 4 mM Mg²⁺.

The reaction mixture contained 25 μl Maxima Hot Start PCR Master Mix (2X), 10 μl of tested DNA, 0.5μM of each primer and water, nuclease-free was added to a PCR mixture to give a final reaction volume of 50 μl. water, nuclease-free was used as a negative control in each PCR run. Primers pairs: UMS 57 (5'-TAAATCTTAGTTCATATTTTTAC-3') / UMA 222 (5'-GTAAGTGCCATATTTCA TG-3') and UMS 170 (5'-GTATTTGCAATCTTATGTTTTTCTG-3') / UMA 263 (5'-TTTGTGGTTGCGTTTTCTG-3') (Biosearch technologies, USA) were used for identification of U. parvum (326 bp) and U. urealyticum (476 bp), respectively.

The reaction mixtures were covered with mineral oil and subjected to the following thermal cycling parameters: 1 cycle of 95°C for 5 min; 35 cycles of 95°C for 30 s, 52°C for 30 s and 72°C for 30 s; and 1 cycle of 72°C for 7 min. PCR products were visualized using 2% agarose gel electrophoresis stained by ethidium bromide and visualized by UV. (Figure 1).

![Figure 1: Ethidium bromide stained gel electrophoresis of U. parvum (326 bp) lanes 2,3,4,5 and 6 and U. urealyticum (476 bp) lanes 7, 8,9, and negative control (lanes 1 and 10).](image)

**Statistical Analysis:**

The data were recorded on an “Investigation report form”. These data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 16, quantitative data were expressed in Mean± Standard deviation (±SD) and qualitative data were expressed in number and percent. In the statistical comparison between the different groups, the significance of difference was tested using Student's t-test to compare between mean of two groups. Inter-group comparison of categorical data was performed by using chi square test (X²-value), Kappa test for agreement is used to detect agreement between two different diagnostic tests, A P value <0.05 was considered statistically significant.
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RESULTS

This study was conducted on 60 premature infants ventilated due to respiratory distress. The results of the study were summarized in the following tables:

33% of premature neonates were colonized with ureaplasma. 30% were high titer ureaplasma and only 3.3% were low titer ureaplasma as detected by Mycoplasma Duo kit (Table 1 and Figure 2).

Table (2), shows that: 35% premature neonates were colonized with ureaplasma. U. parvum was (25%) and U. urealyticum (10%), as detected by PCR.

There was very good agreement (0.96) between results of PCR and Mycoplasma Duo kit (Table 3).

Table (4), shows: there were statistical significant differences between positive and negative cases as regarding to gestational age and birth weight.

Table 1 and Figure 2: Results of study group according to Mycoplasma Duo kit.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>%</th>
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<tbody>
<tr>
<td>High titre ureaplasma</td>
<td>18</td>
<td>30</td>
</tr>
<tr>
<td>Low titre ureaplasma</td>
<td>2</td>
<td>3.3</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>33.3</td>
</tr>
</tbody>
</table>

Table 2: Results of study group according to PCR.

<table>
<thead>
<tr>
<th></th>
<th>No.</th>
<th>%</th>
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</thead>
<tbody>
<tr>
<td>Ureaplasma Parvum</td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Ureaplasma Urealyticum</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 3: Agreement between results of PCR and Mycoplasma Duo kit.

<table>
<thead>
<tr>
<th></th>
<th>PCR</th>
<th></th>
<th></th>
<th>Total</th>
<th>Kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duo media</td>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>0</td>
<td>0</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>39</td>
<td>39</td>
<td>40</td>
<td>0.96  (very good)</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>39</td>
<td>60</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2a: Negative Mycoplasma DUO kit.
Figure 2b: Positive for ureaplasma with low titre (U well).
Figure 2c: Positive for ureaplasma with high titre (U, U ≥10^4 wells).
DISCUSSION

Respiratory distress remains the most common cause of perinatal morbidity and mortality in preterm infants despite many advances in neonatal intensive care and the introduction of artificial surfactant. It is caused by cardiopulmonary immaturity with a deficiency of surfactant in the alveolar space (Cultrera et al. 2006).

An appreciation for the role of inflammation as a consequence of perinatal infection emerged as an important cause in the pathogenesis of respiratory diseases, leading the way for consideration of perinatal pathogens such as Ureaplasma spp. as causal factors (Lyon, 2000). There is a strong evidence that ureaplasmas induce proinflammatory cytokines production in utero that result in chorioamnionitis and chronic lung injury in neonates (Li, 2000).

The aim of this study was to investigate the colonization of the respiratory tract of preterm newborns by U. urealyticum, U. parvum and Mycoplasma hominis using Mycoplasma Duo kit and PCR to prove a possible association between the colonization of these microorganisms and the development of RDS with associated risk factors. Also to compare the performance of the Mycoplasma Duo kit with PCR for the detection of Ureaplasma spp.

In our study 33.3%, 35% of premature neonates were colonized with ureaplasma as detected by Mycoplasma Duo kit and PCR, respectively (Tables 1, 2 & Figure 2).

This was in agreement with Nelson et al. (1998), who found that 36% of 103 premature neonates were colonized with U. urealyticum as detected by culture and/or PCR. Also Hannaford et al. 1999 found that ureaplasma was isolated from 39 (27%) aspirates out of 143 ventilated premature neonates by culture on ureaplasma broth and A8 agar.

Cultrera et al. (2006) found that, the detection rate of U. urealyticum and U. parvum were 38% in preterm infants during respiratory distress by PCR. Bayrakta et al. (2009) detected ureaplasma in 27 (27%) cases out of 100 neonatal aspirate samples by culture on A7 agar medium.

Ureaplasmas can be isolated from endotracheal aspirations in up to 40% of newborn infants within 30 minutes to 24 hours after delivery (Colaizy et al. 2003). This provided evidence that vertical Ureaplasma transmission and neonatal infection may occur in newborn infants. Additionally, recovery of Ureaplasma from the chorion increased with the duration of membrane rupture, suggesting a ascending route of infection (Kundsin et al. 1996).

<table>
<thead>
<tr>
<th>Table 4: Descriptive data of the studied premature neonates.</th>
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<tbody>
<tr>
<td><strong>Positive cases (n=21)</strong></td>
</tr>
<tr>
<td>--------------------------</td>
</tr>
<tr>
<td><strong>Gestational age</strong></td>
</tr>
<tr>
<td>(mean ± SD)</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td><strong>Birth weight</strong></td>
</tr>
<tr>
<td>(mean ± SD)</td>
</tr>
<tr>
<td><strong>Apgar 1</strong></td>
</tr>
<tr>
<td>(mean ± SD)</td>
</tr>
<tr>
<td><strong>Apgar 5</strong></td>
</tr>
<tr>
<td>(mean ± SD)</td>
</tr>
<tr>
<td><strong>Mode of delivery</strong></td>
</tr>
<tr>
<td>NVD</td>
</tr>
<tr>
<td>CS</td>
</tr>
<tr>
<td><strong>PROM</strong></td>
</tr>
<tr>
<td>Positive</td>
</tr>
<tr>
<td>Negative</td>
</tr>
</tbody>
</table>

NVD: normal vaginal delivery CS: caesarean section
PROM: premature rupture of membrane

In our study, 33.3%, 35% of premature neonates were colonized with ureaplasma as detected by Mycoplasma Duo kit and PCR, respectively (Tables 1, 2 & Figure 2). This was in agreement with Nelson et al. (1998), who found that 36% of 103 premature neonates were colonized with U. urealyticum as detected by culture and/or PCR. Also Hannaford et al. 1999 found that ureaplasma was isolated from 39 (27%) aspirates out of 143 ventilated premature neonates by culture on ureaplasma broth and A8 agar.

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In contrast to our study Fook-Choe et al. (2005) reported that rate of Ureaplasma Species in endotracheal aspirates from premature infants was 17.9% by PCR and 19.4% by Mycoplasma Duo kit.

Our result not in agreement with Kafetzis et al. (2004) who reported that by using a culture based method 73% of preterm neonates colonized by U. urealyticum and U. parvum had RDS.

In our study U. parvum was (25%) more than U Urealyticum (10%), (Table2) and data from our study support those found by Waites et al. 2005, De Francesco et al. 2009, Okogbule et al. 2011 and Sung et al. 2011 who found that of the two Ureaplasma species known to infect humans, U. parvum is the most commonly isolated from clinical samples, typically representing 48 to 86% of human Ureaplasma spp.

In contrast, Heggie et al. (2001) reported a similar distribution of the two species by species-specific PCR of culture-positive broths from endotracheal aspirates. Differences in study results may be due to differences in study populations or in selection bias of the DNA source. By performing PCR only on culture-positive broths, Heggie et al. (2001) may have missed PCR-positives of culture-negative aspirates and broth medium may have supported differential growth of one or the other of the two species.

Our study revealed very good agreement (0.96) between results of PCR and Mycoplasma Duo kit (Table3). This results coincide with that reported by Fook-Choe et al. 2005 who found that there was 96% agreement between the Mycoplasma Duo and PCR assays for detection of Ureaplasma spp. in endotracheal samples.

This level of agreement is similar to that recorded between culture and PCR by Blanchard et al 1993 who examined 95 samples by culture and PCR, found that 11 of 12 samples positive by culture were also positive by PCR. The single sample which was positive by culture and negative by PCR was shown to contain only a small number of organisms. There were no samples in their study that were negative by culture but positive by PCR. This may be partly explained by the use of a more sensitive culture technique in their study, as both liquid and solid media were used for isolation.

In the study by Cunliffe et al. 1996 who found overall agreement between the results obtained by both methods was 93%. Of the six specimens negative by culture but positive by PCR, two were aspirates taken from infants receiving erythromycin, an agent which is bacteriostatic for ureaplasmas. This explains the negative culture in the presence of the positive PCR result, as the DNA of non-dividing organisms could still be detected. The other four culture-negative, PCR-positive aspirates maybe explained by the greater sensitivity of PCR compared with culture.

According to different risk factors, it was found that there is no statistical significant difference between positive and negative cases for ureaplasma as regards sex differences, Apgar score, PROM and mode of delivery (Table 4). This agrees with the results of Panero et al. 1995 and Hannaford et al. 1999 who reported that there was non-significant difference between positive and negative cases for ureaplasma as regards their sex differences. also Cultrera et al. 2006 found no link between method of delivery, Apgar score and PROM and colonization of preterm infant with Ureaplasma during respiratory distress.

...but other studies were done by Hassanein et al. 2012 and Alexandra et al. 2013 found that colonization the preterm with these organisms during pregnancy has been increase with vaginal delivery and premature rupture of membrane.

Regarding the average gestational age and birth weight of study group there were a statistical significant difference between positive and negative cases (Table 4).

This agrees with Panero et al. 1995 who reported that there was a highly significant difference (P value =0.001) between positive and negative cases for ureaplasma as regards their average gestational age. Kafetzis et al. (2004) also reported that vertical transmission and isolation rates of ureaplasma spp. varied inversely with gestational age and the overall ureaplasma colonization rate was 10% for full-term infants versus 24% of preterm infants.

Those born weighing less than 1,000 g are at higher risk of infection when the mother is colonized at up to 90% of the infection rate. The possible pathogenesis involves fetal exposure to ascending ureaplasmal intrauterine infection, passage through an infected birth canal, hematogenous dissemination through the placenta into umbilical vessels and colonization of the skin, mucosal membranes, respiratory tract and dissemination into the blood and central nervous system (Schelonka and Waites, 2007).

Klein and Gibbs (2004) reported that isolation rates of ureaplasma spp. from the chorioamnion...
were higher in infants who were less than 1500 g at birth and were born before 32 weeks of gestation.

On the other hand Panero et al. 1995 and Cultierra et al. 2006 reported that there was no statistically significant difference between positive and negative cases of ureaplasma in premature neonatal aspirate samples as regards average birth weight and gestational age.

CONCLUSIONS

*U. parvum* more than *U. urealyticum* were found in the respiratory secretions of preterm newborns with respiratory distress syndrome (RDS) indicating a possible role of these organisms in the pathogenesis of RDS. Our findings support the evidence that *The Mycoplasma Duo* assay is a commercially available kit that is rapid and simple to use and has a sensitivity comparable to PCR for the detection of *Ureaplasma* spp. in neonatal endotracheal aspirates. These characteristics make this test suitable for use in diagnostic laboratories. Also PCR could be a highly sensitive and specific technique for detecting *Ureaplasma* and for distinguishing *U. urealyticum* from *U. parvum* directly in clinical specimens.

REFERENCES


DETECTION OF UREAPLASME SPECIES IN ENDOTRACHEAL ASPIRATES FROM PRETERM INFANTS ...
ملخص البحث

الكشف عن أنواع اليوريابلازما في عينات الأنبوبي الحنجري في الأطفال الذين يعانون من متلازمة الضائقة التنفسية

أنهاد أحمد فؤاد، عفت حسين عسر، ونهاد احمد فؤاد

قسم الكيمياء الطبية، قسم الأطفال وقسم الميكروبيولوجي الطبي، كلية الطب، جامعة بنها

المتلازمة التنفسية القصبيّة - أو RDS - هي اضطراب تنفسي يصيب الرضع غالباً. ويكون الاضطراب أكثر شعوًع عند الأطفال الخجيج الذين يولدون قبل موعدهم بنحو 3 أسابيع أو أكثر. ولا تكون رائحة قادرة على صنع ما يكفي من السورفاكتانت. مما يجعلها أكثر الامراض شيوعا في حديث الامراض والأمراض بين هولاء الأطفال. وقد ارتبطت كل من U. urealyticum و U. parvum في الجهاز التنفسي لدى الأطفال حديثي الولادة المبكرة المصابين بالضائقة التنفسية. وهذا هدفت هذه الدراسة إلى البحث عن وجود كلا من U. urealyticum و U. parvum في الجهاز التنفسي لدى الأطفال حديثي الولادة. والولادة المبكرة المصابين بالضائقة التنفسية. ولذا استخدم كل من طريق الزرع على الميكوبلازما ديو كيت (Mycoplasma Duo kit) وتفاعل البلمر المتسلسل ومقارنه حساسية كل منهما في الكشف عن أنواع الميكروب.

وكشفت الدراسة ان الكشف عن اليوريابلازما بطريق الزرع على الميكوبلازما ديو كيت هو أكثر انتشار من U. urealyticum و U. parvum. وكانت هناك اختلاف بين الامراض الالتهابية والحساسية. وقد اوضح هذه الدراسة أن الكشف عن اليوريابلازما بطريق الزرع على الميكوبلازما ديو كيت هو طريق سرعة وسهولة الاستخدام، ولولأ حساسية مماثلة لتفاعل البلمر المتسلسل للكشف عن اليوريابلازما في افرازات القصبة الهوائية لديديثي الولادة. وأيضاً تفاعل البلمر المتسلسل ذو حساسية عالية للغاية ويمكنه التعرف على أنواع اليوريابلازما مباشرة في عينات الجهاز التنفسي العنف.