

Use of Ligase Chain Reaction and Polymerase Chain Reaction on Urine Specimens to Detect *Chlamydia trachomatis* Infections in a Sexually Transmitted Diseases Clinic in Singapore

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Abstract

This study was done to assess the specificity and sensitivity of the DNA amplification assays of ligase chain reaction (LCR) and polymerase chain reaction (PCR) on urine specimens to detect *Chlamydia trachomatis* infections in both male and female patients seen at a sexually transmitted diseases (STD) clinic in Singapore, compared with other diagnostic methods currently in use.

A total of 100 patients were selected; 50 male patients diagnosed with non-gonococcal urethritis based on symptoms and a positive Gram-stained urethral smear and 50 female asymptomatic sex workers were assessed. Automated assays using LCR and PCR were used, and compared to enzyme immunoassays, chlamydial cell cultures and PCR of urethral and endocervical swab specimens.

In male patients, LCR and PCR of urine specimens had sensitivities of 100%, compared to 87.0% for PCR of urethral swab specimen, 82.4% for enzyme immunoassay (EIA) and 91.3% for cell cultures. In female patients, LCR and PCR of urine samples achieved sensitivities of 77.8% and 88.9% respectively, compared with 55.6% for PCR of endocervical swab specimens, 22.2% for EIA and 66.7% for cell cultures.

LCR and PCR of urine samples provided higher sensitivity compared to cell cultures, EIA and PCR of urethral and endocervical swab specimens. The use of LCR and PCR on urine as a non-invasive means of detecting chlamydial infections is viable, and may have a role to play in population-based screening programmes.

Ann Acad Med Singapore 1999; 28:245-51

Key words: Diagnosis, DNA amplification, Screening

Introduction

Infections caused by *Chlamydia trachomatis* are amongst the most common bacterial sexually transmitted diseases (STDs) in the world, causing substantial morbidity in young sexually-active people.¹ *C. trachomatis* causes a variety of clinical syndromes in males (including urethritis and epididymitis), females (including cervicitis and pelvic inflammatory disease), and new-borns (including conjunctivitis and pneumonia).

At the Department of STD Control (DSC) clinic in Singapore, non-gonococcal urethritis (NGU) is the commonest STD seen.² Among the reasons why effective control of STDs caused by *C. trachomatis* has been hampered are the lack of rapid, inexpensive and non-invasive sampling and diagnostic methods. Cell culture was originally considered the "gold standard" for detection of *C. trachomatis* but is generally considered to be only 70% to 80% sensitive,³ with a specificity of close to 100%.⁴ The disadvantage of cell culture is that it is labour intensive, requires two to seven days to isolate the organism, and also requires special transport media to

process the samples. Storage requirements for cell cultures are stringent, particularly in maintaining a cold chain in transport and storage.

In recent years, the recognition of these deficiencies in cell culture has resulted in the development of alternative tests which include enzyme immunoassays (EIA), direct fluorescent antibody assays (DFA), and rapid antigen tests.³⁻⁵ However, most of these antigen detection methods are even less sensitive than cell cultures, and hence may miss a large number of chlamydia infected individuals. Within the last several years, the use of molecular probes and DNA hybridisation assays has dramatically increased the sensitivity of detecting chlamydial infections.⁶ These assays exist in formats that can utilise non-invasive specimens such as urine, can provide results within 24 hours, do not require special storage conditions, and are relatively inexpensive compared to cell cultures. Two automated applications of amplification assays for detection of *C. trachomatis*, the Roche Cobas Amplicor polymerase chain reaction (PCR) and Abbott LCx assay which uses ligase chain reaction

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(LCR), have recently been introduced. As they utilise urine specimens, they offer greater convenience and patient acceptability and may offer a unique sensitive method for widespread population-based screening for *C. trachomatis* infections. We evaluated these assays by using urine samples from male and female attendees of the DSC clinic, and compared these with the automated Amplicor PCR assay on urethral and cervical swab specimens, EIA of urethral and cervical specimens as well as cell cultures.

The aims of this study were:

- (1) to determine the sensitivity and specificity of the amplification assays of LCR and PCR on urine specimens in comparison with PCR, EIA and cell cultures of urethral and cervical swab specimens, and to assess the viability of using urine specimens to screen for *C. trachomatis* infections, and
- (2) to determine the prevalence of *C. trachomatis* infections amongst symptomatic male patients with NGU and female sex workers seen in the clinic.

Materials and Methods

The patients consisted of 50 men and 50 women. Male patients were selected from the attendees of the DSC clinic with symptoms and signs of NGU confirmed by the presence of 5 or more white blood cells (WBC) per high power field (hpf) in a microscopic examination of a Gram-stained urethral sample. The 50 female sex workers were randomly selected from those seen at the clinic. Patients who had received any form of antimicrobial therapy within the last 4 weeks were excluded, as were those who were diagnosed as having gonorrhoea. A standardised sexual history was collected from all patients and the last time to voiding of urine documented to the nearest quarter-hour. In all patients, 30 ml of first-void urine (FVU) was collected in a sterile container without preservatives. All urine specimens were collected with a minimum of 4 hours from the last void to ensure that adequate bacterial antigen was present. For male patients, urethral specimens were collected by means of a cotton-tipped urethrogenital swab inserted 3 cm into the urethra. Endocervical swabs were taken from female patients after wiping the cervical os with a cotton swab. The sequence of collection was rotated so that each swab specimen had an equal chance of being collected first.

The urine LCR assay, the LCx assay (Abbott Laboratories, Chicago), was performed according to the manufacturer's instructions. The urine samples were vortexed, and 1 ml centrifuged at 12,000 g for 10 minutes. The pellet was then suspended in urine resuspension buffer and heated to 95°C for 15 minutes. Processed urine samples not analysed immediately were frozen at -20°C. Amplification was accomplished by the LCx system thermocycler. After the amplification, the tubes contain-

ing the amplified nucleotide sequences were pulse centrifuged and manually transferred to LCx reaction cells and detection was performed by the analyser.

The Cobas Amplicor *C. trachomatis* PCR assay (F Hoffmann-La Roche Ltd, Illinois) with urine samples was performed according to the manufacturer's instructions. In brief, the prepared specimens were transferred into amplification tubes containing primers for *C. trachomatis* nucleotides, the internal control and DNA polymerase. Amplification was accomplished by the built-in thermocycler. The internal control of amplification in the Cobas Amplicor is a sequence of plasmid DNA with primer binding regions identical to those of the *C. trachomatis* target sequence. A unique probe-binding region differentiates the internal control from the target amplicon. The internal control is introduced into each amplification reaction and is co-amplified with the possible target DNA from the clinical specimen. After the amplification, the amplified nucleotide sequences for the internal control and *C. trachomatis* were automatically detected using target specific DNA probes.

The Cobas Amplicor PCR system was also used to analyse urethral and cervical swab specimens. A PCR-ready specimen is prepared using the Amplicor kit and the samples and reagents loaded. Once the system is programmed, the transfer mechanism handles the delivery of reagents, the shifting of reaction-mixtures and the movement of reaction cups. Amplicon is taken from the thermal cycler by directly piercing the individual amplification tubes. Hybridisation with specific probe coated magnetic particles occurs in the 37°C heat block. The reaction mixture is washed at an automatic wash station using a magnet to separate the particles. Avidin-HRP conjugate and substrate incubation and washsteps follow sequentially. Colorimetric detection is carried out with an on-board photometer.

For EIA tests, the Abbott IMx SELECT (Abbott Laboratories, Chicago) diagnostic kit was used. This utilised a microparticle enzyme immunoassay for the qualitative detection of chlamydial antigen on the analyser. The IMx probe/electrode assembly dispenses microparticles to the sample well which contains the sample. The pre-extracted lipopolysaccharide (LPS) antigen of the sample binds to the microparticles forming a complex. An aliquot of the reaction mixture containing this complex is transferred to the glass fibre matrix of the reaction well, and the microparticle-LPS antigen complex binds irreversibly to the glass fibre matrix. Rabbit anti-chlamydial antibody is then dispensed into the matrix and binds with the microparticle-LPS complex, followed by the dispensing of biotinylated goat anti-rabbit antibody to bind with the microparticle-LPS-rabbit antibody complex. Rabbit anti-biotin:alkaline phosphatase conjugate is dispensed onto the matrix and after washing to remove unbound materials, the substrate 4-

methylumbelliferyl phosphate is added to the matrix and the fluorescent product is measured by the optical assembly.

Chlamydial cultures were performed by inoculating specimens onto cycloheximide treated monolayers of McCoy cells on coverslips in glass vials. Culture was considered positive if the monolayers contained one or more chlamydial inclusions, characterised by their typical crescent shape and intracytoplasmic location on Giemsa staining.

Amplification reactions such as LCR and PCR are sensitive to accidental introduction of product from previous amplification reactions, and to prevent contamination due to aerosols formed during pipetting, pipettors and pipette tips with aerosol barriers were used at all times, and sample amplification and detection were done in separate areas of the laboratory.

For the analysis of results, a positive cell culture was taken to indicate *C. trachomatis* infection. For resolution of discrepant results, any specimen with a positive result corroborated by any of the other 4 tests was considered a true positive. Because this was a pilot study with relatively limited materials, results that were unreadable ("invalid") initially were repeated once and recorded as "invalid" if no interpretation could be made after the second run.

Results

Chlamydial Prevalence, Demographic Characteristics, and Behavioural Characteristics of Study Population

Amongst the 50 male patients with NGU, there were 25 Chinese (50%), 9 Bangladeshi (18%), 7 Malays (14%), 6 Indians (12%) and 3 belonging to other races (6%). All of these patients had symptoms of NGU, which included dysuria, urethral tip discomfort or itch, and urethral discharge. The ages ranged from 20 to 64 years, with an average age of 31.7 years. Chlamydial infection was detected in 23 of the patients, giving a prevalence rate of 46%. There were no statistically significant differences in the prevalence of chlamydial infection amongst the various racial groups. Forty-four per cent of the patients had preceding sexual contact with prostitutes; 32% had sexual relations with a regular partner in the previous 6 months and 24% had sexual contact with a casual partner. Only 14 out of the 50 patients (28%) used condoms during their last exposure.

Amongst the 50 female commercial sex workers in the study, there were 23 Chinese (46%), 15 Thais (30%), 7 Malays (14%) and 5 Filipinos (10%). Their ages ranged from 19 to 43 years, with an average age of 26 years. Only slightly more than half (54%) of these sex workers used condoms regularly. The female patients were all asymptomatic. Nine were detected to have chlamydial infection, giving a prevalence of 18%.

Comparison of Results with the Various Diagnostic Methods

The results of all five tests were identical for 61 (61%) of the 100 patients studied. This figure would be much higher if not for the high number of invalid results obtained with the method of PCR identification of *C. trachomatis* from urethral and endocervical swab specimens. Overall, 32 (32%) of the patients had chlamydial infection by definition; there were 23 males and 9 females. Among the 32 with chlamydial infection, the tests were uniformly positive in 18 of the patients. The discrepant results for the other 14 patients confirmed to be having the infection are presented in Table I. Comparisons of the different tests between male and female patients are summarised in Tables II and III, with a summary of the combined results for males and females in Table IV.

Ligase Chain Reaction of Urine Specimens

Overall, the LCR assay detected 30 positive results. The LCR assay detected all 23 cases of *C. trachomatis* infections in the male patients, giving it a sensitivity and specificity of 100% for males, with positive and negative predictive values of 100%. Among the 9 female sex workers with confirmed infection, the LCR assay detected 7 cases, giving a sensitivity of 77.8%, a specificity of 100%, and positive and negative predictive values of 100% and 95.3%, respectively. It failed to detect *C. trachomatis* in 2 female patients; 1 in whom only cell culture was positive, and 1 where cell cultures and PCR of urine and endocervical swab specimens were positive.

Polymerase Chain Reaction of Urine Specimens

The Cobas Amplicor PCR assay on urine specimens detected 31 positive samples. There were no false positive results. In the male patients, PCR of urine samples detected all 23 cases of confirmed infections, giving a sensitivity and specificity of 100%, with positive and negative predictive values of 100%. PCR assay of urine samples in the female sex workers detected 8 of the 9 confirmed cases of chlamydial infection, thus giving a sensitivity of 88.9% and a specificity of 100% in females. Six samples (4 from females) could not be interpreted as the result was reported as invalid by the automated system. These 6 samples were from patients confirmed to be negative for chlamydial infection by the other diagnostic methods. We were thus able to perform PCR of urine successfully on 94% of all samples.

Polymerase Chain Reaction on Urethral and Endocervical Swab Specimens

PCR of urethral and endocervical swab specimens were successful in 76% of the samples tested. It was positive in 25 of the 32 confirmed cases of chlamydia infections. Among the male patients, PCR assay of a urethral swab specimen detected 20 cases out of the 23 with confirmed infection. There was one false negative

TABLE I: ANALYSIS OF DISCREPANT TEST RESULTS BY ROCHE AMPLICOR PCR ON URINE, ABBOTT LCx URINE, EIA/CELL CULTURE/ AMPLICOR PCR ON URETHRAL AND CERVICAL SWAB SPECIMENS

No. of specimens	Amplicor PCR on urine	LCx on urine (LCR)	Amplicor PCR urethral and cervical swabs	EIA urethral and cervical swabs	Cell culture	Final result and conclusion
4	+	+	+		+	Positive; infection not detected by EIA
2	+	+	invalid	+	+	Positive; results not interpretable by PCR of swab specimens
1	+	+	+	+		Positive; infection not detected by cell culture
1	+	+	+			Positive; infection not detected by EIA and culture
1	+	+	invalid		+	Positive; result not interpretable by PCR of swab specimen; infection missed by EIA
3	+	+				Positive; infection detected only from PCR and LCR of urine
1	+		+			Positive; infection not detected by LCR urine, EIA and culture
1					+	Positive; only cell culture detected infection (Gold standard)

+ = positive; - = negative; invalid = result unreadable

PCR: polymerase chain reaction; LCR: ligase chain reaction; EIA: enzyme immunoassay

TABLE II: COMPARISON OF RESULTS IN MALE PATIENTS

Test (specimen and result)	No. of patients in whom <i>C. trachomatis</i> was present (n = 23)	No. of patients in whom <i>C. trachomatis</i> was absent (n = 27)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
PCR (urine)						
Positive	23	0	100	100	100	100
Negative	0	25				
Invalid	0	2				
LCR (urine)						
Positive	23	0	100	100	100	100
Negative	0	27				
Invalid	0	0				
PCR (swab)						
Positive	20	0	87.0	100	100	91.7
Negative	1	11				
Invalid	2	16				
EIA (swab)						
Positive	19	0	82.6	100	100	87.1
Negative	4	27				
Invalid	0	0				
Cell culture (swab)						
Positive	21	0	91.3	100	100	93.1
Negative	2	27				
Invalid	0	0				

PCR: polymerase chain reaction; LCR: ligase chain reaction; EIA: enzyme immunoassay

result, and in the other 2 cases of *C. trachomatis* infections in males, the results were reported as “invalid” by the system as they could not be analysed. This gives a sensitivity of 87% and a specificity of 100% for male patients, with a positive predictive value of 100% and a negative predictive value of 91.7%. Swab samples from male patients produced the highest number of invalid results (16 from confirmed non-chlamydial cases, 2 from confirmed cases), this was most probably due to the repeated sampling which resulted in trauma to the urethral lining. Among the female patients, PCR assay of

endocervical swab specimens detected 5 of the 9 cases of *C. trachomatis* infections (2 false negatives, 2 cases of “invalid” results), achieving a sensitivity of 55.6% and a specificity of 100%, with positive and negative predictive values of 100% and 94.9%, respectively.

Enzyme Immunoassay of Urethral and Endocervical Specimens

EIA of swab specimens from the urethra and endocervix was positive in 21 of the 32 confirmed cases of *C. trachomatis* infection. EIA was falsely negative in 11

TABLE III: COMPARISON OF RESULTS IN FEMALE PATIENTS

Test (specimen and result)	No. of patients in whom <i>C. trachomatis</i> was present (n = 9)	No. of patients in whom <i>C. trachomatis</i> was absent (n = 41)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
PCR (urine)						
Positive	8	0	88.9	100	100	97.4
Negative	1	37				
Invalid	0	4				
LCR (urine)						
Positive	7	0	77.8	100	100	95.3
Negative	2	41				
Invalid	0	0				
PCR (swab)						
Positive	5	0	55.6	100	100	94.9
Negative	2	37				
Invalid	2	4				
EIA (swab)						
Positive	2	0	22.2	100	100	85.4
Negative	7	41				
Invalid	0	0				
Cell culture (swab)						
Positive	6	0	66.7	100	100	93.2
Negative	3	41				
Invalid	0	0				

PCR: polymerase chain reaction; LCR: ligase chain reaction; EIA: enzyme immunoassay

cases. Among males, 19 cases were detected, with 4 false negative results. EIA had a sensitivity of 82.6% and a specificity of 100% in the male patients, with a positive predictive value of 100% and a negative predictive value of 87.1%. Among females, EIA detected only 2 of the 9 cases of *C. trachomatis* infections, and was false negative in the other 7 cases. In females, EIA had a sensitivity of 22.2%, with a specificity of 100%. Positive predictive value of EIA in the female patients was 100%, with a negative predictive value of 85.4%. There were no invalid or false positive results.

Chlamydia Trachomatis Cell Cultures of Urethral and Endocervical Specimens

This used to be the gold standard for detection of *C. trachomatis* infections. It was positive in 27 of the 32 cases of confirmed infection. In males, cell cultures were positive in 21 cases, with 2 false negative results. Cell cultures in male patients had a sensitivity of 91.3%, with a specificity of 100%. Positive predictive value was 100%, with a negative predictive value of 93.1%. Among the female patients, there was one specimen which was positive only by cell culture, with the other methods all being negative. This was considered a true positive by our definition. Cell cultures detected 6 cases of *C. trachomatis* among the female sex workers, achieving a sensitivity of 66.7%, with a specificity and positive pre-

dictive value of 100%, and a negative predictive value of 93.2%.

Discussion

To detect genital *Chlamydia trachomatis* infections, the traditionally accepted specimen for laboratory diagnosis requires either a cervical swab from female patients or a urethral swab from male patients. Adger and colleagues⁷ first reported the use of urine samples on male patients for the diagnosis of this STD, and since then, this less invasive method has attracted much interest. Early studies have shown that urine was inappropriate for chlamydial isolation;⁸ however, first void urine (FVU) acts as a urethral washout and was felt to be suitable for detecting STD antigens by enzyme immunoassays. EIAs have been evaluated for use with FVU by a number of researchers.⁹⁻¹² Chernesky et al⁹ found that FVU collected from mostly symptomatic males attending a STD clinic was useful for diagnosing chlamydial infections using EIAs, and this finding was consistent with the experience of most researchers. However, Schwebke et al¹¹ found the urine EIA to be relatively insensitive compared to culture, and Kok and coworkers¹² concluded that EIAs on urine samples could not replace urethral swabs for the laboratory diagnosis of *C. trachomatis* infections. The development of techniques using molecular probes and DNA hybridisation over the

TABLE IV: COMPARISON OF ROCHE AMPLICOR PCR AND ABBOTT LCx ON URINE SAMPLES WITH ROCHE AMPLICOR PCR, EIA AND CELL CULTURES ON URETHRAL AND CERVICAL SWAB SPECIMENS (COMBINED RESULTS FOR BOTH MALES AND FEMALES)

Test (specimen and result)	No. of patients in whom <i>C. trachomatis</i> was present (n = 32)	No. of patients in whom <i>C. trachomatis</i> was absent (n = 68)	Sensitivity (%)	Specificity (%)	Positive predictive value (%)	Negative predictive value (%)
PCR (urine)						
Positive	31	0	96.9	100	100	98.4
Negative	1	62				
Invalid	0	6				
LCR (urine)						
Positive	30	0	93.8	100	100	97.1
Negative	2	68				
Invalid	0	0				
PCR (swab)						
Positive	25	0	89.3	100	100	94.1
Negative	3	48				
Invalid	4	20				
EIA (swab)						
Positive	21	0	65.6	100	100	86.1
Negative	11	68				
Invalid	0	0				
Cell culture (swab)						
Positive	27	0	84.4	100	100	93.2
Negative	5	68				
Invalid	0	0				

PCR: polymerase chain reaction; LCR: ligase chain reaction; EIA: enzyme immunoassay

last few years has dramatically increased the sensitivity of detecting chlamydial infections; these assays have now been applied to non-invasive specimens such as urine, can provide results within 24 hours, do not require special storage conditions, and are comparable in costs to cell cultures. A study on the use of DNA amplification methods in FVU from asymptomatic male military recruits showed that the performance of LCR assays and PCR assays on urine was markedly better than that of EIA antigen detection methods.¹³ A Finnish study on the use of Cobas Amplicor PCR and LCx assays on urine specimens in women also showed that the amplification techniques on urine were superior to cell culture with cervical swab specimens for detecting *C. trachomatis* infections in women.¹⁴

The results of this study show that urine is a superior sample material for the detection of *C. trachomatis* in both men and women. In this study, the LCR assay on urine detected 30 of the 32 confirmed cases of *C. trachomatis* infection while the PCR assay detected 31 cases. Both methods, with sensitivities of 93.8% and 96.9%, respectively, and specificities of 100%, proved superior to EIAs, cell cultures and PCR of urethral and cervical swab specimens. There was no statistically significant difference between LCR and PCR assays on urine in our study. These figures are comparable to overseas studies; in the Austrian study on asymptomatic military recruits, the

sensitivity of the LCR assay on urine was 93.1% and 62.1% for PCR assay,¹³ with three false positive PCR assay results and no false positive LCR results, while the Finnish study using urine in women showed sensitivities of 94% each for LCR and PCR assays, with specificities of 100% and 99.2%, respectively.¹⁴ A study using LCR on urine in Malaysia showed a sensitivity and specificity of 100% and 99.6%, respectively, in males, and 100% and 98.5%, respectively, in females.¹⁵ Other studies on LCR assays using FVU report sensitivities of 77% to 94% for men and 78% to 96% for women, with specificities of 97% to 100% for men and 99% to 100% for women.¹⁶⁻²⁰

The relatively poorer performance of PCR on urethral and cervical swab specimens was due mainly to technical failures on swab specimens that contained too much mucus or blood. This was due largely to the numerous specimens required in our study, which required exclusion of gonorrhoea as well as a preliminary urethral smear for men before the other samples are taken for cell cultures, EIA and PCR. The multiple samplings could have traumatised the genital tract lining and contributed to the large number of invalid results of the Cobas Amplicor on these samples.

This study has identified *C. trachomatis*, as detected by the various diagnostic methods, as accounting for close to half (46%, 23 out of 50 cases) of all cases of NGU seen in male patients attending a STD clinic in Singapore.

Two previous local studies which yielded rates of 13.8%²¹ and 30.2%²² were based on culture results. The two automated DNA amplification assays on urine samples increases the rate of detection of *C. trachomatis* infections. The lower rate of isolation in the female sex workers (18%, 9 out of 50 cases) was due to the fact that these women were not selected based on the likelihood of their having active infection; they were all asymptomatic. The EIA, which is the method currently employed for screening, detected only 2 out of the 9 women with *C. trachomatis* infections (22.2%), in contrast to cell cultures, which detected 6 cases (66.7%). LCR of urine detected 7 cases (77.8%), and PCR of urine detected 8 cases (88.9%). Both LCR and PCR failed to detect evidence of infection in 1 woman who was positive only on cell culture; this was considered a true positive and she was treated based on the culture results. The use of urine as a diagnostic sample for screening of asymptomatic sex workers appears to have potential for increased rates of detection and thus improve the effectiveness of screening measures at the DSC. With regards to the costs in implementing this diagnostic method on a widespread basis for the purposes of mass screening, there is recent evidence to suggest that pooling of urine specimens for PCR testing may result in cost savings compared with testing specimens individually, with minimal reductions in sensitivity, depending on the prevalence of the infection.²³

In conclusion, the automated DNA amplification methods using LCR and PCR on urine samples proved to be highly sensitive and specific and superior to cell cultures and EIAs with cervical and urethral swab specimens for the detection of *C. trachomatis* infection in both men with NGU and asymptomatic women with high-risk behaviour. Both automated systems, the Cobas Amplicor PCR and LCx assays, are likely to make testing based on nucleic acid amplification more reliable and less laborious, by offering higher output and a shorter turn-around time. The use of urine as a non-invasive means of screening infection in our clinic is viable, and promises to become the test of choice for screening large numbers of men and women in our public health clinics. LCR and PCR can offer chlamydia control programmes a unique sensitive method for widespread population-based screening for *C. trachomatis* infections, a necessary tenet of chlamydial control programmes.

Acknowledgements

This study was supported by a grant from the National Medical Research Council. The authors would also like to thank Roche Laboratories, Singapore for the loan and technical support of the Cobas Amplicor machine, and Abbott Laboratories, Singapore, for the loan and technical support for the LCx system. We would also like to acknowledge the contributions of the staff at the DSC clinic, Kelantan Lane, Singapore.

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