

A Validated *In-House* PCR Method for the Detection of *Chlamydia trachomatis* from Urine and Swabs Using Automated Sample Preparation.

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Abstract

Chlamydia trachomatis is a major cause of sexually transmitted disease in humans. Sensitive and reproducible detection of *C. trachomatis* is essential to monitor and help prevent further spread of this pathogen. Our department is a NS-EN 45001 certified clinical laboratory for routine diagnostic testing of a wide range of human pathogens. We validate our in-house methods in compliance with the NS-EN 45001 standard. Molecular techniques, and especially PCR, have become an important tool for detecting bacteria and viruses in our laboratory. Real-time PCR is currently our method of choice as it provides an excellent means of multiplexing and detection. We had a need for a high throughput, cost effective diagnosis of *Chlamydia trachomatis*, and in response we developed a method for automated DNA isolation and subsequent PCR detection.

The PCR combines one set of primers, a synthetic IC (IC) and two dual labelled probes in a mastermix from Eurogentec. The IC is longer than the *C. trachomatis* amplicon and in a very low concentration so as not to inhibit positive samples. DNA was purified from urine and swabs using a fully automated protocol on the BioRobot[®] M48 workstation together with the MagAttract[®] DNA Mini M48 Kit.

The primers were designed against *truncated inclusion membrane protein A*. 37 different bacteria, human DNA and viruses were tested against our primers. All tested negative. Using commercially quantified DNA, we were able to reproducibly detect 1 DNA copy / μ l. No inhibition has been seen after testing 168 swabs and 557 urine samples.

Our PCR method was compared to ROCHE's COBAS Amplicor using a panel of 282 clinical samples, were 34 (12 %) was true positives. Samples showing divergent results were tested using Becton Dickinson strand displacement amplification (SDA) at an external laboratory. The in-house PCR showed 97 % sensitivity, 100 % specificity, 100% positive predictive value (PPV) and 99 % negative predictive value (NPV). The Cobas Amplicor showed 97 % sensitivity, 99 % specificity, 97 % PPV and 99 % NPV.

Based on our results we are confident that our method performs equally to the COBAS Amplicor by Roche. In addition, the in house method had higher throughput at a lower cost.

Introduction

Chlamydia trachomatis is a major cause of sexually transmitted bacterial disease in Norway. More than 15000 are diagnosed each year, mostly women. Our laboratory tested 17826 samples for *C. trachomatis* in 2003. 77 % of the samples originated from women.

Table 1. Distribution of Gender and specimen collected for Chlamydia trachomatis diagnosis with Cobas Amplicor in our lab in 2003.

Distribution %		
Specimen	Women	Men
Urine	8 %	78%
Conjunctival secretion	0 %	1 %
Swab	91 %	20 %

Table 2. Positivity of different specimens diagnosed in 2003 on the Cobas amplicor.

Distribution % positivity		
Specimen	Women	Men
Urine	7 %	15 %
Conjunctival secretion	3 %	2 %
Swab	6 %	12 %

An undetected *C. trachomatis* infection may cause ectopic pregnancy, pelvic inflammatory disease (PID) and salpingitis with secondary tubal scarring and infertility². This entails a need for a fast, easy, cost effective method that can be automated. Such a method warrants screening in low-prevalence populations with high predictive values. This is valuable because *C. trachomatis* often causes asymptomatic infections¹.

Methods

Automated DNA isolation

DNA was purified from urine and swabs using a fully automated protocol on the BioRobot[®] M48 workstation (Qiagen, Oslo Norway) together with the MagAttract[®] DNA Mini M48 Kit and a new bacteria DNA protocol.

Primers

We designed primers that targets *truncated inclusion membrane protein A (incA)*. The primers were designed using Primer Express 2.0 (Applied Biosystems Foster City).

Probes

Two dual labelled probes were designed; one against *incA* and one IC probe, targeting a specific sequence on the pGEM[®]-*luc* plasmid (Promega, Maddison WI). The *incA* probe is labelled with FAM, the IC probe with Yakima yellow; both of them are quenched with Eclipse DarkQuencher. Primers and probes were supplied by Medprobe, Norway

Internal Control

An IC was constructed by using Composite³ primers that has *C. trachomatis* specific 5`-end and a pGEM[®]-*luc* plasmid specific 3`-end. When using such primers in a PCR, the product

wil have ends that are recognized by the *C. trachomatis* primers, and an internal part that is recognized by the IC probe (figure 2).

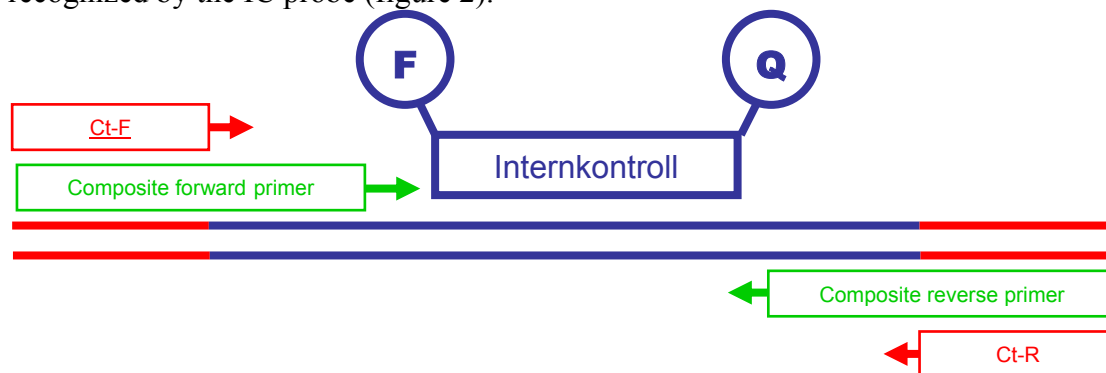


Figure 1. A schematic view of the IC, the probe and the *Chlamydia trachomatis* primers. F: Fluorophore, Q: Quencher.

Multiplex

The PCR is a multiplex where we employ one primer-set and two different probes. This makes it a less complex PCR that is very robust and easier to design. The IC amplicon is longer than the target amplicon, making it less effective in the PCR. The IC is mixed with primers and probes in the qPCR™ mastermix Plus (Eurogentec, Belgium) and stored at 4°C.

Results

Isolation

All heterogeneous controls tested negative, meaning a very specific method (table 2).

Table 3. Heterogeneous controls. The 31 bacterial, viral and human DNA isolates tested against the *C. trachomatis* PCR to ensure specificity.

Gram negative bacteria	
ATCC 35372 <i>Escherichia coli</i>	ATCC 8467 <i>Bordetella pertussis</i>
ATCC 13883 <i>Klebsiella pneumoniae</i>	AR 39 <i>Chlamydia pneumoniae</i> ¹
CCUG 35039 <i>Haemophilus influenzae</i>	ATCC 33153 <i>Legionella pneumophila</i>
ATCC 15531 <i>Mycoplasma pneumoniae</i>	ATCC 19424 <i>Neisseria gonorrhoeae</i>
ATCC 13090 <i>Neisseria meningitidis</i>	Vr 9018 <i>Chlamydia trachomatis</i>
ATCC 9913 <i>Neisseria sicca</i>	19243 <i>Neisseria subflava</i>
13090 <i>Neisseria meningitidis</i> gr. B	13102 <i>Neisseria meningitidis</i> gr C
Gram Positive bacteria	
ATCC 19409 <i>Corynebacterium diphtheriae</i>	ATCC 25923 <i>Staphylococcus aureus</i>
ATCC 19433 <i>Enterococcus faecalis</i>	ATCC 12228 <i>Staphylococcus epidermis</i>
ATCC 19433 <i>Enterococcus faecalis</i>	ATCC 25175 <i>Streptococcus mutans</i>
ATCC 49608 <i>Enterococcus gallinarum</i>	ATCC 6305 <i>Streptococcus pneumoniae</i>
ATCC 43076 <i>Enterococcus saccharolyticus</i>	ATCC 19615 <i>Streptococcus pyogenes</i>
ATCC 9341 <i>Micrococcus luteus</i>	ATCC 13419 <i>Streptococcus salivarius</i>
ATCC 4008 <i>Lactobacillus plantarum</i>	ATCC 19423 <i>Mycobacterium ulcerans</i>
ATCC 25590 <i>Micrococcus diversus</i>	
Others	
Human DNA ²	ATCC 14018 <i>Gardnerella vaginalis</i>

¹ Gift from Kenneth Persson

² Anonymous laboratory specimen from healthy volunteer.

Using commercially quantified *C. trachomatis* DNA (tebu-bio, France), we were able to reproducibly detect 1 DNA copy / μ l.

When we supply small amounts of IC in the mastermix, no reduction in *C. trachomatis* sensitivity compared to similar mastermix without IC is observed. When using the GenoM48 workstation, no inhibition is observed after testing 168 swabs and 557 urine samples.

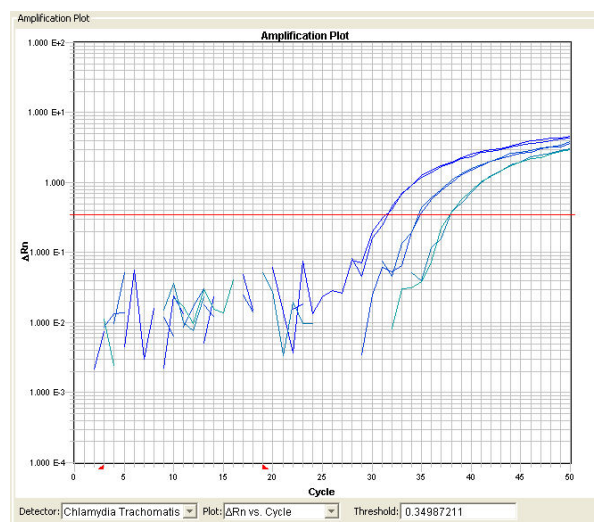


Figure 1. A dilution series of *Chlamydia trachomatis* was run on the ABI 7900 HT and analysed using the SDS 2.1.1 software.

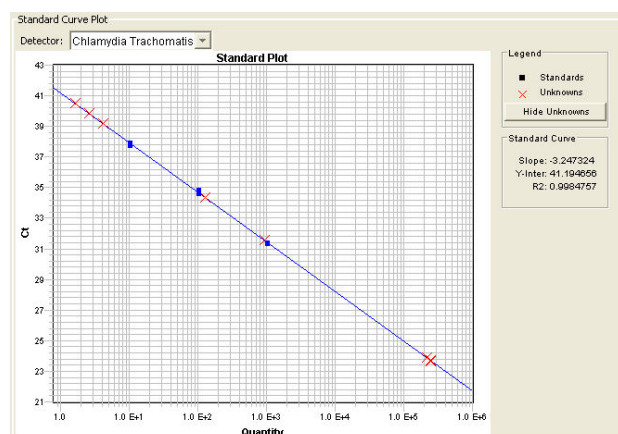


Figure 2. The standard plot for the *Chlamydia trachomatis* analysed using the SDS 2.1.1 software. The slope is -3,247.

The samples with diverging results were tested by Becton Dickinson strand displacement amplification (SDA)⁴ at an external laboratory (*Akershus University hospital, Norway*).

Table 4. Comparison of the to methods

Method	Sensitivity	Specificity	PPV	NPV
In-house	97 %	100 %	100 %	99 %
Roche	97 %	99 %	97 %	99 %

Discussion

We describe a new in-house PCR coupled with automated DNA isolation that is as sensitive and specific as the COBAS amplicor. The great achievement for us is increased throughput and decreased expenses. Another important issue is that the skills and equipment we introduce to the laboratory, can be used to develop PCR detection for commercially non-interesting pathogens.

The implementation of an IC is an effective means of monitoring inhibition.

When we try to run PCR on swabs without any DNA isolation, we see inhibition in some samples. This is seen as an increase in Ct-value for the IC, or a loss of signal from the IC (fig 5). The

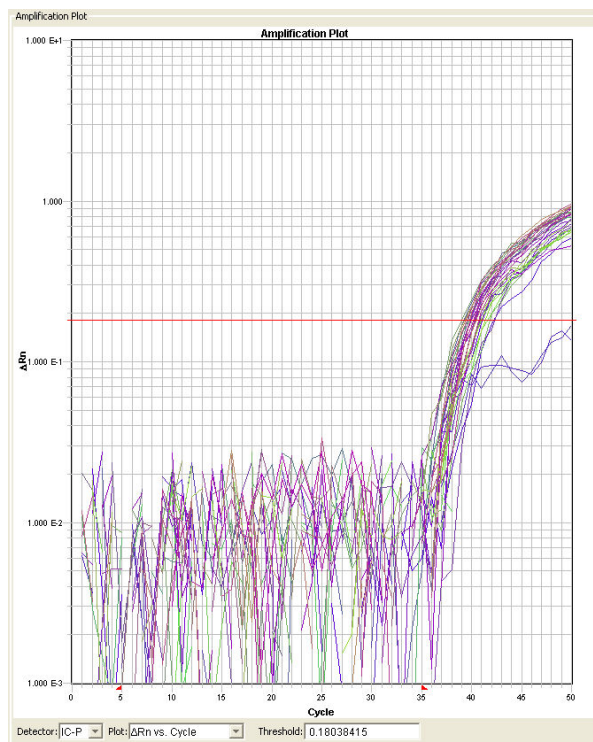


Figure 5. The amplification plot for several negative samples and one low copy number *C. trachomatis*.

Based on our results we are confident that our method performs equally to the COBAS Amplicor by ROCHE. Our method is now implemented in routine laboratory diagnosis and is continuously being optimized. We are currently developing interface software that will monitor the DNA isolation step, a pipetting robot, the PCR step, and finally communicating the results back to the laboratory information system. This will reduce labour and give us optimal traceability of our specimens.

Literature

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