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Comparison of nested and ELISA based polymerase chain reaction assays for detecting *Chlamydia trachomatis* in pregnant women with preterm complications

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Abstract. Identification of pregnant women infected with *Chlamydia trachomatis* is essential to allow early antibiotic treatment in order to prevent adverse pregnancy outcomes. In this study, two nucleic acid amplification tests (NAAT) namely nested PCR (BioSewoom, Korea) and Amplicor CT/NG (Roche Diagnostic, USA) were evaluated in terms of sensitivity and specificity for the detection of *C. trachomatis* DNA in pregnant women with preterm complications. A cross-sectional study was carried out in two public hospitals in Southern Selangor, Malaysia. Endocervical swabs obtained were subjected to DNA amplification using nested PCR (BioSewoom, Korea) and Amplicor CT/NG (Roche Diagnostic, USA). A total of 83 endocervical swabs obtained from pregnant women of less than 37 weeks gestation and presented with preterm complications were subjected to chlamydial DNA detection using both assays. The study shows that Amplicor CT/NG assay is more effective in the detection of *C. trachomatis* DNA from endocervical swabs compared to Biosewoom nested PCR kit. Agreement between the two assays were poor (kappa=0.094) with nested PCR showing a low sensitivity of 10.81% and a 97.83% specificity when compared to Amplicor CT/NG. The results obtained indicated that BioSewoom nested PCR was less sensitive than Amplicor CT/NG for detecting *C. trachomatis* in endocervical specimens and that another more reliable test is required for confirmatory result.

INTRODUCTION

Chlamydia trachomatis is known as the chief etiological agent of sexually transmitted diseases (STDs) in women, which leads to urogenital infections. *Chlamydia trachomatis* infections are asymptomatic in 70% of women, and untreated infection can cause serious sequelae, such as pelvic inflammatory disease, ectopic pregnancy, infertility and reactive arthritis. In the past few decades, there has been an increasing emphasis on bacterial infections as a probable etiology of preterm delivery and its classical clinical manifestations such as preterm contractions, preterm premature rupture of membranes (PPROM) and preterm labour among pregnant women (Vaitkiene *et al.*, 2002; Pararas *et al.*, 2006; Hitti *et al.*, 2007; Baud *et al.*, 2011). Presence of *C. trachomatis* has been associated with the risk of preterm birth and PPROM among pregnant women in Hungary and Rotterdam (Nyári *et al.*, 2001; Rours *et al.*, 2011). Nonetheless, there has been no information as yet on *C. trachomatis* infection in pregnant women with preterm complications in Malaysia.

In most countries, opportunistic chlamydial testing is offered to sexually active adolescents and young adolescents with multiple sexual partners (Maymon *et al.*, 2000). The Malaysian Guidelines in the Treatment of STI (2008) recommends screening for *C. trachomatis* in pregnant women aged 25 years and younger presented with muco-purulent discharge, dysuria and lower abdominal pain to prevent neonatal conjunctivitis and pneumonia. Routine laboratory tests used for *C. trachomatis* screening include gram stain to exclude gram-negative intracellular diplococci, cell culture which detects *C. trachomatis* elementary bodies after 24-72 hours of growth and direct fluorescent antibody which utilizes fluorescein-conjugated monoclonal antibodies that bind specifically to *C. trachomatis* antigen (Malaysian Guidelines in the Treatment of STI, 2008). However, *C. trachomatis* is not routinely screened by nucleic acid amplification among high-risk pregnant women in Malaysia.

Chlamydia trachomatis is best detected using nucleic acid amplification tests (NAATs) such as hybrid-capture system (HCS) and transcription-mediated amplification (TMA). Most studies defined culture as the gold standard due to its high specificity (100%) and widely varying sensitivity to detect *C. trachomatis* inclusion bodies (CDC, 2002). Despite the longer time required (72 hours), culture technique depends on experience and skilled laboratory personnel whereby performance may vary widely ranging from 50-80% sensitivity (Black *et al.*, 2002).

The development of NAATs has resulted in a significant increase in sensitivity and specificity of chlamydial diagnosis (Masek *et al.*, 2009). Dean and coworkers (2008) compared the three most widely used fully automated NAATs in United Kingdom diagnostic laboratories for *C. trachomatis* detection which include the COBAS Amplicor CT/NG, Becton-Deckinson Probe Tec (strand displacement analysis) and Gene APTIMA Combo (transcription mediated amplification) using urine specimens for both men and women. Sensitivities and specificities of all three assays range between 94.94%–99.4% and 97.94%–99.81% respectively. Gene APTIMA Combo was considered as the most sensitive assay while Probe Tec was found to be the most specific assay in their study (Dean *et al.*, 2008). However, most of the fully automated NAATs are usually more expensive than the more rudimentary tests such as semiautomated AMPLICOR CT/NG test, making test performance from the economic perspective a key consideration (CDC, 2002). Thus, Amplicor CT/NG may be the best choice as it was proven to exhibit equal sensitivity and specificity as COBAS AMPLICOR CT/NG for detection of *C. trachomatis* with endocervical and urine specimens (Pol *et al.*, 2000).

Hitherto, there is no report yet on the use of Amplicor CT/NG nor Biosewoom nested PCR kit to detect *C. trachomatis* infections in the specific subset of pregnant patients who have preterm contractions and other complications. This implies that our study is the first which evaluated the Biosewoom PCR kit in comparison to Amplicor CT/NG focusing on pregnant patients who have the conditions of preterm contractions or premature rupture of placental membrane. Most previous studies on *C. trachomatis* screening among pregnant women had utilized urine samples as the test specimens; however, there was a report by Mahony and co-investigators (1998) which stated that urine samples from both pregnant and non-pregnant women contained substances that were inhibitory to PCR. Therefore, we chose to use endocervical swabs instead of urine samples. In short, to the best of our knowledge, our paper is the first to report the use of Amplicor CT/NG assay to detect *C. trachomatis* infection among pregnant women with preterm complications, and to evaluate the utility of the Biosewoom PCR assay in comparison to the former.

MATERIALS AND METHODS

Settings and sample collection

This cross-sectional study was conducted between August 2008 and June 2010 on 83 consecutive pregnant women with less than 37 weeks of pregnancy presented with preterm contractions, preterm premature rupture of membranes (PPROM) and stillbirths in two public hospitals in the state of Selangor, Malaysia. The study protocol had been approved by the University Putra Malaysia Medical Research Ethics Committee and the Medical Research and Ethics Committee Ministry of Health, Malaysia (Ethic approval number NMRR-08-1125-2280). Informed consent was obtained from each woman, who had to undergo routine pelvic examination for collection of endocervical swab. Inclusion criteria were pregnant women with age ranging from 15-45 years with gestational age of less than 37 weeks (preterm) and diagnosed with preterm contractions, PPROM and stillbirths. Exclusion criteria were pregnant women at term pregnancy (≥37 gestation weeks), antibiotic therapy at the time of screening and insufficient volume of endocervical specimen. Two endocervical swabs were obtained from each respondent using sterile cotton swabs in which one was sent for routine bacterial identification where as the other was placed in transport buffer for *C. trachomatis* DNA detection.

Sample size calculation for sensitivity and specificity testing

The sample size calculation for sensitivity and specificity testing was determined based on the rough estimate of sensitivity and specificity (p) of the test under evaluation (Banoo *et al*., 2010). Frost *et al.* (1993) reported the sensitivity and specificity of nested PCR using cell culture as the gold standard for chlamydial detection was 97.2% and 50% respectively. Hence, the minimum sample size to be recruited was determined to be 97 respondents. In order to measure the sensitivity and specificity of nested PCR within ±10% confidence interval, approximately 97 samples that are positive by the 'gold standard' test were needed. In this study, only 83 samples tested by both assays were available for analysis.

Sample preparation and DNA extraction

Presence of *C. trachomatis* was determined using commercially available nested polymerase chain reaction kit (BioSewoom, Korea) and Amplicor CT/NG (Roche Diagnostic, USA) as the gold standard (Jenson *et al*., 2013). Endocervical swab obtained for *C. trachomatis* DNA detection was placed in a universal container containing 20 ml of sucrose phosphate transport media at 4°C and sent to the laboratory. The transport buffer is commonly used for better survival of *C. trachomatis* inclusion bodies which contains 0.2 M sucrose in 0.02 M phosphate buffer supplemented with 1% serum albumin and 4 µg/ml ketoconazole as described by Tjiam *et al.* (1984).

Cotton swab was transferred into a sterile 20 ml falcon tube and spun at 1252 x g for 20 minutes. Supernatant was removed and the pelleted cells were resuspended in 2 ml phosphate buffered saline (PBS). An aliquot of the suspension was transferred into two separate 1.5 ml tubes labeled with respondent's identification number and centrifuged at 14,000 x g for 1 min. The pelleted samples were stored at -20°C up to six months. DNA extraction was performed using nested PCR (BioSewoom, Korea) and Amplicor CT/NG (Roche Diagnostic, USA) specimen preparation kits according to the respective manufacturer's instructions.

Nested Polymerase Chain Reaction (PCR)

Nested PCR assay was carried out according to the manufacturer's instructions (Biosewoom, Korea) using outer and inner primers targeting on *C. trachomatis omp1* gene. The first primer pair was NLO (5'-ATGAAAAAACTCTTGAAATCG-3') and NRO (5'-CTCAACTGTAACTGCGTATTT-3') while the second primer pair was PCTM3 (5'-TCCTTGCAAGCTCTGCCTGTGGGGA ATCCT-3') and SERO2A (5'-TTTCTAGA(T/C)TTCAT(T/C)TTGTT-3') (Lan *et al*., 1994).

PCR mixtures were prepared in total volume of 20 µl containing 4.5 µl isolated DNA with concentration ranging between 50-700 ng/µl, 15 µl master mix mixture containing 3.0 mM of $MgCl_2$, 200 mM of dNTPs, 10 pmol of each primer, 10x PCR buffer and 0.5 µl (1U) of Taq polymerase. The amplification was carried out with 5 min of initial denaturation at 95°C followed by 30 cycles of amplification. Each cycle consisted of denaturation at 94°C, annealing at 61°C and extension at 72°C for 30 s each. Second round-nested PCR was carried out in a total volume of 20 µl comprising of 1.5 µl of first round PCR product, 18 µl of the same PCR master mix and 0.5 µl of 1U Taq polymerase. The PCR results were visualized using 1.5% (w/v) agarose gel electrophoresis. Expected size of the amplified products was 288 bp for *omp1* gene.

AMPLICOR CT/NG Assay

Chlamydia trachomatis DNA was also detected using Amplicor CT/NG (Roche Diagnostic, USA) assay to amplify *C. trachomatis* cryptic plasmid using CP24 forward primer (5'-GCTGTGGTTGAGC TTTATACAGACAC-3') and CP27 reverse primer (5'-TTTAGGTTTAGATTGAGCATA TTGGA-3') (Solomon *et al*., 2003). Fifty microlitres of processed specimens were added with 50 µl of working master mix (master mix added with internal control) into each reaction tube. Positive and negative controls provided with the kit were run in duplicate. Amplification was carried out with initial denaturation at 93°C followed by 36 cycles of amplification. Each cycle consisted of denaturation at 94°C for 20 sec, annealing at 61°C for 1 min and extension at 71°C for 40 sec. There was a final hold at 72°C for 5 min. Detection was done by denaturing the amplified products into single-strands for hybridization with *C. trachomatis* oligonucleotide probes bound in wells of the 96-well microwell plate followed by absorbance reading at 450 nm. Specimens above the cutoff signal ($A_{450} \geq 0.8$) were interpreted as positive for *C. trachomatis* regardless of the internal control result (Rosentraus *et al*., 1998). Specimens yielding below cutoff signal ($A_{450} < 0.8$) were interpreted as negative for *C. trachomatis* provided the internal control signal were above $A_{450} \geq 0.2$. Specimens yielding signal below cutoff value for both target ($A_{450} < 0.8$) and internal control ($A_{450} < 0.2$) were interpreted as inhibitory.

Discrepancy analysis

All samples (two swab specimens per patient) were tested once initially by both assays as described earlier. In the case of discrepant results, both tests were repeated. We are aware that ideally we should use multiple molecular assays to provide a reference or standard for assessing the performance of a newly introduced assay, however due to the lack of sufficient funding, we could not carry out investigation with other assays. Furthermore, a previous study by Jenson *et al.* (2013) also compared only one single test (Cepheid's GeneXpert kit) against Amplicor CT/NG, in which they did not include any third-party test nor cell culture as a reference test for discrepancy result. Doornum *et al.* (2001) also did not use nor define any gold standard reference test.

Definition of truly infected patients

Chlamydia trachomatis infection status for a particular sample was determined as true positive when tested positive for both Amplicor CT/NG and nested PCR methods. True negative was also confirmed when tested negative by both methods. Jalal *et al.* (2007) had defined true positive when *C. trachomatis* DNA was detected by at least two of the four assays (comparison between in-house real-time PCR (IHRT-PCR), in-house nested cryptic plasmid PCR and in-house nested major outer membrane protein PCR with Amplicor CT/NG test), while a sample was defined as true negative if *C. trachomatis* DNA was detected in only one or none of the assays. We take note that they had not defined true positive as positive culture or positive for Amplicor assay alone. Indeed, owing to the differing sensitivity and specificity of different tests, we can no longer rely on one single assay as the gold standard.

Table 1. Comparison for *C. trachomatis* results from endocervical specimens

Number of specimens (n=83)	Result of:		
	Nested PCR	Amplicor CT/NG	
4	+	+	True positive
33	–	+	False Negative
1	+	–	False Positive
45	–	–	True Negative

Statistical analysis

SPSS test was used to calculate the 95% confidence interval of sensitivity and specificity. *P* value of <0.05 is considered to be statistically significant.

RESULTS

***Chlamydia trachomatis* DNA detection from cervical samples using nested PCR and Amplicor**

A total of 83 pregnant women presented with preterm complications and stillbirths were enrolled in the study. The mean age of women who participated in this study was 28.02 (SD=6.071) ranging from 15 to 42 years old. DNA extracted from 83 endocervical swab specimens were tested for *C. trachomatis* by Biosewoom's nested PCR assay and Roche Amplicor assay.

Table 1 shows five out of 83 specimens (6%) were detected positive for *C. trachomatis* using nested PCR while 37 out of 83 specimens (43%) were detected positive by Amplicor. Of the five specimens positive by nested PCR, one sample was detected negative by Amplicor. On the other hand, 33 specimens positive by Amplicor were detected negative by nested PCR. The proportion of agreement between the two assays were 0.59 and the kappa value was 0.094 (p>0.05).

Test performance for *C. trachomatis* detection

The accuracy of Biosewoom's nested PCR to detect *C. trachomatis* DNA in infected and uninfected pregnant women was based on two important aspects namely sensitivity and specificity of the test. Amplicor (Roche Diagnostic, USA) was used as the reference test for comparison due to its high sensitivity and specificity (Lehmusvuori *et al.*, 2010). A sensitivity of 10.81% showed that of 37 pregnant women who were detected positive for *C. trachomatis* by Amplicor, nested PCR was likely to detect only four of them (true-positives of 10.81%). Thirty three specimens missed by nested PCR were said to be false-negative results. In order to avoid false negative results due to contamination, PCR mixtures were set up in a laminar flow workstation (Holten Laminar Air) using aerosol-resistant microppipette tips at an area distinct from the DNA extraction work (Chong *et al.*, 2010). Negative and positive controls were also valid.

Meanwhile, high specificity of 97.83% showed that of 46 pregnant women detected as not infected with *C. trachomatis* by the reference test, nested PCR was likely to detect 45 of them (true negatives of 97.83%). One sample was detected positive by nested PCR but negative by Amplicor due to inhibitory specimen. In Amplicor assay, inhibitory specimen can be identified by

Table 2. Test performance of nested PCR using Amplicor CT/NG as the gold standard

	Percentage (%)
Sensitivity	10.81
Specificity	97.83
Positive Predictive Value	80
Negative Predictive Value	57.7

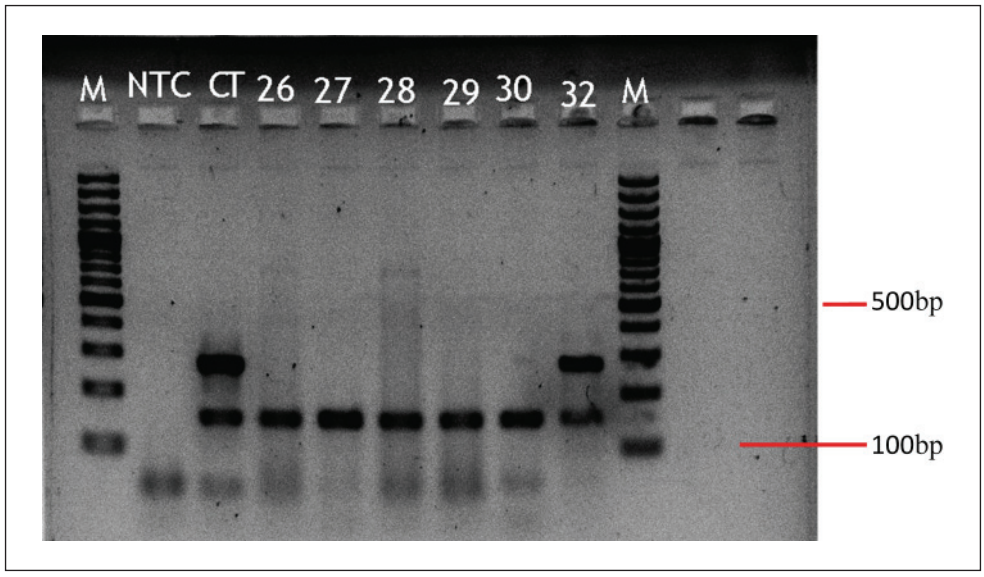

Figure 1. Results from second round nested PCR for selected samples
After the second round nested PCR, one sample was identified as *Chlamydia* positive (sample 32). The bands generated were 154bp for internal control and 288bp for the expected size of the amplicon. M: 100bp ladder marker, NTC: Non-template control, CT: positive control, 26-32: Endocervical samples

monitoring the amplification of both *C. trachomatis* target and unique internal control DNA sequence. Specimens yielding signal below 0.8 A_{450} for *C. trachomatis* and below 0.2 A_{450} for internal control are classified as inhibitory and were excluded from the study, thereby maximizing the sensitivity (Rosenstraus *et al.*, 1998). Presence of *C. trachomatis* DNA was detected in 47 out of the 106 (44.3%) endocervical specimens using nucleic acid amplification tests.

DISCUSSION

In this study, DNA-based PCR was used to detect *C. trachomatis* in endocervical specimens. Nested PCR method was chosen because it has been widely used to detect and genotype *C. trachomatis* in clinical specimens (Zheng *et al.*, 2007), although the Biosewoom nested PCR kit is not widely in use. The Biosewoom nested PCR test utilizes two steps of PCR targeting on *C. trachomatis*. In the AMPLICOR CT/NG test, a single PCR is needed to detect *C. trachomatis* cryptic plasmids. Both nested PCR and Amplicor CT/NG assays may be performed by qualified personnel and does not require a specialized analyzer.

One reason why one would want to compare two different detection methods is to find out the sensitivity and specificity of the two methods in order to produce an unbiased data as a guideline for clinical microbiologists when choosing an economical, easy-to-use yet accurate test in clinical practice. The nested PCR test developed by Biosewoom, Korea is relatively new in Malaysia and the region, as it was brought in by a local distributor. To our knowledge, there is no published report in PubMed regarding the evaluation of the performance of this nested PCR kit (a search in PubMed with the term "Biosewoom AND *Chlamydia*" yielded no result). Hence, we sought to evaluate the effectiveness of this test against the more widely known and used Amplicor CT/NG test from Roche.

Amplicor CT/NG which utilized PCR ELISA based assay was used as the gold standard rather than the cell culture to evaluate the performance of Biosewoom nested PCR. Gold standard refers to a reference standard that is without error,

whereas reference standard refers to the best available method to determine the presence or absence of the condition or disease of interest (Rutges *et al.*, 2007). Cell culture technique did not meet the demands of a reference method for chlamydial nucleic acid amplification technique because it depends on experience and skilled laboratory personnel whereby performance may vary widely ranging from 50-80% sensitivity (Pasternack *et al.*, 1996; Black *et al.*, 2002).

In this study, nested PCR appeared to be less sensitive (10.81%) but highly specific (97.83%) for detection of *C. trachomatis* with 33 false-negative results (39.8%). The high false negative results could be due to the presence of inhibitors in the sample such as heme in blood cells, loss of nucleic acids during specimen processing (Chong *et al.*, 2003) and some degree of degradation of DNA in storage (Jalal *et al.*, 2007). In order to overcome the possibility of false positive results, stringent controls were performed in the sampling and experimental procedures such as the use of sterile disposable specula and cotton swabs during endocervical swab sampling and the use of sterile transport medium transported on ice to the laboratory. Each PCR reaction was also performed in duplicates in a UV-exposed alcohol sterilized laminar flow whereby DNA extraction area was separated from the PCR work area as well as the gel electrophoresis area. Chong *et al.* (2010) also highlighted the crucial step during DNA extraction which is the use of aerosol-resistant filter tips to avoid cross-contamination between samples.

Thirty three specimens negative by nested PCR but positive by Amplicor were not retested for confirmation using other nucleic acid amplification tests (NAATs) such as strand displacement amplification test (SDA) and transcription mediated amplification test (TMA) due to the fact that the population employed in this study was high-prevalence group. Centers for Disease Control and Prevention, USA (2002) states that repeat testing should be performed on positive samples generated from NAAT in population with a low prevalence of *C. trachomatis*. In the early studies, culture was used as the gold standard, despite its lack of sensitivity (50-80%) and some previous studies used culture as a definitive standard. However, the results of culture are personnel-dependent and skill-dependent and can vary from laboratory to laboratory. To date, there is no universally agreed "gold standard" for detection of *C. trachomatis*. Thus, when there is a discrepant result between two or more tests, repeat testing has been the advocated practice (Rutjes *et al.*, 2007).

On the other hand, we managed to detect 33 *C. trachomatis*-positive specimens through Amplicor CT/NG, wherein these specimens were missed by Biosewoom nested PCR. The nested PCR method employed in this study was designed to amplify the single-copy major outer membrane protein (*omp 1* gene) while Amplicor CT/NG was targeted on the multi-copy cryptic plasmids. Hence, this major difference in the respective target genes copy number in the bacterial genome could be one of the contributory factors for the lack of sensitivity observed in the nested PCR. Besides the low copy of *omp 1* gene present in the elementary body (Freise *et al.*, 2001), low sensitivity of nested PCR may be due to the lack of specific nucleic acid extraction method provided in the kit. Additionally, cryptic plasmid is present in the elementary body in approximately 10-20 copies per elementary body (Mahony *et al.*, 1993). This may explain why Amplicor CT/NG that targeted on chlamydial plasmid was more sensitive compared to nested PCR assays (Ripa & Nilsson, 2007; Zheng *et al.*, 2007).

Nevertheless, despite its limitation on sensitivity, some specific nested PCR assays have an advantage in their applicability to detect *C. trachomatis* serovar L2 directly from the clinical specimen. (Frost *et al.*, 1993; Zheng *et al.*, 2007). Some nested PCR methods have the ability to further genotype *C. trachomatis* genovars directly from the clinical specimen, but this is not the case for the Biosewoom nested PCR kit.

Accurate reporting of *C. trachomatis* infection status plays an utmost important role not just in the treatment and management, but also in the psychosocial impact of the patients. False negatives can result in pregnant women being denied of appropriate

treatment and going on to suffer from chronic sequelae (Pasternack *et al*., 1996). At the same time, multiple examinations with different NAATs are not always practical due to increased turnaround time and are too costly to maintain (Jalal *et al*., 2007).

In conclusion, *C. trachmatis* detection in endocervial swabs from patients with preterm complications using the Biosewoom nested PCR kit is less sensitive than Amplicor and that another more reliable test is required for confirmatory result.

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REFERENCES

Banoo, S., Bell, D., Bossuyt, P., Herring, A., Mabey, D., Poole, F., Smith, P.G., Sriram, N., Wongsrichanalai, C., Linke, R., O'Brien, R., Perkins, M., Cunningham, J., Matsoso, P., Nathanson, C.M., Olliaro, P., Peeling, R.W. & Ramsay, A. (2010). Evaluation of diagnostic tests for infectious diseases: general principles. *Nature Reviews Microbiology*: S16-S28.

Baud, D., Goy, G., Jaton, K., Osterheld, M.C., Blumer, S., Borel, N., Vial, Y., Hohlfeld, P., Pospischil, A. & Greub, G. (2011). Role of *Chlamydia trachomatis* in miscarriage. *Emerging Infectious Diseases* **17**(9): 1630-1635.

Black, C.M., Marrazzo, J., Johnson, R.E., Hook, E.W. 3rd, Jones, R.B., Green, T.A., Schachter, J., Stamm, W.E., Bolan, G., St Louis, M.E. & Martin, D.H. (2002). Head-to-head multicenter comparison of DNA probe and nucleic acid amplification tests for *Chlamydia trachomatis* infection in women performed with an improved reference standard. *Journal of Clinical Microbiology* **40**(10): 3757-3763.

Centers for Disease Control and Prevention. (2002). Screening tests to detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Infections. *Morbidity and Mortality Weekly Report* **51** (No. RR-15).

Chong, P.P., Lee, Y.L., Tan, B.C. & Ng, K.P. (2003). Genetic relatedness of Candda strains isolated from women with vaginal candidiasis in Malaysia. *Journal of Medical Microbiology* **52**(8): 657-666.

Chong, P.P., Asyikin, N., Rusinahayati, M., Halimatun, S., Rozita, R., Ng, C.K., Hamilton, W.H., Tan, B.C., Noraihan, N., Rohani, A., Faezah, H., Latiffah, L., Maha, A. & Sabariah, A.R. (2010). High prevalence of human papillomavirus DNA detected in cervical swabs from women in southern Selangor, Malaysia. *Asian Pacific Journal Cancer Prevention* **11**: 1645-1651.

Dean, L., Perry, K., Arnold, E. & Charlett, A. (2008). Four nucleic acid amplification tests for *Chlamydia trachomatis* in urine specimens. London: Centre for Evidence based Purchasing.

Doornum, G.J.J., Schouls, L.M., Pijl, A., Cairo, I., Buimer, M. & Bruisten, S. (2001). Comparison between the LCx Probe system and the COBAS AMPLICOR system for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in patients attending a clinic for treatment of sexually transmitted diseases in Amsterdam, The Netherlands. *Journal of Clinical Microbiology* **39**(3): 829-835.

Freise, J., Gérard, H.C., Bunke, T., Whittum-Hudson, J.A., Zeidler, H., Köhler, L., Hudson, A.P. & Kuipers, J.G. (2001). Optimised sample DNA preparation for detection of *Chlamydia trachomatis* in synovial tissue by polymerase chain reaction and ligase chain reaction. *Annals of the Rheumatic Diseases* **60**: 140-145.

Frost, E.H., Deslandes, S. & Bourgaux-Ramoisy, D. (1993). Sensitive detection and typing of *Chlamydia trachomatis* using nested polymerase chain reaction. *Genitourinary Medicine* **69**: 290-294.

Hitti, J., Nugent, R., Boutaina, D., Gardella, C., Hillier, S.L. & Eschenbach, D.A. (2007). Racial disparity in risk of preterm birth associated with lower genital tract infection. *Paediatric and Perinatal Epidemiology* **21**: 330-337.

Jalal, H., Al-Suwaine, A., Stephen, H., Carne, C. & Sonnex, C. (2007). Comparative performance of the Roche COBAS Amplicor assay and an in-house real-time PCR assay for diagnosis of *Chlamydia trachomatis* infection. *Journal of Medical Microbiology* **56**: 320-322.

Jenson, A., Dize, L., Mkocha, H., Munoz, B., Lee, J., Gaydos, C., Quinn, T. & West, S.K. (2013). Field evaluation of the Cepheid GeneXpert *Chlamydia trachomatis* assay for detection of infection in a trachoma endemic community in Tanzania. *PLoS Neglected Tropical Diseases* **7**(7): e2265.

Lan, J., Ossewaarde, J.M., Walboomers, J.M., Meijer, C.J. & van den Brule, A.J. (1994). Improved PCR sensitivity for direct genotyping of *Chlamydia trachomatis* serovars by using a Nested PCR. *Journal of Clinical Microbiology* **32**(2): 528-530.

Lehmusvuori, A., Juntunen, E., Tapio, A.H., Rantakokko-Jalava, K., Soukka, T. & Lövgren, T. (2010). Rapid homogeneous PCR assay for the detection of *Chlamydia trachomatis* in urine samples. *Journal of Microbiological Methods* **83**: 302-306.

Mahony, J.B., Luinstra, K.E., Sellors, J.W. & Chernesky, M.A. (1993). Comparison of plasmid- and chromosome-based polymerase chain reaction assays for detecting *Chlamydia trachomatis* nucleic acids. *Journal of Clinical Microbiology* **31**(7): 1753-1758.

Mahony, J., Chong, S., Jang, D., Luinstra, K., Faught, M., Dalby, D., Sellors, J. & Chernesky, M. (1998). Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: Identification of urinary substances associated with inhibition and removal of inhibitory activity. *Journal of Clinical Microbiology* **36**(11): 3122-3126.

Masek, B.J., Arora, N., Quinn, N., Aumakhan, B., Holden, J., Hardick, A., Agreda, P., Barnes, M. & Gaydos, C.A. (2009). Performance of three nucleic acid amplification tests for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by use of self-collected vaginal swabs obtained via an internet-based screening program. *Journal of Clinical Microbiology* **47**(6): 1663-1667.

Maymon, E., Romero, R., Pacora, P., Gervasi, M.T., Bianco, K., Ghezzi, F. & Yoon, B.H. (2000). Evidence for the participation of interstitial collagenase (matrix metalloproteinase 1) in preterm premature rupture of membranes. *American Journal Obstetric Gyneacology* **183**(4): 914-920.

MOH. (2008). Malaysian guidelines in the treatment of sexually transmitted infections. Kuala Lumpur: Ministry of Health.

Nyári, T., Woodward, M., Mészáros, G., Karsai, J. & Kovács, L. (2001). *Chlamydia trachomatis* infection and the risk of perinatal mortality in Hungary. *Journal of Perinatal Medicine* **29**: 55-59.

Pararas, M.V., Skevaki, C.L. & Kafetzis, D.A. (2006). Preterm birth due to maternal infection: causative pathogens and modes of prevention. *European Journal of Clinical Microbiology and Infectious Disease* **25**: 562-569.

Pasternack, R., Vuorinen, P., Kuukankorpi, A., Pitkajarvi, T. & Mietinen, A. (1996). Detection of *Chlamydia trachomatis* infections in women by Amplicor PCR: comparison of diagnostic performance with urine and cervical specimens. *Journal of Clinical Microbiology* **34**(4): 995-998.

Pol, B.V.D., Quinn, T.C., Gaydos, C.A., Crotchfelt, K., Schachter, J., Moncada, J., Junkind, D., Martin, D.H., Turner, B., Peyton, C. & Jones, R.B. (2000). Multicenter evaluation of the AMPLICOR and automated COBAS AMPLICOR CT/NG tests for detection of *Chlamydia trachomatis*. *Journal of Clinical Microbiology* **38**(3): 1105-1112.

Ripa, T. & Nilsson, P.A. (2007). A *Chlamydia trachomatis* strain with a 377-bp deletion in the cryptic plasmid causing false-negative nucleic acid amplification tests. *Sexually Transmitted Diseases* **34**(5): 255-256.

Rours, G.I., de Krijger, R.R., Ott, A., Willemse, H.F., de Groot, R., Zimmermann, L.J., Kornelisse, R.F., Verbrugh, H.A. & Verkooijen, R.P. (2011). *Chlamydia trachomatis* and placental inflammation in early preterm delivery. *European Journal Epidemiology* **26**: 421-428.

Rosenstraus, M., Wang, Z., Chang, S.Y., Debonville, D. & Spadoro, J.P. (1998). An internal control for routine diagnostic PCR: Design, properties, and effect on clinical performance. *Journal of Clinical Microbiology* **36**(1): 191-197.

Rutjes, A., Reitsma, J., Coomarasamy, A., Khan, K. & Bossuyt, P. Evaluation of diagnostic tests when there is no gold standard. A review of methods. (2007) *Health Technology Assessment* **11**(50).

Solomon, A.W., Holland, M.J., Burton, M.J., West, S.K., Alexander, N.D., Aguirre, A., Massae, P.A., Mkocha, H., Muñoz, B., Johnson, G.J., Peeling, R.W., Bailey, R.L., Foster, A. & Mabey, D.C. (2003). Strategies for control of trachoma: observational study with quantitative PCR. *Lancet* **362**: 198-204.

Tjiam, K.H., van Heijst, B.Y., de Roo, J.C., de Beer, A., van Joost, T., Michel, M.F. & Stolz, E. (1984). Survival of *Chlamydia trachomatis* in different transport media and at different temperatures: Diagnostic implications. *British Journal of Venereal Diseases and Genitourinary Medicine* **60**: 92-94.

Vaitkiene, D., Bergstrom, S. & Cigriejiene, V.M. (2002). Antenatal risk factors associated with preterm prelabour rupture of membranes. *Acta Medica Lituanica* **9**(3): 203-209.

Zheng, H.P., Jiang, L.F., Fang, D.Y., Xue, Y.H., Wu, Y.A., Huang, J.M. & Ou, Z.Y. (2007). Application of an oligonucleotide array assay for rapid detecting and genotyping of *Chlamydia trachomatis* from urogenital specimens. *Diagnostic Microbiology and Infectious Disease* **57**: 1-6.