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## Comparison of DNA Extraction from Cervical Cells Collected in PreservCyt Solution for the Amplification of Chlamydia Trachomatis

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# Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*

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## Comparison of DNA extraction from cervical cells collected in PreservCyt solution for the amplification of *Chlamydia trachomatis*

**Objective:** The aim of this study was to compare and evaluate three methods of DNA extraction for the amplification of *Chlamydia trachomatis* in uterine cervical samples collected in PreservCyt solution. ThinPrep is the trade name for the slide preparation.

**Methods:** Thirty-eight samples collected in LCx buffer medium, which were identified as *C. trachomatis* infected by ligase chain reaction (LCR), were selected for this study. DNA from the PreservCyt samples was extracted by three methods: (i) QIAamp kit, (ii) boiling in Tris-EDTA buffer with Chelex purification, and (iii) Proteinase K digestion with Chelex purification. Sample DNA was tested for the presence of *C. trachomatis* by PCR using cryptic plasmid research (CTP) primers and major outer membrane protein research *momp* gene (MOMP) primers. Real-time (LightCycler) PCR for relative *C. trachomatis* quantification following DNA extraction was performed using primers (Hsp 60) for the 60 kDa heat-shock protein *hsp60* gene.

**Results:** Amplification using CTP primers was the most successful with each of the extraction protocols. Boiling in buffer was the least successful extraction method. QIAamp was the best extraction method, yielding the most positives with both the CTP and MOMP primers. Proteinase K-Chelex extraction gave similar sensitivity to QIAamp extraction with CTP primers but lower for MOMP primers.

**Conclusions:** The DNA extraction method must be carefully selected to ensure that larger PCR amplicons can be successfully produced by PCR and to ensure high sensitivity of detection of *C. trachomatis*. In this study it was found that the QIAamp extraction method followed by PCR with the CTP primers was the most successful for amplification of *C. trachomatis* DNA.

**Keywords:** DNA extraction, cervical cells, PreservCyt, *Chlamydia trachomatis*, PCR, LightCycler

## Introduction

Gynaecological cytology is rapidly changing due to the development of new technologies for smear preparation, automated evaluation and molecular analysis of cervical smear samples.<sup>1,2</sup>

Liquid-based cervical smear preparation is overtaking conventional smear preparation as the method

of choice for cytological evaluation due to the improvement in rates of detection of preneoplastic lesions, ease of slide interpretation and decrease in number of unsatisfactory (repeat) smears.<sup>3,4</sup> These liquid-based cervical samples are rich in cellular material, with thousands of residual cells remaining in the solution after the preparation of a monolayer smear. This cellular material is ideal for molecular analysis of both host cell genome and for detection and quantification of micro-organisms,<sup>5-7</sup> thus offering the possibility of additional molecular tests for patients who have abnormal epithelial cells or features of inflammation on routine cytology.

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Detection of human papillomavirus in cervical samples has been exhaustively studied in both conventional and liquid-based cytology samples,<sup>8,9</sup> however, detection and quantitation of *Chlamydia trachomatis* has been less thoroughly explored.<sup>10,11</sup> *Chlamydia trachomatis* is an obligate intracellular bacterium which may be present in between 2% and 17% of cervical smear samples from healthy asymptomatic females depending on the population studied.<sup>12</sup> Infection with *C. trachomatis* may result in pelvic inflammatory disease, infertility and is a major cause of ectopic pregnancy. Numerous studies have suggested that screening of young women for *C. trachomatis* would be cost effective due to the ultimate burden on the health service of the complications of this infection.<sup>13–15</sup>

The main detection methods for *C. trachomatis* include culture, enzyme immunoassay and nucleic acid-based techniques (NAAT).<sup>16</sup> Recent studies indicate that the NAAT methods, which are mostly based on PCR amplification of the *C. trachomatis* cryptic plasmid sequences, offer the highest sensitivity for detection of the organism.<sup>17,18</sup> A hybrid capture (HC) system has also been developed for *Chlamydia* detection, similar to the HCII system for HPV detection (Digene Corporation).<sup>19</sup>

Methods for DNA extraction from cervical cells are varied, mostly relying on some form of Proteinase K extraction, with or without a further purification step.<sup>20,21</sup> Other protocols have been applied to cervical cell samples, including simple boiling in buffer, microwaving and automated DNA extraction techniques.<sup>22,23</sup> In this study three methods of DNA extraction from uterine cervical cell samples collected into PreservCyt solution were evaluated, by the sensitivity of detection and relative quantitation of *C. trachomatis* by molecular (PCR) amplification methods compared to a gold standard of routine cervical samples tested by LCR.

## Methods

### *Study population and clinical specimens*

Cervical samples were taken from women attending a genitourinary medicine clinic at St James' Hospital, Dublin, Ireland, and placed in LCx transport medium (LCx; Abbott Laboratories, Chicago, IL, USA). These were tested for *C. trachomatis* using the Ligase Chain Reaction assay (LCx; Abbott Laboratories). A second cervical sample was taken on the same visit and placed

in a vial of PreservCyt medium and transported to the cytology laboratory of the hospital where a cervical smear was made. Specimens were then kept at room temperature and the DNA was extracted within 6 weeks. The sample population in this study consisted of 38 women who tested positive for *C. trachomatis* by the LCx.

### *DNA extraction*

Fifteen millilitres of PreservCyt specimen was vortexed briefly and divided into three 5-ml aliquots. These were then centrifuged at 13000 *g* and the pellet was washed twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and resuspended in a final volume of 200 µl TE buffer. In extraction method A (TE-Chelex), the cell suspension was boiled for 10 minutes with 0.1% Chelex solution (Sigma-Aldrich, St. Louis, MO, USA). In method B (Proteinase K-Chelex), the cells were resuspended in 200 µl of cell lysis buffer (10 mM Tris-HCl, 400 mM NaCl, 2 mM Na<sub>2</sub>EDTA, pH 8.2), with 20 µl of Proteinase K (20 mg/ml) and 0.5% sodium dodecyl sulphate and incubated at 65 °C for 2.5 hours. This was then boiled for 20 minutes with 0.1% Chelex-100 solution. In method C (QIAamp), the QIAamp DNA Mini Kit (Qiagen Ltd, Crawley, UK) was used according to the manufacturer's instructions. DNA was extracted from a single sample using the three extraction methods within the same 48-hour period. Following DNA extraction samples were stored at -20 °C, until required for PCR.

### *PCR amplification of C. trachomatis plasmid and omp gene*

The following primer sets were used for detection of *C. trachomatis*: a plasmid primer set (CTP)<sup>24</sup> (201 bp), and a primer set (MOMP)<sup>25</sup> for the major outer membrane protein gene (540 bp), (Table 1, Figure 1). PCR was performed in 20 µl reaction volume, containing 2 µl PCR buffer (Invitrogen Ltd, Renfrew, UK), 1.5 mM MgCl<sub>2</sub> 200 µM of each dNTP, 25 pmol of each primer set, 1 U of *Taq* DNA polymerase (Invitrogen Ltd) and 5 µl of DNA sample. The PCR reaction mixture was performed in a Hybaid Omni-E thermal cycler (Hybaid Ltd, Ashford, UK), with an initial denaturation of 95 °C for 5 minutes followed by 40 cycles of 95 °C for 1 minute, 55 °C for 1 minute and 72 °C for 2 minutes. After 40 cycles, a further elongation step was carried out at 72 °C for 5 minutes. The products were run on a 1% agarose gel containing 0.5 µg/µl ethidium bromide.

Gene target	Primer name	Primer sequence	Fragment amplified (bp)
Plasmid	CTP 1	5'-TAGTAACTGCCACTTCATCA-3' <sup>24</sup>	201
	CTP 2	5'-TTCCCCTTGTAATTCGTTGC-3'	
<i>momp</i>	MOMP A	5'-TATACAAAAATGGCTCTCTGCTT-3' <sup>25</sup>	540
	MOMP B	5'-CCCATTGGGAATCTTTATTCACATC-3'	
<i>hsp60</i>	Hsp 60 F	5'-GATGGTGTACCGTTGCGA-3' <sup>26</sup>	650
	Hsp 60 R	5'- CCTCCACGAATTCTGTTCAC-3'	

**Table 1.** DNA oligonucleotides used for polymerase chain reaction

#### *Real-time PCR detection and quantitation of C. trachomatis DNA*

Real-time PCR for detection and quantitation of *C. trachomatis* was performed on all DNA samples using a primer set (Hsp 60)<sup>26</sup> specific for the heat-shock protein 60 gene (Table 1). An aliquot of 1 µl of each sample was added to 9 µl of a reaction mixture containing 3 mM MgCl<sub>2</sub>, 0.5 µM primers and 1 µl of LC DNA FastSTART Master SYBR Green I enzyme mix (Roche Biochemicals, Mannheim, Germany). Samples were amplified on a LightCycler (Roche) under the following cycle conditions: an initial 10 minutes at 95 °C for activation of the FastSTART *Taq* DNA polymerase, followed by 40 cycles of 5 seconds of denaturation at 95 °C, 10 seconds of annealing at 68 °C, decreasing to 65 °C at a rate of 1 °C/cycle and 30 seconds of extension at 72 °C. Data were obtained after the extension period in the single acquisition mode. The Hsp 60 PCR product was cloned into a pBSII vector and serially diluted cloned copies were used to create a standard curve (10<sup>5</sup> to 10<sup>2</sup> copies) for quantitation of chlamydial copy numbers. These standards were run with each LightCycler run and a calculation of *C. trachomatis* copy numbers was taken by the machine at the crossing point of each sample during the exponential phase of amplification. A melt curve step was included to confirm the amplification. Samples which gave copy numbers outside the range of the standards or whose melt-temperature (*T<sub>m</sub>*) was outside those of the standards had their real-time PCR product analysed by agarose gel electrophoresis. A sample was positive if amplification of the 650 bp product occurred during the amplification programme of 40 cycles.

## Results

DNA isolated from the 38 LCx-positive samples by the three extraction protocols was analysed for *C. trachomatis* positivity by PCR and real-time PCR and quantified using real-time PCR. A positive result

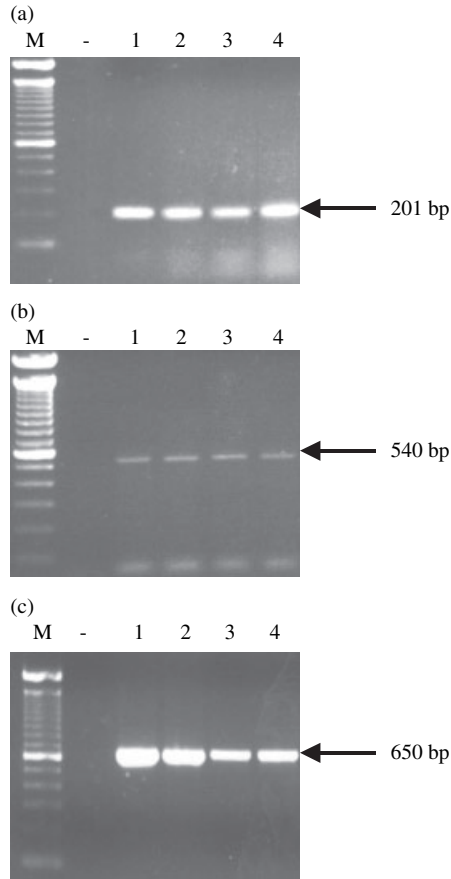
for either the CTP or the MOMP primer set was determined by the presence of 201 bp product for the CTP primer set and a 540-bp product for the MOMP primer set on agarose gel electrophoresis. The Hsp 60 real-time PCR assay was positive if quantified *C. trachomatis* copy numbers were inside the range of the standards or if running of the product on an agarose gel gave the required 650 bp product size. 5 TE-Chelex, 5 Proteinase K-Chelex, and 2 QIAamp samples had copy numbers below the range of the standards (<100) but were positive on gel electrophoresis of the PCR product.

#### *Detection of C. trachomatis by plasmid, major outer membrane protein and heat-shock protein 60 real-time PCR*

Ninety-five percent (36/38) of samples extracted using either the TE-Chelex method or the Proteinase K-Chelex method were positive for *C. trachomatis* by either of the three PCRs. The QIAamp samples gave a slightly higher overall positivity rate of 97% (37/38) with a sensitivity of 95% for detection using plasmid PCR, 90% by MOMP PCR and 95% by Hsp 60 real-time PCR (Table 2). Of the QIAamp samples 90% (34/38) amplified for each of the three PCRs as opposed to 71% (27/38) of the Proteinase K-Chelex samples and only 50% (19/38) of the TE-Chelex samples (Table 2). The plasmid primers were the most successful for the amplification of *C. trachomatis* DNA extracted by each of the three methods, followed by the Hsp 60 primer set and then the MOMP primer set (Table 2).

#### *Real-time LightCycler PCR analyses of C. trachomatis copy numbers*

Real-time quantitation of *C. trachomatis* copy numbers in samples was carried out to determine if reduced sensitivity of detection by PCR was associated with low copy numbers of the organism and to determine the relative yield of *C. trachomatis* copy numbers for each extraction method.



**Figure 1.** (a) Examples of PCR amplification of a region of *Chlamydia trachomatis* plasmid (201 bp) using the CTP primer set. (b) Examples of PCR amplification of *C. trachomatis* major outer membrane protein gene (540 bp) using the MOMP primer set. (c) Examples of PCR amplification of the *C. trachomatis* heat-shock protein 60 gene (650 bp) using the Hsp 60 primer set. M = 100 bp DNA marker; -: negative control; 1: positive control; 2–4: different samples.

The QIAamp and the Proteinase K-Chelex samples most commonly gave copy numbers of  $10^4/\mu\text{l}$  of extracted DNA as opposed to the TE-Chelex samples which gave a lower yield of  $10^2/\mu\text{l}$  of extracted DNA (Table 2). The distribution of copy numbers was evenly spread for the Proteinase K-Chelex samples, when compared with the TE-Chelex and QIAamp samples, which had copy numbers concentrated at the lower and upper end of the range respectively (Table 2).

Negative results by the plasmid and the MOMP PCRs were frequently associated with low copy numbers. Of the four TE-Chelex extracted samples negative by plasmid PCR, three were below the

**Table 2.** Comparison of positive PCR results for each extraction method using the CTP, MOMP and Hsp 60 primers, with quantification of *Chlamydia trachomatis* copy numbers by Hsp 60 real-time PCR

Primer set	No. of positive tests*		
	TE-Chelex (%) <sup>†</sup>	PK-Chelex (%) <sup>‡</sup>	QIAamp (%) <sup>§</sup>
CTP	34 (90)	35 (92)	36 (95)
MOMP	23 (61)	28 (74)	34 (90)
Hsp 60	28 (74)	32 (84)	36 (95)

Chlamydial copy/ $\mu\text{l}$	No of positive tests in each copy number level		
<100	5	5	2
$10^2$	14	6	8
$10^3$	6	6	11
$10^4$	2	8	13
$10^5$	1	6	2
$10^6$	0	1	0

\*All samples in the study were *C. trachomatis* positive by the LCx assay.

<sup>†</sup>Boiling in Tris-EDTA buffer followed by Chelex purification.

<sup>‡</sup>Proteinase K digestion followed by Chelex purification.

<sup>§</sup>QIAamp DNA extraction kit.

detection level of 100 copies of *C. trachomatis*/ $\mu\text{l}$  of extracted DNA and of the 15 MOMP-negative samples three samples had <1000 copies and 12 had <100 copies of *C. trachomatis* by quantitative PCR. Of the four QIAamp samples which did not amplify for all three genes, one sample was positive by Hsp 60 real-time PCR only, with a copy number of  $10^2$ . The other two amplified for the plasmid but not the *momp* gene; the real-time PCR result was negative for one and copy numbers <100/ $\mu\text{l}$  DNA for the other).

**Discussion**

The advent of liquid-based cervical cytology may enable gynaecologists to screen for both cervical preneoplastic lesions and sexually transmitted infectious agents from the same sample. This is based on the ability of liquid-fixed cells to yield nucleic acids suitable for molecular-based assays.<sup>27,28</sup> There are a growing number of publications using PreservCyt specimens for molecular detection of organisms including HPV, herpes simplex viruses, *Trichomonas vaginalis* and *C. trachomatis*.<sup>29,30</sup> As DNA extracted from PreservCyt specimens may be used to screen for multiple organisms there is a need for the development of simple rapid inexpensive DNA extraction methods,



which can readily be applied in the diagnostic setting. The main aim of this study therefore was to compare the efficiency of two inexpensive in-house DNA extraction procedures (Tris-EDTA-Chelex and Proteinase K-Chelex) and one kit-based method (QIAamp) for the detection of *C. trachomatis* by PCR.

The results of this study indicate that the commercial QIAamp extraction kit was the most successful extraction method for amplification of the three different target genes, but that the Proteinase K-Chelex method had a similar success rate for *C. trachomatis* amplification when the plasmid primers were used. However, amplification of larger PCR products was less successful from Proteinase K-Chelex than with the commercial extraction kit which would have implications for application of other molecular methods, particularly restriction enzyme digestion for restriction fragment length polymorphism analysis.

In this study there was 90%, 92% and 95% sensitivity of *C. trachomatis* detection by plasmid PCR amplification by TE-Chelex, Proteinase K-Chelex and QIAamp respectively. Koumans *et al.* also analysed PreservCyt samples for *C. trachomatis* detection using a commercial extraction and detection system and reported 97% sensitivity for *C. trachomatis* detection.<sup>11</sup> On comparison of commercial DNA extraction kits QIAamp has been reported as the most successful.<sup>31</sup> In this study amplification for the plasmid gene was the most successful method for detection of *C. trachomatis* and showed the greatest concordance for the three extraction methods. Other studies have shown that plasmid primers are more sensitive than MOMP primers for the detection of *C. trachomatis* due to the presence of multiple plasmids per organism.<sup>32,33</sup>

Some studies have reported lack of reproducibility of *C. trachomatis* detection, even with commercial systems, and recommend duplicate samples or the application of two NAAT detection methods.<sup>34</sup> In this study all samples were amplified for three different genes to increase the specificity of detection. This study recommends the combined use of the plasmid and heat-shock protein 60 gene primers for PCR detection of *C. trachomatis*.

Real-time PCR is a fast and effective way for the detection and quantitation<sup>35</sup> of bacterial load in clinical samples and for validation of DNA extraction methods. Real-time PCR quantitation was used in this study to show that a drop off in sensitivity of detection, particularly for the larger PCR products, was associated with low copy numbers of *C. trachomatis*.

This study shows that a single PreservCyt cervical specimen can be used as a source of high quality DNA for testing for sexually transmitted infections. Optimization of the method of DNA extraction from PreservCyt is essential to avoid false negatives and ensure adequate sensitivity of detection. Careful selection of genes to be amplified and the PCR product size for the detection of *C. trachomatis* is recommended. Real-time PCR quantitation is a valuable method for validation of the sensitivity of PCR detection methods.

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