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Chronic Lung Disease of the Malaysian Premature Neonates is not Associated with *Ureaplasma urealyticum* and *Mycoplasma*

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Abstract: Mortality rate in the preterm neonate is believed to be associated with the chronic lung disease (CLD) and CLD is mainly caused by the infections of either *Ureaplasma urealyticum* or *Mycoplasma horminis* based on other studies. Therefore in this research, endotracheal aspirates of 57 intubated Malaysian premature neonates were used to study the association between colonization of *Ureaplasma urealyticum* or *Mycoplasma* in the respiratory tract of preterm neonates with subsequent development of chronic lung disease using Mycofast® screening kit and nested PCR approaches. Overall, only 1 out of 57 samples was detected to have a mixture of 2 different *Mycoplasma* species using the later approach. Therefore, we conclude that development of chronic lung disease in Malaysian premature neonates is not due to colonization of *Ureaplasma urealyticum* or *Mycoplasma* but other factors.

Key words: *Mycoplasma*, *Ureaplasma urealyticum*, chronic lung disease, nested PCR

INTRODUCTION

The name *Mycoplasma* was derived in the 1950s from the Greek term mykes to describe fungus and plasma-formed organisms (Waites *et al*., 2005). *Mycoplasma* and *Ureaplasma* are members of the family Mycoplasmataceae of the class Mollecutes (Hashimoto *et al*., 2006). More than 200 known species have been detected in humans, animals, arthropods, vertebrates and plants (Waites *et al*., 2005). Among these, fourteen *Mycoplasma* species (i.e., *M. amphoriforme*, *M. buccale*, *M. faucium*, *M. fermentans*, *M. genitalium*, *M. hominis*, *M. lipophilium*, *M. orale*, *M. penetrans*, *M. pirum*, *M. pneumoniae*, *M. primatum*, *M. salivalium* and *M. spermatophilium*) as well as two *Ureaplasma* species (i.e., *U. parvum* and *U. Urealyticum*) have been well-documented in humans, primarily localized in the urogenital and respiratory tracts. Human *Mycoplasma* are mostly commensals that play no role in disease development; however several species have been identified as pathogens, e.g., *M. pneumoniae* which is found in the respiratory tract and *M. genitalium*, *M. hominis*, *U. parvum* and *U. urealyticum* in the urogenital tract. In some circumstance, *M. fermentans*, *M. penetrans* and *M. amphoriforme* are thought to be associated with human disease (Waites *et al*., 2005).

To date, several studies have shown the possible link between vaginal flora such as *M. hominis* and *U. urealyticum*, that are transmitted from mother to infant during delivery, with the development of neonatal respiratory diseases. Chronic lung disease (CLD) (Wang *et al*., 1995; Agarwal *et al*., 2000; Kotecha *et al*., 2004) bronchopulmonary dysplasia (BPD) (Abele-Horn *et al*., 1998; Katz *et al*., 2005) and respiratory distress syndrome (RDS) (Cultrera *et al*., 2006) are the most common respiratory diseases that have become a major cause of morbidity and mortality in premature newborns.

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Previously, there was a lack of routine screening for detection of *U. urealyticum* and various *Mycoplasma* species amongst Malaysian premature neonates due to the absence of a specific and standardized test. However, with increasing advances in molecular approaches, the rapid screening of *U. urealyticum* and various *Mycoplasma* species in clinical specimens has become possible. Generally, the aim of the present study was to screen and identify various *Mycoplasma* species and *U. urealyticum* in the respiratory secretions of premature newborns and to evaluate any possible association with the development of respiratory diseases in these newborns. A culture identification assay and two commercially available kits were used, i.e., the Mycofast® Screening Evolution 3 diagnostic kit (International Microbio, France) and the PCR Mycoplasma Detection Set (TaKaRa Bio Inc, Japan).

MATERIALS AND METHODS

Clinical Specimens

Endotracheal aspirates from 57 intubated Malaysian premature neonates were collected soon after birth but prior to surfactant therapy, within the first 12 h of admission to the University Malaya Medical Centre (UMMC) Neonate Unit. The samples were collected with informed consent from the neonats' parents. Endotracheal aspirates were collected during suction of a neonate's endotracheal tubes by passing a sterile catheter into the distal tracheal and using a non-bacteriostatic saline lavage. The specimens were inoculated into 2 mL of an adapted transport media supplied by the Mycofast® kit manufacturer and immediately transported to the laboratory for culture identification and PCR assays.

Culture Identification Assay

The presence of *M. hormonis* and *U. urealyticum* would be determined by the Mycofast® Screening Evolution 3 diagnostic kit (International Microbio, France), which relied on the ability of *M. hormonis* and *U. urealyticum* to hydrolyse arginine and urea, respectively, in A7 agar. In general, this kit can only be used to culture and detect *U. urealyticum* and *M. hominis*. Specimens were processed according to the manufacturer's recommendations and results were read after incubation for 24 to 48 h at 37°C.

Nested PCR Assay

Specimen collected were cultured on Mycoplasma Agar (Oxoid, England) with appropriate conditions and incubated at 37°C for 7 days. The culture was isolated by rinsing with 100 μL of sterile distilled water. The supernatant of the samples were collected and kept at - 70°C until PCR screening. Five microliter of the sample were used directly as the template in the PCR assay. The presence of various *Mycoplasma* species and *U. urealyticum* would be detected using the commercially available PCR Mycoplasma Detection Set (TaKaRa Bio Inc, Japan). The nested PCR reactions contained a 0.1 mM concentration of dNTP mixture, 5 ρmol of each respective MCGp F1 (5'-ACACCATGGGAGCTGGTAAT-3') and MCGp R1 (5'-CTTCWTCGACTTYCAGACCC AAGGCAT-3') primers (for the first PCR reaction) and MCGp F2 (5'-GTTCTTTGAAAACTGAAT-3') and MCGp R2 (5'-GCATCCACCAWAWACTCT-3') primers (for the second PCR reaction) and 2.5 U of TaKaRa Taq™ DNA polymerase (TaKaRa Bio Inc, Japan). All reactions in a final reaction volume of 25 μL were performed in a PCR Mastercycler® gradient (Eppendorf, Germany) under the following conditions: 1 cycle of 30 sec at 94°C, followed by 35 three step cycles of 94°C for 30 sec, 55°C for 60 sec and 72°C for 60 sec, followed by 10 min at 72°C. The PCR products were separated on ethidium bromide stained 1.5 % (w/v) agarose gel (Merck, USA). In a separate reaction, each sample of DNA was tested by adding supplied control DNA and running a PCR to determine if amplification inhibitors were present.

RESULTS AND DISCUSSION

No bacterial growth was observed in the Mycofast® diagnostic kit assay. Meanwhile, the PCR Mycoplasma Detection kit detected neither *U. urealyticum* nor *M. hominis* in 57 tested samples, although other *Mycoplasma* species could be present with an approximately 500 bp band shown in one of the 57 samples (Fig. 1). The second PCR of the nested PCR analysis confirmed that the particular sample might have contained mixed cultures of *M. neurolyticum* and *M. pulmonis* (Fig. 2).

Mycoplasma and *Ureaplasma*, especially *M. hominis* and *U. urealyticum* are two common sexually transmitted genital microflora found in sexually active adults (Heggie *et al*., 2001). Most colonized women are asymptomatic; however, vaginal colonization associated with preterm delivery has been linked to high mortality rates in newborn neonates (Kafetzis *et al*., 2004; Kataoka *et al*., 2006). The microbes may be parentally transmitted to the newborns from the mother at the time of delivery or by direct invasion of the fetus *in utero* (Waites *et al*., 1993; Lyon, 2000; Pandey *et al*., 2007).

In recent years, several studies attempted to relate the possibility of an association between *M. hominis* and *U. urealyticum* in respiratory colonization with the development of CLD in preterm neonates (Iles *et al*., 1996; Agarwal *et al*., 2000; Kafetzis *et al*., 2004; Kotecha *et al*., 2004). On the other hand, there have been studies that failed to detect any association (Heggie *et al*., 2001; Ollikainen *et al*., 2001; Pandey *et al*., 2007). Thus, to date, the role of *M. hominis* and *U. urealyticum* in the pathogenesis of CLD remains controversial. The great variations in sample selection, sampling methods and identification assays applied might explain the different results observed in those studies.

Endotracheal aspirates are valuable markers of lower respiratory tract infections (Cassell *et al*., 1998); therefore, it could be beneficial to perform quantitative cultures for detection of *Mycoplasma* species and *U. urealyticum* in lower respiratory secretions soon after birth. In the past, a culture would

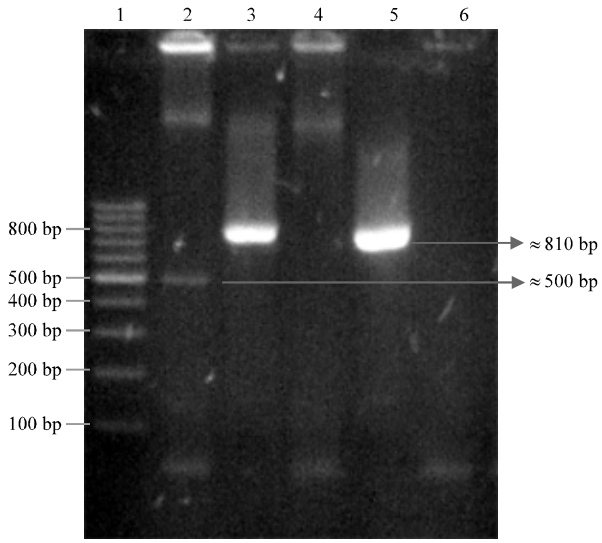

Fig. 1: First PCR result of nested PCR assay for identification of *Mycoplasma* species and *Ureaplasma urealyticum*. Lane 1: 100-bp ladder (Fermentas, Maryland); Lanes 2 and 4: tested samples; the ~500 bp band in Lane 2 indicates the presence of *Mycoplasma* species; Lanes 3 and 5: tested samples with added internal control DNA (~ 810 bp); Lane 6: DNA blank (sterile distilled water)

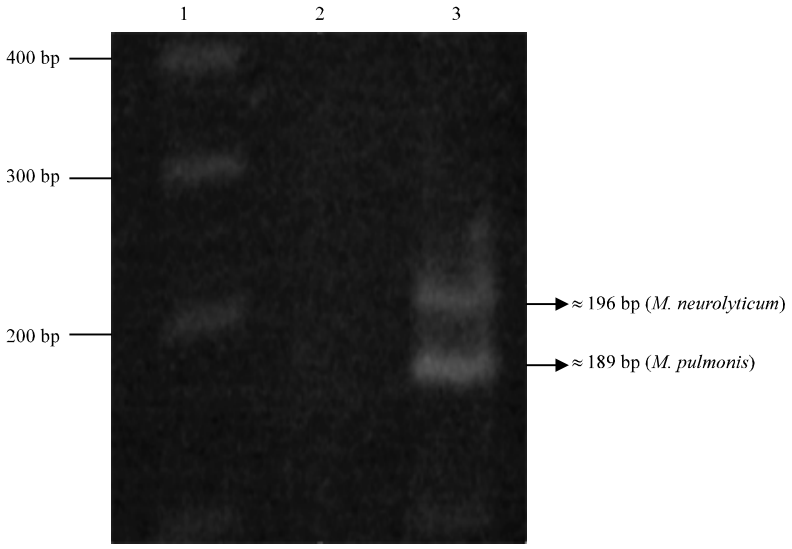

Fig. 2: Second PCR result of nested PCR assay. Lane 1, 100-bp ladder (Fermentas, Maryland); Lane 2, DNA blank; Lane 3, Result after PCR using amplicon obtained from first PCR. Band with 196 bp indicated the presence of *M. neurolyticum* and 189 bp for the *M. pulmonis*

be the only assay for detection of *Mycoplasma* and *Ureaplasma*. As a consequence, *M. hominis* and *U. urealyticum* were among the least frequently diagnosed respiratory pathogens in clinical specimens. In addition, culture identification is slow; it can take up to 2-5 days to culture *Ureaplasma* and *M. hominis* and possibly up to 8 weeks to culture *M. genitalium* (Waites *et al*., 2005). Historically, culture identification is considered definitive; colony identification is difficult, subjective and lacks sensitivity, since it is based on observation under a light microscope. It is also vulnerable to cross-contamination with other microorganisms that might generate false-positive results.

Now, with advancements in molecular techniques, there have been evaluations of different PCR systems, commercial as well as in-house PCR assays, that showed high sensitivity, specificity, rapidity and the prospect of direct identification of many *Mycoplasma* species as well as *Ureaplasma* species in clinical specimens (Abele-Horn *et al*., 1996; Luki e*t al*., 1998; Stellrecht *et al*., 2004; Dhawan *et al*., 2006; Hashimoto *et al*., 2006). A PCR assay would have better diagnostic potential compared to cultures in the detection of *Mycoplasma* and *Ureaplasma* in neonatal respiratory samples. The enhanced sensitivity and specificity of the PCR also suggest that PCR methods could be suitable as a routine diagnostic tool in identification of *Mycoplasma* and *Ureaplasma*, especially in diagnostic laboratories that do not currently test for *Mycoplasma* and *Ureaplasma*.

In the present study, Mycofast Screening kit was unable to detect other *Mycoplasma* and *Ureaplasma* species besides *M. hominis* and *Ureaplasma urealyticum* and this could explain the failure of detection of other *Mycoplasma* species in one of our specimens by using this kit. However, we used a second approach, PCR to screen all the samples again. Commercially available PCR *Mycoplasma* Detection Set used in this study relied on the principle of PCR for rapid identification of various *Mycoplasma* species and *U. urealyticum* (Uemori *et al*., 1992). From the results obtained, neither *M. hominis* nor *U. urealyticum* was detected in any of the 57 tested samples. Only one of 57 specimens was found to carry mix infection of *M. neurolyticum* and *M. pulmonis*. In general, false-negative results might have occurred if the specimens contained PCR amplification inhibitors; however, our samples with additional control DNA shown absence of inhibitors with all samples generating an approximately 810 bp amplicon (Fig. 1) in the internal control test.

Generally, inability to detect *Ureaplasma* and *Mycoplasma* by either serological or molecular method might also be attributed to the length of time the tracheal aspirate specimens had been stored; a period of several months to a year before laboratory analysis began. Tracheal aspirates were collected in normal saline only, without being inoculated in any storage media. Some degree of deterioration may have occurred to a point where no bacteria could be detected at all. Therefore, analysis should be carried out as soon as possible once the samples are collected.

In conclusion, as only a single sample was detected to have a mix culture of *Mycoplasma* species by means of the nested PCR approach, this did not give a significant association between *U. urealyticum* and *Mycoplasma* species infection with CLD. Therefore, we conclude CLD in Malaysian preterm neonates is not due to colonization of *U. urealyticum* or *Mycoplasma* species in their respiratory tract.

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