Minimal inhibitory effect of male urine on detection of Chlamydia trachomatis by Roche Amplicor PCR

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A total of 1120 specimens of fresh urine from male patients was tested for Chlamydia trachomatis with the Roche Amplicor PCR Kit and an in-house PCR assay. The in-house PCR had an internal control to monitor inhibitory effects of clinical specimens on the PCR assay. All urine samples were processed within 24-48 h of collection and DNA was extracted on the same day that the assays were performed. Specimens that gave discrepant PCR results were tested by a reference laboratory with both the Roche Amplicor kit and their in-house PCR assay. Of the 1120 samples, 174 gave positive results in both assays and 942 gave negative results in both assays. Only one specimen showed an inhibitory effect on the in-house PCR assays, as indicated by failure to produce the internal control PCR product. This specimen gave negative results by both assays. There were four discrepant results in the two PCR assays. One was a false negative result obtained with the Roche Amplicor kit and the remaining three discrepant results could not be resolved because there was an insufficient quantity of specimen. This study demonstrated that the Roche Amplicor kit could effectively detect C. trachomatis in urine specimens from this population of male patients with negligible inhibition of PCR.

Introduction

Chlamydia trachomatis is the most common bacterial sexually transmitted disease (STD) in both men and women, with an estimated 4 million infections in the USA annually. The prevalence of C. trachomatis infection is 15-20% in men attending STD clinics [1]. Studies have shown that 4-10% of males are asymptomatic carriers who could continuously infect their sexual partners [2, 3]. These asymptomatic males are very difficult to identify because most of them do not seek medical care as they lack symptoms. Also, obtaining urethral swabs from men for detection of chlamydia is not a pleasant procedure and taking the swab could discourage patients from seeing their doctor and affect follow-up. In addition, up to 30% of the swabs are inadequately obtained [4], which may lead to false negative results because of poor quality specimens. Traditionally, C. trachomatis was detected by enzyme immunoassay (EIA) [5], but PCR has been shown to be more sensitive and specific than EIA [6]. The sensitivity and specificity of PCR on urine specimens from men and women ranged from 90% and 99% to 100%, respectively [6]. However, recent publications suggest that clinical samples might contain factors that inhibit the PCR process and potentially give false negative results with fresh urine [7, 8]. Freezing and thawing or dilution of the sample usually restores the PCR process [6]. The extent of inhibition varies with the specimen type, but can be as high as 27% in female cervical specimens [8]. To assess the reliability of the Roche Amplicor assay as a routine test for the detection of C. trachomatis in male urine in our clinical laboratories, the present study evaluated the extent of inhibition caused by urine from males in the Roche Amplicor assay by comparing it with the in-house PCR assay, which uses an internal control.

Materials and methods

Patient specimens

Urine specimens (1120) from male patients attending three STD clinics and physicians' offices were sent to the Provincial Laboratory for detection of chlamydia by
All specimens were transported to the laboratory at room temperature and processed within 24–48 h of collection. DNA extractions and testing for both assays were done on the same day.

**Roche PCR assay**

The Roche Amplicor *C. trachomatis* PCR assay (Roche Diagnostic Systems, Mississauga, Ontario, Canada) was performed according to the manufacturer's instructions. The urine samples were extracted with the Amplicor™ kit and tested for the presence of *C. trachomatis* with strict adherence to the Amplicor protocol, with the exception that any samples with A_{450} between 0.2 and 0.8 were considered equivocal samples. Equivocal samples were tested twice, by repeating the amplification and detection on the stored extract and on the original sample. DNA extracts and the original specimens were stored at 4°C until the results were finalised.

**Internal control production**

Low stringency PCR was used to generate non-specific amplification products from *Physcomitrella patens* DNA with the *C. trachomatis*-specific primers KL-1 (unlabelled) and KL-2 (see below). The reduced stringency PCR was achieved with a low annealing temperature (25°C) in the thermal cycling and with excessive concentrations of both primers (2.0 μM) and *Taq* polymerase (5 units/100 μl). An amplicon was selected on the basis of molecular size (i.e., smaller and readily distinguishable from the *C. trachomatis* 241-bp specific product). The potential internal control was also subjected to amplification with each of the KL-1 and KL-2 primers alone to ensure that no amplification occurred with each primer in isolation. The chosen 174-bp PCR product was excised and purified by standard agarose gel and column purification techniques utilising Wizard™ PCR Preps (Promega, Madison, WI, USA). The purified amplicon was quantified with the DNA Purification Kit (Invitrogen, Carlsbad, CA, USA) and cloned into pGEM-T™ vector (Promega). Large quantities of the internal control were produced by a standard large-scale plasmid preparation protocol and purified with Wizard™ MegaPreps (Promega).

**In-house PCR assay**

The DNA target for amplification was a 241-bp sequence of the genetically conserved cryptic plasmid. The plasmid primers that were used were designated KL-1 (5′-TCC ggA gCg AgT TAC gAA gA-3′) and KL-2 (5′-AAT CAA TgC CCg ggA TgT gT-3′) and have been described previously [9]. The primer KL-1 was labelled at the 5′ end with the fluorescent molecule 6-FAM (National Biosciences, Plymouth, MN, USA). The template for the in-house PCR (5 μl) was taken from the same Amplicor extraction used in the corresponding Roche Amplicor PCR. The in-house PCR amplifications were performed with a PE9600 thermal cycler (Perkin-Elmer, Mississauga, Ontario, Canada) in a final reaction volume of 25 μl. The reactions contained: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 3.5 mM MgCl₂, 250 μM each dNTP, 10 pmol of each primer, 1.25 units of *Taq* DNA polymerase (Gibco BRL, Burlington, Ontario, Canada), 0.275 μg of *TaqStart™* antibody (CloneTech, Palo Alto, CA, USA) and 2.5 μl (0.3 fg) of internal control. DNA amplification was achieved with in-house PCR thermal cycling parameters which consisted of an initial heat-shock at 94°C for 2 min, followed by 20 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, with an additional 20 cycles of 92°C for 15 s, 55°C for 15 s, 72°C for 30 s, with a final extension at 72°C for 10 min and then held at 4°C until detection.

The fluorescent PCR products were detected by PCR fragment analysis on an ABI PRISM™ 377 DNA Sequencer (PE Applied Biosystems, Mississauga, Ontario, Canada). After thermal cycling, 1 μl of the PCR reaction mixture was mixed with 0.25 μl of GENESCAN-500 TAMRA internal lane standard (PE Applied Biosystems) and 1.75 μl of loading buffer (seven parts de-ionised formamide to one part of a stock solution of blue dextran 50 mg/ml, and 25 mM EDTA, pH 8.0). The prepared samples were immediately heat-shocked at 94°C for 5 min and then transferred to a Labtop™ Cooler (Nalgene, Baxter Diagnostics, Mississauga, Ontario, Canada) to snap cool them at −20°C. Samples were electrophoresed at 350 bases/h with 750 V, 200 W and 60 mAmp on acrylamide 4%, cross-linked 5%, 6 M urea gels at a run temperature of 51°C with a gel thickness of 0.2 mm and a well-to-read distance of 12 cm. The fluorescent images were captured with virtual filter set A and 2400 scans/h with ABI PRISM™ Collection Software v1.1.0 (PE Applied Biosystems). The raw data were processed by multi-component analysis, baseline subtraction and scaling with ABI PRISM™ GENESCAN Analysis Software v2.0.2 (PE Applied Biosystems). Analysis parameters within GENESCAN software were customised to remove background fluorescence by setting peak amplitude threshold to 70 units, and size calling was effected with third order least squares giving a sizing accuracy within ±2 bp. All peaks were confirmed manually by visual inspection of the electropherograms.

**PCR inhibitor determination**

An empirically derived dilution of the prepared plasmid containing the internal control estimated at 90 copies was included in every in-house PCR. This ensured the production of low levels of internal control amplicon after PCR amplification. Negative results that may have been caused by PCR inhibitory substances in the specimen extracts were identified by amplification failure of the 174-bp internal control or reduced amounts of this product as measured by peak area on the electropherograms. Any PCR reactions showing
reduced internal control amplification were deemed indeterminate and the specimens were re-tested in both PCR assays with both a fresh extract and the stored extract.

**Analysis of discrepant results**

Any specimens that gave discordant results in the in-house and Roche PCR assays were re-tested with a fresh extraction of the urine stored at 4°C and the previous extract which had been stored at 4°C. Specimens that required re-testing were typically done within 3 days to minimise storage time between extraction and PCRs. Specimens with discordant results which could not be resolved in this manner were sent to a reference laboratory (National Laboratory for Sexually Transmitted Diseases, Winnipeg, Manitoba, Canada) for repeat testing by both the Roche Amplicor *C. trachomatis* PCR assay and their in-house PCR with primers for the MOMP gene [10].

**Statistical analysis**

Sensitivity, specificity, positive predictive value and negative predictive value were calculated after resolution of discrepant results by the reference laboratory.

**Results and discussion**

The use of commercial PCR assays for the detection of *C. trachomatis* from urine specimens was first reported around 1993 [6]. Use of the 'Expanded gold standard' (which is based on a combination of two or more assay results and not on the culture results alone), has demonstrated that PCR assays have sensitivity and specificity in the range 97–100% [6]. Several studies have suggested that there may be inhibitors in fresh urine that may render DNA amplification assays such as PCR [7] or ligase chain reaction (LCR) [11] falsely negative. The original Roche Amplicor kit did not have an internal control to monitor the PCR process, causing some uncertainty in all the negative results. An internal control is now available in the automated Cobas Amplicor kit. Freezing and thawing, or dilution of the original samples, is able to reverse most of the false negative PCR results to positive [6], but such treatments involve additional testing of specimens and provide no indication as to which false negative results may be due to inhibition of PCR. Based on these reports and concerns, the present study aimed to evaluate the utility of the Roche Amplicor PCR for male urine specimens and to determine if PCR inhibition is a problem in our patient population that could compromise the sensitivity of the assay, and if so, how much this could affect our detection capacity for urine specimens from male patients.

Of 1120 urine samples tested in the study's male patient population, only one specimen was inhibitory to PCR, as suggested by its failure to amplify the internal control in the in-house PCR assay. All the other negative urines gave the 174-bp PCR product, which demonstrated that the PCR process was intact and no inhibitor was present to give a false negative result. There was one false negative specimen by Roche Amplicor, which was positive by the in-house PCR assay and the reference laboratory. No inhibition was detected in this specimen by the in-house assay, indicating that the false negative result was caused by other factors. There were three unresolved specimens; one was shown to be positive by the in-house assay, but not by Roche or by the reference laboratory. The in-house PCR had a plasmid-based primer set which has been demonstrated to be at least 10 times or 1000 times by weight more sensitive than primers directed towards the major outer-membrane protein gene as used by the reference laboratory [12]. Therefore, it was felt that this may not be a false positive but could be another false negative result by Roche PCR. However, it was not possible to study the specimen further because of the limited volume of the sample. The other two specimens could not be resolved by the reference laboratory because of insufficient quantity. Excluding these three unresolved specimens from the data for analysis, the Roche Amplicor PCR gave a sensitivity and specificity of 99.42% and 100%, respectively (Table 1).

This study has answered our concern regarding the inhibitory effect of male urine on the Roche Amplicor PCR assay. This is especially important in decision-making at this laboratory, as it was planned to use the Cobas Amplicor PCR assay for all male patients in place of urethral EIA swabs. This minimal inhibition of male urine in the Roche Amplicor assay of the patient population gave confidence that the number of false negative results caused by inhibitory factors in the specimens is negligible and will not significantly compromise the sensitivity of the Amplicor assay.

| Table 1. Performance of Amplicor PCR and in-house PCR |

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<th>PCR results</th>
<th></th>
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<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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<tr>
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<td>175</td>
<td>942</td>
<td>100</td>
<td>100</td>
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*PPV, positive predictive value; NPV, negative predictive value; three unresolved specimens are not included in this table.

*Includes one false negative result.
the present study, the in-house PCR also acted as a confirmation assay for the Roche assay, because a different primer set was used to eliminate system error. Similar findings were presented by Toye et al. [13] at the 97th Annual Meeting of the American Society for Microbiology, with 1.1% inhibition in urine specimens. This is contrary to other findings that male urine has an inhibitory effect on PCR assay of 3–31% [3,9]. This difference could be due to the different populations of patients being studied. It will be advisable for each laboratory to test their patient population to determine the level of inhibition in their PCR assay and to determine the cost-effectiveness of PCR assays in their laboratory. Since the change to the urine PCR detection in this laboratory, the positive rate for male patients has increased from 9%, with EIA and urethral swabs, to 13.2%. In contrast to LCR, which requires specimens to be transported cold (4°C), urine samples for the PCR assays used in the present study can be transported at room temperature; a distinct advantage for specimens coming from sites some distance from the laboratory. Furthermore, the number of specimens received from male patients in this area has increased, because of the non-invasive nature of the test. This should have a major impact on the public health effort to reduce STD by detecting more asymptomatic patients.

In conclusion, there is minimal inhibitory effect of male urine on the Roche Amplicor PCR assay in this male patient population. This was confirmed by running the in-house PCR assay, which contains an internal control, in parallel with the Roche Amplicor kit on 1120 male urines. The change to a urine-based PCR assay encourages more patient testing and detects more positive results, thus having a positive impact on the public health control of STD.

This work was presented in part at the 97th American Society for Microbiology General Meeting, Miami Beach, Florida, 4–8 May 1997, poster #C-391. The authors wish to thank Janette Romanuik for her excellent work in preparation of the manuscript.

References


