The Myanmar Health Sciences Research Journal, Vol. 19, No. 2, 2007

Optimization of a multiplex polymerase chain reaction assay and its application for simultaneous detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in clinical specimens

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Chlamydia trachomatis and *Neisseria gonorrhoeae* infections are common sexually transmitted infections with a similar pattern of clinical presentation and related sequelae such as urethritis, cervicitis, pelvic inflammatory disease, ectopic pregnancy and infertility. Chlamydial co-infections are also frequently found in patients with gonorrhoea. This study was conducted to optimize a multiplex polymerase chain reaction (M-PCR) assay which could be applied to detect *N. gonorrhoeae* and *C. trachomatis* simultaneously from genitourinary specimens. The procedure of a M-PCR assay, developed by Mahony *et al.* (1995), was modified by incorporating lambda phage-Neisseria hybrid DNA as an internal control for the detection of amplification inhibitors. Thermal cycling parameters were optimized by varying annealing temperatures and using different concentrations of magnesium chloride, dNTPs and primers. The optimized M-PCR assay had a detection limit of 2.885 x 10^3 genomecopies for *C. trachomatis* and 8.995x10^2 genome copies for *N. gonorrhoeae.* It was used to detect *N. gonorrhoeae* and *C. trachomatis* in 90 endocervical swab specimens from women with vaginal discharge. The specimens were also tested for chlamydial antigen using Clear View test kit (Unipath, UK) and for *N. gonorrhoeae* by conventional culture. The sensitivity and specificity of the M-PCR assay were found to be superior to those of gonococcal culture and chlamydia antigen testing, making it a potentially useful test for the diagnosis of *N. gonorrhoeae* and *C. trachomatis* in lower genital tract infections in symptomatic women.

INTRODUCTION

Neisseria gonorrhoeae and *Chlamydia trachomatis* infections are common bacterial causes of sexually transmitted infections. They have a similar pattern of clinical presentation and related sequelae such as urethritis, epididymitis, cervicitis, pelvic inflammatory disease, ectopic pregnancy and infertility. Both organisms colonize the mucosal epithelium of the genital tract with the cervix as the primary site in women and urethra in men. Concomitant gonococcal and chlamydial infections are frequently found in men and women [1].

Multiplex Polymerase Chain Reaction (M-PCR) assays permit simultaneous screening for multiple pathogens that might be causing a disease condition. In multiple-agent infections, all the pathogens causing

the disease can be detected in a single specimen subjected to a single reaction [2]. This makes M-PCR a useful tool for the determination of the prevalence of sexually transmitted infections in the community, thus enabling further interventions for the prevention and management of these infections [3].

Numerous studies have demonstrated that a small, but significant, proportion of clinical specimens contain substances that interfere with the nucleic acid amplification process. Unless inhibitory specimens are identified, negative amplification test results do not exclusively indicate the absence of infection. Inhibitory specimens can be identified by monitoring the amplification of a second target nucleic acid which serves as an internal control [4]. The inclusion of an internal control (IC) in a PCR assay may decrease the sensitivity of the test because of the competition between the IC and the target DNA. Hence, thermal cycling conditions have to be optimized to overcome this competition and ensure adequate test sensitivity.

General objective

- To optimize a multiplex PCR assay which can detect *N. gonorrhoeae* and *C. trachomatis* (Ng-Ct M-PCR) simultaneously from genitourinary specimens

Specific objectives

- To determine the optimal conditions for a Ng-Ct M-PCR assay which was modified by incorporating an internal control to identify inhibitory specimens
- To assess the applicability of the optimized Ng-Ct M-PCR assay in clinical specimens.

MATERIALS AND METHODS

Laboratory tests were carried out from January 2005 to October 2006 at the Molecular Diagnostics and Research Laboratory, University of Malaya, Malaysia and Bacteriology Research Division, Department of Medical Research (LM).

Optimizing the Ng-Ct M-PCR Assay

a. Construction of a hybrid internal control (IC) for Ng-Ct M-PCR assay

A 650 base pair (bp) lambda phage DNA segment (# SD 0011) was selected for use in a hybrid internal control, as its G+C content (51%) and melting temperature were similar to those of the *N. gonorrhoeae cpp* gene.

The hybrid IC was created in a single PCR with GCIC 1 and GCIC 2 primers with the following DNA sequences:

- GCIC1 primer (sense)
5'GCTACGCATACCCGCGTTGCTTCCGGTTTAAGG CGTTTCC 3'

- GCIC 2 (anti-sense)
5'CGAAGACCTTCGAGCAGACATCATCCAGCGCG GCTGCTTT 3'

The resultant 730 bp hybrid IC DNA, consisting of the 650 bp lambda phage segment flanked by 2 short sequences complementary to HO 1 and HO 3 primers of *N. gonorrhoeae* [5], was purified with QIA Quick PCR Purification Kit (Qiagen).

A series of 10-fold dilutions of hybrid DNA was amplified with HO 1 and HO 3 primers and the dilution presenting the faintest positive band was selected as the optimal amount of hybrid internal control to minimize the competition between *N. gonorrhoeae* target DNA and hybrid IC DNA.

b.Optimizing thermal cycling conditions for Ng-Ct M-PCR with hybrid IC incoporated

Primers KL1 and KL2 were used to amplify 241 bp fragment of ORF 2 of a 7.5 kb genetically conserved plasmid in *C. trachomatis* as described in Mahoney JB *et al.*, 1992[6]

- KL 1 5' TCCGGAGCGAGTTACGAAGA 3'
- KL 2 5' AATCAATGCCCGGGATTGGT 3'

Primers HO1 and HO3 were used for the amplification of a 390 bp fragment of the *cpp* gene of a 4.2 kb *N. gonorrhoeae* cryptic

plasmid as described in Ho BSW *et al*., 1992[5]:

- HO 1 5'GCTACGCATACCCGCGTTGC 3'
- HO 3 5'CGAAGACCTTCGAGCAGACA 3'

N. gonorrhoeae ATCC 49226 and *C. trachomatis* L2 strain were used as positive DNA controls.

With the incorporation of the hybrid IC into the PCR mixture, thermal cycling conditions were modified and optimized by varying the annealing temperature and using different concentrations of magnesium chloride, dNTPs and primers.

Sensitivity of Ng-Ct M-PCR Assay

N. gonorrhoeae DNA and *C. trachomatis* DNA were estimated using optical density measurement at 260 nm in a spectrophotometer (Eppendorf, Hamburg). The sensitivity of Ng-Ct M-PCR was assayed with 10-fold dilutions of *N. gonorrhoeae* DNA and *C. trachomatis* DNA.

Specificity of Ng-Ct M-PCR Assay

Ng- Ct M-PCR assays were performed with the DNA of non-target bacterial strains (*Escherichia coli, Klebsiella* spp, *Staphylococcus aureus, Gardnerella vaginalis*) which can be found in the female genital tract.

Detection of N. gonorrhoeae and C. trachomatis in clinical specimens

DNA Extraction

DNA was extracted from 90 endocervical swabs which were collected from women presenting with vaginal discharge. This method involved cell digestion by lysis buffer which contained Proteinase K, Nonidet and Tween-20 followed by DNAzol treatment [7].

Ng-Ct M-PCR assay

Ng-Ct M-PCR assay was carried out using a thermal cycler (Perkin Elmer 480,USA) and two sets of primers: HO1-HO3 and KL 1-KL 2. PCR products were analyzed by electrophoresis on a 1.5% agarose gel in 1x TBE buffer system.

Detection of C. trachomatis antigens

C. trachomatis was detected from endocervical swabs using Clear View test kits (Unipath, UK) according to the manufacturer's instructions. This is a solid phase sandwich immunoassay using chromatography and monoclonal antibodies to a genus-specific LPS antigen.

Detection of N. gonorrhoeae by culture

N. gonorrhoeae was isolated from endocervical swabs by inoculating onto Thayer Martin and chocolate agar and incubating up to 48 hr in 5% CO_2. The isolates were confirmed by gram staining, oxidase test and Phadebact GC monoclonal antibody test.

Analysis of the results obtained by Ng-Ct M-PCR, antigen detection test and culture

The M-PCR results were compared with those of conventional culture and antigen detection methods. The specimens with discrepant results were retested with single PCR assays for *N. gonorrhoeae* and *C. trachomatis*, using HO1-HO3 and KL1-KL2 primers respectively.

Ethical consideration

Strict confidentiality is maintained with regard to the identity of the subjects.

RESULTS

Optimization of the M-PCR

10-fold dilutions of the hybrid IC generated with HO1 and HO3 primers were visualized in a gel electrophoresis with ethidium bromide staining. The faintest positive band was obtained for the10^{11} dilution. Thus 1 µl of 10^{-11} hybrid IC was used in the M-PCR assay. Following optimization, PCR components used in the reaction mixture for the Ng-Ct M-PCR are shown in Table 1.

The thermal profile for the Ng-Ct M-PCR consists of 1 cycle of 95˚C x 5 min followed by 40 cycles of 95˚C x 1 min, 57˚C x 1 min and 72˚C x 2 min, and ending with a final extension at 72˚C x 7 min.

Table 1. PCR components in the reaction mixture for Ng-Ct M-PCR

PCR Components	Volume (µl)
Sterile double distilled water	25.0
10 x PCR Buffer	5.0
dNTPs mix (10mM)	2.0
50 mM Magnesium chloride	4.0
5 U/µl Taq polymerase	0.4
HO 1 primer (5 pmole/µl)	0.5
HO 3	0.5
KL 1 primer (8 pmole/µl)	0.8
KL 2	0.8
DNA Template	10.0
Internal control (10^{-11}dilution)	1.0
TOTAL VOLUME	50.0

The detection limit of M-PCR was calculated at the lowest dilution showing positive band for both *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Both *N. gonorrhoeae* and *C. trachomatis* were detected up to 10^{-4} dilution (Fig.1).

Sensitivity(Detection Limit)of Ng-Ct M-PCR

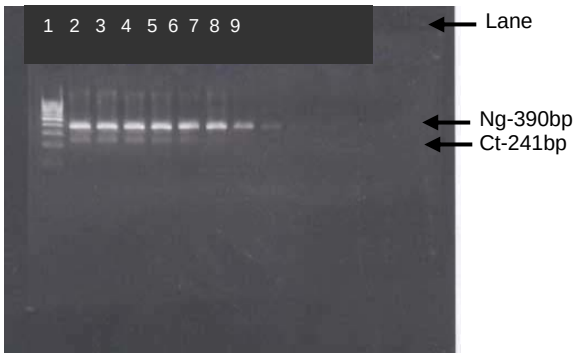

Fig.1. Ng-Ct M-PCR assay using serial dilutions of Ng and Ct DNA

L1 – 100 bp DNA ladder
L2 – Neat Conc. of Ng and Ct (+) for both (Ng- 390 bp and Ct- 241 bp)
L3 – Ng 10^{-1} and Ct 10^{-1} (+) for both
L4 – Ng 10^{-2} and Ct 10^{-2} (+) for both
L5 – Ng 10^{-3} and Ct 10^{-3} (+) for both
L6 – Ng 10^{-4} and Ct 10^{-4} (+) for both
*(Lowest dilution showing both bands)
L7 – Ng 10^{-5} and Ct 10^{-5} (+) for Ng only
L8 – Ng 10^{-6} and Ct 10^{-6} (+) for Ng only
(L=Lane)

As a starting preparation of *N. gonorrhoeae* had $8.995x10^6$ genomes copies, the detection limit of it was 8.995×10^2 genome copies. Similarly, *C. trachomatis* which contained $2.885x10^7$ genomes copies in an initial preparation giving a detection limit of 2.885×10^3 genomes.

Specificity of Ng-Ct M-PCR

None of the non-target bacterial strains were amplified, indicating that the primers used in Ng Ct M-PCR had a high specificity for *N. gonorrhoeae* and *C. trachomatis*.

Detection of N. gonorrhoeae and C. trachomatis in endocervical swabs by M-PCR

The M-PCR was positive for *N. gonorrhoeae* in 11 endocervical swabs and for *C. trachomatis* in 14, giving detection rates of 12.2% and 15.6% respectively. Specimens were classified as M-PCR negative only if the IC was amplified, indicating the absence of amplification inhibition (Fig. 2).

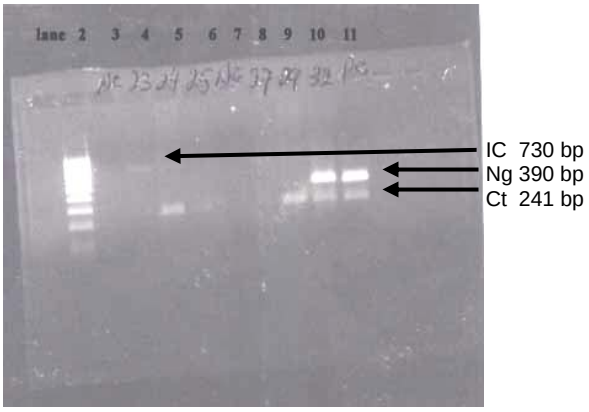

Fig. 2. Examples of Ng-Ct M-PCR results on endocervical swabs from patients with vaginal discharge

L2 –100 bp DNA Ladder
L3 –NC (-)
L4 –S no.23 (-) for Ng and Ct,(+)IC(730 bp)
L5 – S no. 24 (+) for Ct (241 bp)
L9 – S no. 29 (+) for Ct
L10 – S no.32(+) for Ng (390 bp)+Ct (241 bp)
L11 – PC(+) for Ng +Ct
(L= Lane, S= Specimen,NC= Negative Control, PC= Positive Control)

Analysis of results obtained by Ng-Ct M-PCR, antigen detection test and culture

The results of tests performed on the 90 endocervical swabs are summarized in Table 2. There were a total of 25 M-PCR

positive results, and 8 and 16 positives for Ng culture and Ct antigen testing respectively. Discrepant results were observed for 5 samples only, giving a concordance rate of 94.4% for paired M-PCR/Ng culture and M-PCR/Ct antigen tests. All specimens showing discrepant results between M-PCR and either Ng culture or Ct antigen detection was examined by corresponding monoplex PCR assays.

Table 2. Results of M-PCR compared to *N. gonorrhoeae* culture and antigen test for *C. trachomatis*

		Culture for Ng		Ag test for Ct		Total
		+	-	+	-	M-PCR
M-PCR for Ng	+	8	3	-	-	11
	-	0	79	-	-	79
Total cultures		8	82	-	-	90
M-PCR for Ct	+	-	-	14	0	14
	-	-	-	2	74	76
Total Ag tests		-	-	16	74	90

Of 11 M-PCR positives for Ng, 3 were culture negative. However, because the monoplex Ng PCR was also positive in these samples, they were classified as true positives. Similarly, in 2 M-PCR Ct negative but Clear View positive samples, the monoplex Ct PCR was negative, and these samples were classified as true negatives. Four specimens were positive for both Ct and Ng in the M-PCR. They were all positive for Ng by culture and in the CT antigen test.

Defining a true positive as a positive result in 2 different tests performed in a symptomatic patient, the clinical sensitivity and specificity of the M-PCR were found to be both 100%, while the corresponding sensitivities and specificities for Ng culture and Ct antigen test were 72.7, 100%, and 100%, 97.4% respectively.

DISCUSSION

Although conventional culture for *N. gonorrhoeae* and tissue culture for *C. trachomatis* remain as the gold standard methods for the detection of these organisms, there is an increasing use of nucleic acid amplification tests (NAAT) for the screening of sexually transmitted infections. In this study a Ng-Ct multiplex PCR was optimized with the inclusion of a suitable internal control for the detection of amplification inhibition. Its sensitivity and specificity were found to be adequate for the detection of both pathogens simultaneously from endocervical specimens of patients with vaginal discharge.

Many gonococcal and chlamydial infections are asymptomatic and therefore do not present at physicians' clinics. For the screening of these cases, the use of non-invasive specimens like urine and low vaginal swabs is a preferred strategy. This strategy is made feasible with highly sensitive NAAT. The Ng-Ct M-PCR assay established in this study can be further optimized to increase its sensitivity for specimens with low number copies of *N. gonorrhoeae* and *C. trachomatis* so that it can also be used for the detection of asymptomatic infections using non-invasive specimens. This will make it an important tool not only for the diagnosis of overt infection but also for community-based prevalence studies of sexually transmitted infections.

ACKNOWLEDGEMENTS

We are grateful to Mr. Cheong Soon Fatt for helping with the designing of the Ng-lambda phage internal control used in the M-PCR. We also thank WHO HRP for the WHO Fellowship awarded to Wah Wah Aung that provided her the opportunity to conduct this study.

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