

Detection of *Chlamydia trachomatis* in urine samples by polymerase chain reaction and enzyme immunoassay

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Abstract

First-void urine samples collected from sexually transmitted diseases (STD) clinic patients were examined by a nested polymerase chain reaction (PCR) and a commercial enzyme immunoassay (IDEIA Chlamydia) for the diagnosis of *Chlamydia trachomatis* urethritis or cervicitis. The primers for the PCR amplified a target in the major outer membrane protein (MOMP) gene in *C trachomatis* while the IDEIA detected genus-specific chlamydial lipopolysaccharide. Discrepant results were resolved by retesting urine specimens with a second (plasmid-based) PCR and taking urethral or endocervical swab results into consideration.

For 231 men (chlamydial prevalence 20.4%), the sensitivity, specificity, positive and negative predictive values were 59.6%, 99.5%, 96.6% and 90.6% for urine IDEIA, 68.1%, 99.5%, 97% and 92.4% for urethral swab IDEIA and 97.9%, 99.5%, 97.9% and 99.5% for urine PCR. The corresponding rates for 66 women (chlamydial prevalence 54.6%) were 19.4%, 100%, 100% and 50.8% for urine IDEIA, 86.1%, 96.7%, 96.9% and 85.3% for endocervical swab IDEIA and 91.7%, 93.3%, 94.3% and 90.3% for urine PCR. Hence, in a high prevalence population, the urine IDEIA was a suitable alternative to the male urethral swab IDEIA but significantly less sensitive than the endocervical swab IDEIA. The urine PCR was, however, much more sensitive than the urine IDEIA for both men and women and could replace the endocervical swab IDEIA for the diagnosis of chlamydial cervicitis.

Key words: Chlamydia, urine, enzyme immunoassay, polymerase chain reaction.

INTRODUCTION

Chlamydia trachomatis genital tract infections are the most prevalent sexually transmitted diseases (STD) in the developed world. In Malaysia, there is also evidence that these infections are an important cause of morbidity¹ but accurate epidemiological data is lacking which is partly due to difficulties with laboratory confirmation.

The traditional approach to the collection of specimens for the diagnosis of chlamydial genital tract infections is swabbing the urethra or endocervix. In recent years there has been much interest in the use of urine as an alternative to the urethral swab. The use of urine which is easily obtained with non-invasive means would greatly facilitate the screening of difficult-to-reach or asymptomatic populations. However, urine is not suitable for chlamydial culture which is usually regarded as the “gold standard” for diagnosis. Non-culture methods such as the direct fluorescent antibody test (DFAT) and the

enzyme-linked immunosorbent assay (ELISA) have been widely used on urethral and cervical swabs but have not been adequately evaluated for urine. The most promising diagnostic tests are DNA amplification techniques which have the potential of detecting even one copy of chlamydial DNA. Commercially available DNA tests based on the polymerase chain reaction (PCR)² and ligase chain reaction (LCR)³ have been shown to be suitable for urine specimens from both men and women. This paper reports the results of a study comparing the performance of a nested PCR and a commercially available ELISA for the detection of chlamydia in urine samples from STD clinic patients.

MATERIALS AND METHODS

Patients and specimen collection

Specimens were collected from men and women attending STD clinics for genital tract infections. From each patient, an endocervical or urethral

swab was first collected for Gram staining and gonococcal culture on modified Thayer-Martin agar. Two other swabs were then collected and put into IDEIA (DAKO Diagnostics Ltd, U.K.) transport medium and PCR buffer for chlamydial ELISA and PCR respectively. The patient was then asked to pass the first 15-20ml of urine into a plain sterile bottle for the urine chlamydial ELISA and PCR.

Specimen handling oz

In the laboratory, urine samples were "whirlmixed" with sterile glass beads to break up threads and to distribute the cell contents evenly. One ml of urine was pipetted into an Eppendorf tube and centrifuged at $13000 \times g$ for 10 minutes. The sediment was resuspended in 1ml of distilled water and kept frozen at -20°C until tested by PCR. The remainder of the urine was centrifuged at $3000 \times g$ for 15 minutes. The sediment was resuspended in 1 ml of IDEIA transport medium and used for ELISA within 3 days of storage at 4°C .

Swabs for PCR were similarly vortexed in PCR buffer after which the swab was discarded and the buffer centrifuged at $13000 \times g$ for 10 minutes. The sediment was resuspended in 1ml distilled water for storage at -20°C . Swabs for ELISA were kept at 4°C for up to 3 days before processing.

Chlamydial ELISA

The IDEIA Chlamydia (DAKO Diagnostics Ltd, U.K.) procedure was carried out according to the manufacturer's instructions. Swabs and resuspended urine sediments were boiled in IDEIA transport medium for 15 min, vortexed and added to wells in a microtitre plate coated with a genus-specific anti-chlamydial lipopolysaccharide (LPS) monoclonal antibody. Following incubation and washing, alkaline phosphatase-conjugated chlamydial monoclonal antibody was used to identify the "captured" antigen and subsequently the enzyme substrate NADPH was added to be catalysed into a product which was used in a second enzyme reaction with alcohol dehydrogenase and diaphorase to produce a colour change. Colour development was stopped with dilute sulphuric acid and readings were taken in a spectrophotometer at 492 nm and 650 nm wavelengths. A colour intensity significantly above background levels indicated the presence of chlamydial antigen in the patient's sample.

All specimens giving positive readings were

retested with and without the IDEIA Chlamydia Blocking Reagent (DAKO Diagnostics Ltd, UK) which was a murine monoclonal antibody against chlamydial LPS which blocked the binding of the chlamydial LPS to the capture antibody in the IDEIA Chlamydia test. In the presence of this blocking antibody, the absorbance reading for a positive specimen should be significantly reduced. A positive result was verified if there were a reduction of $>40\%$ for strongly reactive specimens and $>25\%$ for weakly reactive ones.

Chlamydial PCR

Two primer sets were used in a nested PCR for the amplification of a target on the major outer membrane (MOMP) gene of *C trachomatis*. The primers were a gift from Professor ME Ward, Southampton University, UK. The outer primers FLA-FLS amplified a 1175 bp fragment encompassing all 4 variable regions in the MOMP gene. This was then used as the template for the second PCR using the inner primers Nest 2 and Nest 4. The product of the second PCR was a 347 bp fragment in the variable domain VD4. The DNA sequences of these primers are:

FLA 5' - TTAGAAGCGGAATTGTGCA
TTTACGTGAGC - 3'
FLS 5' - CTCTTGAAATCGGTATTAGT
ATTTGCCGCT - 3'
Nest 2 5' - CATGAI TGGCAAGCAAGTTT
A - 3'
Nest 4 5' - GCTCTCTCATCGATCAAGCG
- 3'

Samples (200 μl) were spun at $13000 \times g$ to pellet chlamydiae. The pellet was resuspended in 20 μl of Tween20 (0.5%), Nonidet P-40 (0.5%) and proteinase K (100 mg/l), incubated 1 h at 60°C then boiled for 10 minutes. PCR was performed in 50 μl of PCR solution containing 50 mM KCL, 10 mM Tris-HCL, pH 8.3, 1.5 mM MgCl_2 , 200 μM of each dNTP, 20 μM of each primer, 1 unit of Taq DNA polymerase (Promega, USA) and 10 μl of the patient's sample. The mixture was overlaid with 50 μl of paraffin oil to prevent evaporation and subjected to 30 cycles of amplification in a PE 480 thermal cycler (Perkin-ElmerCetus, USA). Each cycle consisted of a denaturation step at $94^{\circ}\text{C} \times 1$ minute, a primer annealing step at $60^{\circ}\text{C} \times 1$ minute and a chain elongation step at $72^{\circ}\text{C} \times 1.5$ minutes. The second PCR was carried out with an annealing temperature of 46°C .

At the completion of the second PCR, 10 μl of the amplified product were analysed by 1.2%

agarose gel electrophoresis.

Resolution of discrepant results

Urine specimens which were PCR positive but ELISA negative were further tested by a second PCR which amplified a plasmid gene with a primer set designed by Dr Gerald Hamett of the Western Australian Centre for Pathology and Medical Research, Perth, Australia (G Harnett, personal communication). For urines which were ELISA positive but PCR negative, the PCR was repeated at 1:10 dilution to exclude the presence of polymerase inhibitors and nucleases in the urine.

In the absence of chlamydial culture, an "expanded gold standard" was applied which allowed urine specimens positive by two or more different tests to be considered as "true positives". Wherever necessary, the results of swab EIA and PCR were also used to help decide whether the patient had a chlamydial infection. Examples of "true positives" were : (a) urine ELISA and PCR positive; (b) urine ELISA negative but both urine PCRs positive; (c) urine ELISA negative, PCR 1 positive, PCR 2 negative but swab ELISA or PCR positive; (d) urine ELISA positive, PCR negative but swab PCR positive.

Statistical analysis was carried out with the Chi-square test with or without Yates' correction.

RESULTS

Study subjects were all sexually active men and women aged 16-62 years. There were 314 men with urethritis and 151 with other STDs like genital warts and ulcers. Of those with urethritis, 47(15.0%) were culture positive for gonorrhoea (55% beta-lactamase producing) and 60 (19.1%) were chlamydia positive by urethral swab ELISA (10 with concurrent gonococcal urethritis). Six (4%) of the men without urethritis were also chlamydia positive by swab ELISA. There were 189 female patients, 56 presenting with pelvic inflammatory disease (PID) and the rest with vaginal discharge. Chlamydia was detected by cervical swab ELISA in 20 of the PID patients (35.7%) and 25 of those with discharge (18.8%).

Swab versus urine

When the IDEIA Chlamydia for urine and swabs were compared, concordant results were obtained for 154 (81.5%) of female and 445 (95.7%) of male specimens. In women, the cervical swab was positive significantly more

frequently than urine (23.8% vs 6.4%, $p < 0.000000$) while in men, the detection rates by urethral swab and urine ELISA were similar for those with urethritis (19.1% vs 17.2%) and those without urethritis (4% vs 4%). Tables 1a, b and c show the results for swab and urine ELISAs.

TABLE 1a: Comparison of urine and urethral swab ELISAs for men with urethritis

		Swab IDEIA		
		+	-	Total
Urine IDEIA	+	49	5	54
	-	11	249	260
Total		60	254	314

TABLE 1b: Comparison of urine and urethral swab ELISAs for men without urethritis

		Swab IDEIA		
		+	-	Total
Urine IDEIA	+	4	2	6
	-	2	143	145
Total		6	145	151

TABLE 1c: Comparison of urine and cervical swab ELISAs for women with cervicitis/urethritis

		Swab IDEIA		
		+	-	Total
Urine IDEIA	+	11	1	12
	-	34	143	177
Total		45	144	189

Urine ELISA versus urine PCR

Sixty-six female and 231 male urines were examined by both Chlamydia IDEIA and PCR. Discrepant results were obtained for 28 (42.4%) female and 21 (9.1%) male urines. All 28 female urines were PCR positive, ELISA negative. Two of these were regarded as "false urine PCR positives" because their corresponding cervical

swabs and plasmid-based urine PCRs were negative. The other 26 were confirmed positive by a second DNA amplification test on the urine and were associated with positive cervical swabs. These were interpreted as chlamydial cervicitis cases detected by urine PCR but not urine ELISA.

Of the 21 male urines with discrepant results, 19 (urethral swab and two urine PCRs positive) were considered "false urine ELISA negatives"; one (swab and second urine PCR negative) was a "false urine PCR positive" and one (urine ELISA positive but negative in swab and urine PCRs) was a "false urine ELISA positive". Hence, after resolution of discrepant results, there were a total of 36 chlamydial cervicitis/urethritis cases in 66 women (54.6% prevalence) and 47 chlamydial urethritis cases in 231 men (20.4% prevalence). Based on these figures, the sensitivity, specificity, positive and negative predictive values were estimated and shown in Table 2. The urine PCR was equally sensitive for male and female urine (97.9% vs 91.7%, $p=0.06$) but much more sensitive than the urine ELISA for both gender ($p<0.000000$). It was as sensitive as the endocervical swab ELISA for the diagnosis of chlamydial cervicitis (91.7% vs 86.1%, $p=0.26$).

DISCUSSION

There are obvious advantages of using first void urine instead of genital swabs for laboratory

diagnosis. However, the performance of routine tests designed for genital swabs may not be optimal for urine. The solid-phase ELISA for the detection of chlamydial lipopolysaccharide has been the most widely used diagnostic test for chlamydial urethritis and cervicitis in the last decade. The application of this technique on urine samples is a logical development. Previous studies have shown the performance of the test to vary with factors like sampling site, specimen handling, differences in commercially available assays and the "gold standard" used for comparison. When compared to genital swab culture or antigen detection by direct immunofluorescence, urine ELISAs have shown consistently high specificities but sensitivities ranging from 42% to 95.8% for men^{4,5} and 37.3% to 87.5% for women.^{6,7} The detection limit of the ELISA has been estimated to be about 1×10^3 elementary bodies (EBs) per assay.⁸ Hence, against highly sensitive DNA amplification tests with detection limits of 2 EBs per assay,⁸ the urine ELISA has scored sensitivities as low as 10%-18.8%.^{3,9}

In this study the urine ELISA was evaluated against a nested PCR and both urine tests were compared to the swab ELISA used for routine diagnosis. When performed with a confirmatory test, the ELISA was as specific as the PCR but significantly less sensitive. In men, the urine ELISA was as good as the urethral swab ELISA for both men with (71% vs 83.9%, $p=0.49$) and

TABLE 2: Performance of IDEIA and nested PCR for 231 male urines and urethral swabs and 66 female urines and endocervical swabs

	Sensitivity	Specificity	PVP	PVN
Male Urine				
IDEIA	59.6 (44.3, 73.3)	99.5 (96.5, 100)	96.6 (80.4, 99.8)	90.6 (85.5, 94.1)
PCR	97.9 (87.3, 99.9)	99.5 (96.5, 100)	97.9 (87.3, 99.9)	99.5 (96.5, 100)
Female Urine				
IDEIA	19.4 (8.8, 36.6)	100 (85.9, 100)	100 (56.1, 100)	50.8 (37.6, 63.9)
PCR	91.7 (76.4, 97.8)	93.3 (76.5, 98.8)	94.3 (79.5, 99.0)	90.3 (73.1, 97.5)
Endocervical swab IDEIA	86.1 (69.7, 94.8)	96.7 (80.9, 99.8)	96.9 (82.0, 99.8)	85.3 (68.2, 94.5)
Urethral swab IDEIA	68.1 (52.7, 80.5)	99.5 (96.5, 100)	97.0 (82.5, 99.8)	92.4 (87.6, 95.5)

(95% confidence intervals)

PVP = Predictive value of positive result

PVN = Predictive value of negative result

without overt urethritis (37.5% vs 37.5%, $p=1$) but the urine PCR was even better than the urethral swab ELISA again for men with (100% vs 83.9%, $p=0.000047$) and without symptomatic urethritis (100% vs 37.5%, $p<0.000000$). In women, the ELISA detected much fewer positives in urine (19.4%) than in the cervical swab (86.1%). This low sensitivity for female urine has also been reported by others⁶ and has been attributed to the fact that chlamydial infection in women is predominantly in the uterine cervix. However, many women are dually infected in the cervix and the urethra and in 5%-30% of infections, only the urethral swab is positive.^{6,10} In the absence of urethral involvement, urine can still be contaminated by cervical discharge. Hence the examination of urine specimens from women can be better than testing cervical swabs alone as it would allow the detection of organisms from both the urethra and the cervix. The data from this study confirmed that the urine ELISA was not satisfactory for screening female infections but the more sensitive urine PCR was at least as good as the cervical swab ELISA.

When comparing two tests in the absence of an undisputed "gold standard", it is generally accepted that a "true infection" can be established with two test positives and discordant results can be resolved by a third test. In this study, the swab ELISA was used to help resolve discrepancies between the urine ELISA and the urine PCR but as the PCR was obviously more sensitive than the ELISA, this approach was likely to result in true PCR positives being wrongly labelled as false PCR positives. This problem was overcome to some extent by retesting all PCR positive, ELISA negative specimens with a second PCR with a different profile to reduce the possibility of amplicon contamination. Overall, evidence of amplicon contamination and PCR inhibition occurred in 3.7% and 1.9% of urine specimens respectively.

The need for a reliable diagnostic test for chlamydia is obvious. False negatives can result in patients, particularly women, being denied of appropriate treatment and going on to suffer from chronic and devastating sequelae. A false positive result for an STD agent like *C. trachomatis* can have a profound psychosocial effect on the patient. It is obvious that at the present state of art, no one laboratory test is able to detect all positive specimens. Multiple examinations of different sites and with different tests may be necessary to establish diagnosis but this is not always practical and is invariably too

costly. When a single test is used, however, the clinician must be aware of the limitations of the test and always interpret results in the light of clinical findings. This study has demonstrated that, for the diagnosis of chlamydial genital infection in a high prevalence population, if the ELISA for antigen detection were the only test available, first void urine can be used as an alternative to the male urethral swab but in female patients the endocervical swab is to be preferred. If PCR technology were available, then the urine sample would be suitable for the diagnosis of both chlamydial urethritis and cervicitis.

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