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ORIGINAL ARTICLES

Multiplex PCR for the detection of urogenital pathogens in mothers and newborns

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Abstract

Two duplex PCR assays were established for the detection of *C. trachomatis* (Ct), *N. gonorrhoeae* (GC), *M. hominis* (Mh), and *U. urealyticum* (Uu). These assays were used on clinical specimens obtained from women with Premature Rupture of Membrane or Post Partum Fever, from preterm infants, as well as from women with uneventful pregnancies and their babies delivered vaginally at term.

The analytical sensitivity of the duplex PCR assays with internal controls incorporated is 7.0, 19.0, $5x10^3$ and $7x10^2$ genome copies per reaction for Ct, GC, Mh and Uu respectively. Specificity was demonstrated by the amplification of only target DNA in the presence of other organisms. Among 40 women with normal, at term, deliveries, there were 6 positives for Ct, 2 for GC and 1 for Uu. None of these women had signs of genital tract infection. The Mh/Uu PCR was positive in 11 of 40 PROM cases, with 7 women positive for Uu, 2 for Mh and 2 others for both organisms. Of 40 blood cultures taken from post-partum maternal infections, 6 were positive for Ct and 1 for Mh. Respiratory secretions from 30 premature neonates yielded 5 positives for Uu and one each for Mh and Ct. In contrast, there was only 1 positive result (for Mh) in 30 mature neonates. With 1 exception, all mycoplasma and ureaplasma positives were confirmed by culture and the concordance between paired tracheal aspirates and nasopharyngeal swabs from neonates was 96.7%. These results show the potential use of the duplex PCR assays for the diagnosis of maternal and neonatal disease caused by the four urogenital pathogens.

Key words: urogenital pathogens, PCR, pregnant mothers and neonates.

INTRODUCTION

Maternal genital tract infection has been associated with complications such as ascending chorioamnionitis, premature rupture of membranes (PROM), preterm delivery, post-abortion or post-partum endometritis, low birth weight babies, and perinatal morbidity and mortality. Major pathogens associated with these complications include *Chlamydia trachomatis, Neisseria gonorrhoeae, Mycoplasma hominis and Ureaplasma urealyticum*[1-4]. Maternal chlamydial and gonococcal cervicitis may be asymptomatic and hence, remain undiagnosed and untreated. *M hominis and U urealyticum* are present in 30-80% of women but are also known causes of neonatal infection, which unfortunately, are often undiagnosed because of low awareness or lack of diagnostic facility. Rapid and reliable tests are required for early detection and treatment to prevent adverse pregnancy outcomes and neonatal infection.

Multiplex PCRs are designed for the simultaneous detection of multiple microbial targets. These assays have been increasingly used for the detection of common etiologic agents in genital discharge and genital ulcers[5-6]. In this study, two duplex PCR assays, one for *C. trachomatis* and *N. gonorrhoeae* and one for *M. hominis* and *U. urealyticum* were optimized for different specimen types frequently collected for the diagnosis of maternal and neonatal infections.

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MATERIAL AND METHODS

This study was approved by the Medical Ethics Committee, University of Malaya Medical Centre, Ref.201.12.

Specimen collection

In order to avoid unnecessary intrusion on routine management, three different specimen types were collected from women in the labour room and maternity wards, after obtaining informed consent: (a) in women with preterm PROM (at less than 37 weeks gestation) from whom a vaginal swab is usually taken for bacterial culture to exclude genital tract infection, an additional swab was taken for PCR assays, (b) in women with normal vaginal deliveries at term (at least 37 weeks of gestation) who normally do not require a vaginal swab to be taken, a swab was taken from the outer surface of the placenta after placental delivery, and (c) in women with post-partum fever (temperature of at least 38°C on at least 2 occasions after the first 24 hours post-partum) who are routinely investigated by a blood culture, an extra 5ml of blood was collected for PCR.

Respiratory secretions were collected by nasopharyngeal swabs from premature (28-36 weeks gestation or birth weight less than 2500g) as well as term babies (birth weight at least 2500g). In addition, tracheal aspirates were collected from babies who required tracheal suction for clinical indications.

Specimens were transported to the laboratory in Amies medium for bacterial culture and in PCR transport medium for storage in -20°C prior to DNA amplification work. A questionnaire was filled up for each mother investigated, to obtain obstetrical history and related risk factors.

Laboratory investigations

Microscopy and culture
All maternal and neonatal specimens were examined for the presence of gonococci by the Gram-stained smear and culture on Modified Thayer-Martin (MTM) agar. Tracheal secretions from babies were also cultured for *M. hominis* and *U. urealyticum* using the Mycofast Evolution 2 kit (International Microbio, France) according to the manufacturer's instructions.

DNA extraction
The method of DNA extraction used varied with the specimen type.

Tracheal aspirates and nasopharyngeal swabs were treated as described in Nelson *et al*., 1998[7]. Briefly, specimens were centrifuged in lysis buffer (Tween-20, Nonidet -P40, Proteinase K 20mg/ml) at 13,000 rpm x 30 min. The supernatant was resuspended in lysis buffer and incubated at 60°C for 1h, then boiled for 10 min. The resulting lysate was mixed with guanidine isothyocianate containing DNAzol (Molecular Research Center, Inc. USA) and centrifuged. DNA was precipitated from the supernatant with 100% ethanol, recovered by spooling, washed in ethanol and solubilized in TE buffer.

For placental and vaginal swabs, the procedure for tracheal aspirates was applied except that DNAzol treatment was replaced by phenol-chloroform extraction as described in Sambrook *et al.*, 2001[8].

Blood in BACTEC blood culture bottles was treated using alkali wash and heat lysis extraction as described in Millar *et al*., 2000[9]. 0.1ml of blood was added to 1 ml of alkali wash solution containing 0.5M NaOH and 0.05 M sodium citate and mixed by inversion for 10 min at room temperature. After centrifuging at 13,000rpm x 5 min, the pellet was resuspended in 0.5ml Tris-HCL (0.5M, pH 8.0) and centrifuged again at 13,000rpm for 5 min. The washing and centrifugation step was repeated 2 more times, after which the pellet was resuspended in 0.1ml Tris-EDTA (10mM Tris-HCL at pH8 with 1mM EDTA) and heated at 100°C for 1 h. The lysate was frozen and thawed twice then centrifuged at 13,000rpm x 15m. The supernatant containing extracted DNA was stored at -20°C until used for PCR.

DNA quantification
DNA concentration in μg/μl was estimated by taking absorbance readings measured at 260nm in a spectrophotometer (Eppendorf, Hamburg)

Polymerase chain reaction
The bacterial strains used as controls were: *C. trachomatis* L2 (from Prof. Akira Matsumoto, formerly from Kawasaki Medical School, Japan); *N gonorrhoeae* clinical isolate confirmed by CTA sugars, Phadebact Gonococcus Test (Pharmacia Diagnostics) and API NH (BioMerieux, France); *M. hominis* and *U. urealyticum,* both from International Microbio, France.

Primers used for the PCR assays were: KL1 and KL2 for *C. trachomatis*[10]*;* HO1 and HO3 for *N. gonorrhoeae*[11]*;* UMS125 and UMA226 for *U. urealyticum*[12], and RNAH1 and RNAH2 for

M. hominis[13]. The DNA sequence of primers is as described in the respective reference articles.

A hybrid internal control (IC) was synthesized for each duplex PCR. For the *M. hominis* and *U. urealyticum* (Mh/Uu) PCR, the IC was a 650bp lambda DNA fragment (sequence positions 40-690) flanked by 2 short *M. hominis* sequences of 42bp and 39bp, that are complementary to the primers RNAH1 and RNAH2 respectively. For the *C. trachomatis* and *N. gonorrhoeae* (Ct/GC) PCR, the IC was the same lambda phage fragment flanked by 40bp sequences complementary to the primers HO1 and HO3. The optimal amount of IC for each duplex PCR was determined prior to its incorporation into the corresponding assay.

Thermal cycling parameters were optimized by varying annealing temperatures and concentrations of magnesium chloride, dNTPs and primers. The analytical sensitivity of of each duplex assay was determined with serial 10-fold dilutions of known concentrations of control strains and compared to the sensitivity of single PCR assays similarly determined. Primer specificity was tested against organisms commonly found in the genital and respiratory tracts, including *E. coli, Klebsiella* species, *G. vaginalis, S. aureus* and *C. pneumoniae*.

PCR on clinical samples

All clinical samples were tested by both duplex PCR assays.

For the GC/Ct assay, each 50 μl reaction mixture contained: 1xPCR buffer, 100 μM of each dNTP, 0.2μM of HO1 and HO3 primers, 0.4 μM of KL1 and KL2 primers, 2U of *Taq* polymerase, 2.5mM of $MgCl_2$, 1 μl of IC and 10 μl of DNA template. Amplification was carried out on a Perkin Elmer 480 thermal cycler with 35 cycles of denaturation (95°C x 1 min.), annealing (55°C x 1 min.), and extension (72°C x 2 min) and ending with a final extension step at 72°C for 7 minutes.

For the Mh/Uu assay, each 50 μl reaction mixture contained: 1xPCR buffer, 100 μM of each dNTP, 0.4 μM of RNAH1 and RNAH2 primers, 0.6 μM of UMS125 and UMA 226 primers, 2U of *Taq* polymerase, 2 mM of $MgCl_2$, 3 μl of IC and 10 μl of DNA template. Amplification was carried out on the same Perkin Elmer 480 thermal cycler with 35 cycles of denaturation (94°C x 1 min. 30s), annealing (55°C x 2 min.), extension (72°C x 1 min. 30s) and ending with a final extension step at 72°C for 7 minutes.

Organism and reagent controls were included in all assays.

To visualize PCR products, gel electrophoresis was carried out on a 1.5% agarose gel in 1xTBE buffer for 45 - 60 min. at 90V, followed by staining with 2 μl of 10mg/ml ethidium bromide solution and viewing under UV light.

RESULTS

Study population

A total of 40 subjects in each group of women (groups a, b and c as described above) was studied, together with 30 premature and 30 term babies. The women in group (a) were mostly Chinese housewives, and primipara, between 24 and 38 years old. Those in groups (b) and (c) were mostly Malay working mothers between 28 and 38 years old. The mean birth weight of term and premature babies was 3.1 kg $\pm$ 540g and 1.6 kg $\pm$ 469g, respectively, and their Apgar scores were 8.8-9.97 and 6.4-8.6. All preterm babies were managed in the special care nursery for 2-6 days, for presumed sepsis, mild to severe respiratory distress syndrome or intra-uterine growth retardation, and were mostly treated with penicillin and gentamicin empirically. All were discharged well within 10 days of admission to the nursery, including those with positive laboratory results.

Culture

None of the specimens cultured for *N. gonorrhoeae* were positive. Mycoplasmal cultures on nasopharyngeal swabs from neonates yielded 5 positives for Uu and 1 for Mh at $>10^5$ and $>10^4$ cfu/ml respectively. These specimens were also positive in the Mh/Uu PCR.

Polymerase Chain Reaction

After optimization and with the inclusion of the hybrid IC, the limits of detection for the Ct/GC PCR were found to be 6.9 and 19.1 genome copies for Ct and GC respectively. For the Mh/Uu PCR, the limits of detection were $5.6x10^3$ genome copies for Mh and $7.6x10^2$ for Uu. The sensitivity of the duplex assays was 10 fold lower than that of corresponding monoplex PCRs for Ct, GC and Mh but 10^3 fold lower for Uu (results not shown).

The specificity of both assays was demonstrated by the failure of primers to amplify organisms other than the target organism.

PCR detection rates for the 4 uropathogens are summarized in Table 1. Of 13 Ct PCR positives,

TABLE 1: Detection rates in maternal and neonatal specimens obtained using duplex PCRs for *C. trachomatis, N.gonorrhoeae, M.hominis* and *U.urealyticum*

		No. (%) Detection			
		Ct	Gc	Mh	Uu
Normal pregnancy	(n=40)	6(15.0)	2(5.0)	0	1(2.5)
PROM	(n=40)	0	0	4(10.0)	9(22.5)
PP fever	(n=40)	6(15.0)	0	1(2.5)	0
Preterm neonate	(n=30)	1(3.3)	0	1(3.3)	5(16.7)
Term neonate	(n=30)	0	0	1(3.3)	0

Ct, *C.trachomatis*; GC, *N.gonorrhoeae*; Mh, *M.hominis*; Uu, *U.urealyticum*
PROM, premature rupture of membranes
PP, post-partum

6 were for placental swabs from women with term deliveries and 6 in the blood cultures of women with PP fever. The GC PCR was only positive in 2 women with term deliveries. There were 7 positive Mh PCR results, of which 4 were in the vaginal swabs of women with PROM. The Uu PCR had the most number of positive results with 9 in women with PROM and 5 in the nasopharyngeal swabs of term babies.

In preterm neonates, paired tracheal aspirates and nasopharyngeal swabs were obtained. These paired specimens showed a 96.7% (29/30) concordance in the PCR results (Fig.1). The 5 positives for Uu and 1 for Mh were positive in both aspirates and swabs. One neonate had Ct detected in his nasopharyngeal swab but not in his tracheal aspirate. This discrepancy could be explained by Ct colonization in the nasopharynx but absence of infection lower down in the respiratory tract.

The high concordance rate for results of paired tracheal and nasopharyngeal specimens indirectly indicates the reproducibility of the duplex PCR assays. Moreover, where specimens were collected from mother-infant pairs, there was good correlation between maternal and neonatal specimens as illustrated in Fig.2.

DISCUSSION

The aim of this study was to establish a rapid diagnostic test for the detection of pathogens associated with maternal and neonatal infections. As convenience specimens were collected to avoid inconvenience to patients and their attending medical personnel, different specimen types were processed, and this provided an opportunity to study the efficacy of various DNA extraction methods. Preliminary experiments identified the highest DNA yield from the

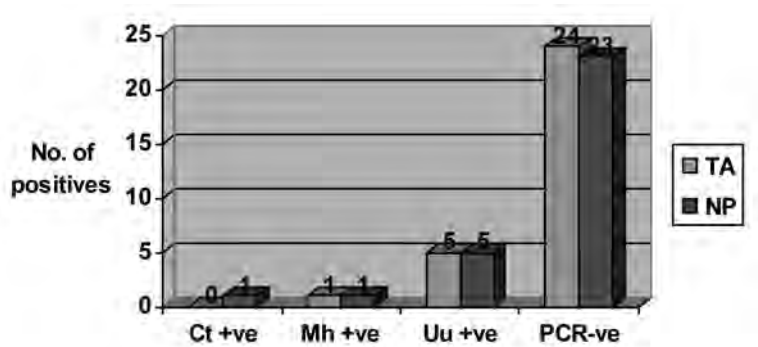

FIG.1: Results of duplex PCR assays for *C.trachomatis, M.hominis* and *U. urealyticum* in paired tracheal aspirates and nasopharyngeal swabs from preterm neonates

Ct, *C.trachomatis;* Mh, *M.hominis ;* Uu, *U. urealyticum*
TA, tracheal aspirate; NP, nasopharyngeal swab

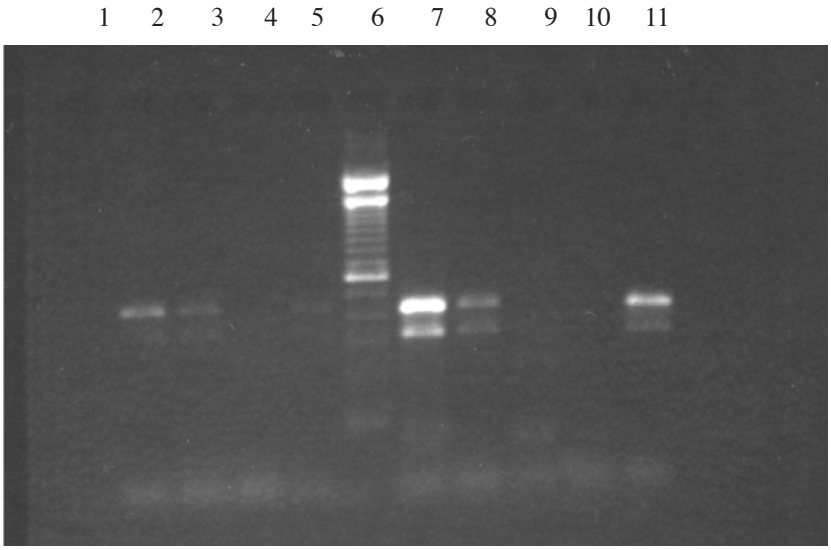

FIG. 2: *Mh-Uu* duplex PCR results for one infant positive for both *M. hominis* (Mh) and *U. urealyticum* (Uu).

Lane 1	: Reagent Control	(-)
Lane 2	: Nasooropharyngeal Swab	(+) for both *Mh* and *Uu*
Lane 3	: Oral Swab	(+) for both *Mh* and *Uu*
Lane 4	: Nasal Swab	(-)
Lane 5	: Tracheal Aspirate	(+) (taken in Special Care Nursery)
Lane 6	: DNA marker 100bp	
Lane 7	: Tracheal Aspirate	(+) (taken in Labour Room)
Lane 8	: Endocervical Swab	(+) (from mother of the infant)
Lane 9	: Placental swab	(-) (from mother of the infant)
Lane 10	: Negative Control	(-)
Lane 11	: Positive Control	(+) for both *Mh* and *Uu*

following protocols: lysis buffer-DNAzol extraction for tracheal secretions, Lysis buffer-phenol-chloroform extraction for vaginal secretions and alkali wash - heat lysis for blood samples. These methods were subsequently used on the various clinical material obtained from women and their newborn babies.

In the PCR assays, the primers used were those described by other workers for single target amplification. For multiplex testing, thermal cycling conditions had to be re-optimized to ensure adequate sensitivity for competing target organisms and the internal control. The limit of detection for all 4 target organisms were reduced at least 10-fold in the duplex assays with internal control incorporated, compared to the limit of detection for corresponding monoplex assays. Nevertheless, the results obtained for the CT/GC PCR suggest that this assay has adequate clinical sensitivity. Previous studies among asymptomatic women attending antenatal clinics in Malaysia reported 0.5% prevalence of gonorrhoea by culture and 2-3% of chlamydia by enzyme immunoassay[14]. As expected of a nucleic acid amplification test, the CT/GC detection rates obtained in this study for women with normal deliveries are higher than that obtained previously with less sensitive non-nucleic acid based tests. The more frequent detection of *C. trachomatis* compared to *N. gonorrhoeae* is again in line with earlier observations that genital chlamydia is more prevalent than gonorrhoea among Malaysians[14].

The Mh/Uu PCR detected very few positives among well mothers and mature neonates. Since genital mycoplasmas are highly prevalent among healthy women, and neonates are often colonized at birth, this low detection rate could be related to the relatively high detection limit of 10^2 and 10^3 genome copies for *U. urealyticum* and *M. hominis* respectively. For the detection of low numbers of these organisms in asymptomatic individuals, the Mh/Uu PCR should be further optimized to improve clinical sensitivity. However, in routine

clinical practice, there will be little need for the use of these tests in asymptomatic subjects.

In contrast, the Mh/Uu assay was more often positive among symptomatic women and premature babies, who, presumably, carried more organisms in their secretions. Genital mycoplasmas have been implicated as the cause of PROM[15,16], PP fever[17] and respiratory disease in premature infants[18]. The Mh/Uu assay should be useful for the diagnosis of these infections, particularly when no other pathogens are detected and when patients are not responding to conventional antibiotic coverage.

The main limitations of this study are the small number of subjects examined, and the lack of chlamydial culture and other nucleic acid amplification tests to confirm Ct/GC PCR results. Nevertheless, the findings suggest the superiority of the CT/GC PCR over gonococcal culture, good correlation of the Mh/Uu PCR with mycoplasmal culture for respiratory secretions, and reproducibility of results for both PCR assays. Hence, these assays are potentially useful tools for the rapid detection of *C. trachomatis, N. gonorrhoeae, M. hominis,* and *U. urealitycum* in female genital and neonatal respiratory secretions.

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