Comparison of different primer sets for detection of *Chlamydia trachomatis* by the polymerase chain reaction

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Summary. The sensitivity and specificity of the polymerase chain reaction (PCR) method was studied *in vitro* with HeLa cells infected with *Chlamydia trachomatis* serovar L2. Three different primer sets were studied; they were derived from the endogenous plasmid, the non-variable part of the MOMP gene and the 16S ribosomal RNA (rRNA) gene. The plasmid primers were the most sensitive in the PCR method and detected at least 0.1 infectious unit of *C. trachomatis* in the presence of a superfluous amount of human DNA. Application of this plasmid PCR to 13 *C. trachomatis* culture-positive cervical smears containing < 10–> 200 inclusion-forming units showed that it was the most sensitive of the three methods and detected *C. trachomatis* in all samples. This correlates with the observation that the plasmid PCR method could detect *C. trachomatis* in cervical smears of four symptomatic patients for up to 3 weeks after the start of treatment with doxycycline. In contrast, the MOMP gene- and rRNA gene-directed PCR, as well as culture and direct immunofluorescence, gave negative results within 1 week. Therefore, we conclude that the plasmid primers are the best candidates for use in the PCR method in *C. trachomatis* screening programmes and clinical follow-up studies.

Introduction

Chlamydia trachomatis is an obligate intracellular gram-negative bacterium. Fifteen different serovars are known and eight of these (D–K) cause urogenital infections, mainly in the western industrialised countries. These infections involve the urethra and cervix and may progress to more serious disorders, such as endometritis, salpingitis and pelvic inflammatory disease, resulting in infertility and ectopic pregnancy.¹

The current standard technique for diagnosis of *C. trachomatis* infection is isolation of chlamydiae in cell culture. However, the viability of *C. trachomatis* may be compromised by collection, transportation and various steps in the culture procedure which can affect the sensitivity of culture methods. Therefore, several alternative methods have been developed. Antigen detection methods such as direct immunofluorescence (DIF) and enzyme-immunoassays (EIAs) are the most widely used tests that do not rely on growth of *C. trachomatis*.

Recently, DNA hybridisation techniques have been introduced. However, when compared to culture under optimal conditions, these tests were less sen-

sitive.² Furthermore, DIF is a laborious technique that requires great experience, and EIAs may lack specificity, due to the detection of cross-reactive antigens. During recent years, a new sensitive and specific technique for the detection of micro-organisms, the polymerase chain reaction (PCR), has become available.^{3,4} This technique is based on the amplification, potentially by at least a million-fold, of specific nucleotide sequences in a sample, by means of specific primers and Taq DNA polymerase.

PCR methods may overcome the shortcomings of the more conventional techniques for detection of *C. trachomatis*. Several studies have been reported on the use of PCR in *C. trachomatis* detection.^{5–17} In these studies, primers were used that had been derived from three different *C. trachomatis*-specific DNA sequences—the gene coding for the major outer-membrane protein (MOMP), the 16S ribosomal RNA gene and the 7.5 kb plasmid. However, in none of these investigations has PCR with primers derived from these three different targets been compared directly. Therefore, we have compared PCR based on primers directed to specific sequences in the MOMP gene, the 16S rRNA gene and the 7.5-kb plasmid for their relative sensitivity in detecting serovar L2 DNA from *C. trachomatis* cultured in HeLa cells. This DNA was isolated from a stock suspension of *C. trachomatis* L2 titrated in HeLa cells, permitting the sensitivity of each

PCR to be expressed in terms of inclusion-forming units (ifu). PCR was also performed on cervical smears that were culture-positive in various degrees for *C. trachomatis*. The diagnostic implications of the results of PCR for *C. trachomatis*, in comparison to those of DIF and culture, were explored by monitoring four symptomatic patients after antibiotic treatment.

Materials and methods

C. trachomatis strains

C. trachomatis LGV strains, L2, E and G, were kindly provided by Dr J. H. T. Wagenvoort, Department of Clinical Microbiology, Erasmus University, Rotterdam.

Cervical swabs

Cervical swabs from women attending the outpatient clinic of the Gemeentelijke Geneeskundige en Gezondheidsdiensten of Amsterdam were kindly provided by Dr G. J. J. van Doornum. They were placed in 2 ml of sucrose phosphate transport medium (2SP) and used for attempted culture of *C. trachomatis* and for DNA extraction. Samples from four patients with cervicitis-like symptoms (e.g., abnormal discharge) were found to be positive by DIF for *C. trachomatis* at our hospital. These patients and their sexual partners were treated with doxycycline (100 mg twice daily for 14 days). Endocervical (cytobrush) and urethral (swab) samples were taken and tested by DIF, culture and PCR at 0, 1, 2, 3, 4, 5 and 8 weeks after starting the treatment. Smears were prepared on glass slides for DIF and thereafter swab and brush were transferred to 2SP for culture and PCR and then stored at 4°C.

Direct immunofluorescence

The cervical smears on glass slides were stained with fluorescent anti-chlamydia monoclonal antibodies (MAbs) (MicroTrak; Syva, USA) according to the manufacturer's instructions.

Culture

C. trachomatis strain L2 was grown for 2 days in 80-cm² flasks containing HeLa 229 cells pre-treated with DEAE dextran 15 µg/ml. The chlamydial culture medium consisted of EMEM complete medium (autopo; Flow Laboratories) pH 7.5, containing NaHCO₃ 0.15% w/v, fetal calf serum (Hyclone, USA) 10% v/v, vitamins (Flow Laboratories) 1% v/v, glutamine (Flow Laboratories) 1% v/v, gentamicin 25 mg/L, vancomycin 25 mg/L, amphotericin B 2.5 mg/L, cycloheximide 0.5 mg/L and glucose 4.5 g/L. The monolayer containing the chlamydia inclusions was disrupted by shaking with glass beads. After sonication twice for 15 s at 7 µm amplitude (Soniprep MSE:

probe 2.5 mm), cell debris was pelleted by centrifugation for 5 min at 100 g. Chlamydiae were concentrated by centrifugation of the supernate at 30000 g for 1 h and then suspended in sucrose phosphate glutamate (SPG) medium. By titration of 10-fold dilution series of HeLa cells in microtitration plates, the suspension was found to contain 6.5×10^5 ifu of chlamydia/ml.

Cervical swabs were cultured for *C. trachomatis* on HeLa 229 cells in flat-bottomed tubes (FBT), according to standard procedures, within 24 h of sample collection. Briefly, HeLa 229 cells were rinsed with 500 µl of DEAE-dextran 15 µg/ml in Hanks's Balanced Salts Solution (Oxoid) with NaHCO₃ 0.15% w/v. After the specimens had been agitated, 300 µl were added to the HeLa 229 cells. The inoculum was centrifuged on to the cells at 1200 g for 60 min and the fluid was replaced by 1 ml of chlamydial culture medium. Samples were incubated for 2 days at 37 °C. The monolayers were fixed with absolute ethanol, stained with fluorescent anti-chlamydia MAbs and examined for fluorescing inclusions. The FBT-culture scores were divided into four groups: < 10, 10–100, 101–200 and > 200 ifu. The 2SP that remained after taking a sample for culture was stored at –70°C.

DNA isolation

DNA was isolated from 1 ml of the cervical samples in 2SP and from 1 ml of the suspension of strain L2 (titre 6.5×10^5 ifu/ml). Cells were lysed in SDS 1% w/v and proteinase K 150 µg/L by incubation for 30 min at 37 °C. After phenol extraction, chloroform-isoamylalcohol extraction and ethanol precipitation,¹⁸ the DNA was suspended in 10 mM Tris, 1 mM EDTA (T₁₀E₁). PCR was performed on 10 µl of concentrated isolated DNA of the patient samples, which corresponded to c. 300 µl of the original cervical smear suspension in 2SP medium used for culture, on 10 µl of serovar L2 DNA, corresponding to 6.5×10^3 ifu in the original serovar L2 suspension, and on 10 µl of dilution series of L2 DNA in twice-distilled water. To determine any possible influence of a superfluous amount of DNA on PCR performance chlamydial DNA was diluted in water containing 100 ng of human placental DNA 100 mg/10 µl and tested by the PCR.

Primers and PCR protocol

Primer sets derived from three different targets were compared for their sensitivity and specificity in amplifying specific *C. trachomatis* sequences. The following primer sets were used: a plasmid primer set generating a 201-bp fragment,⁶ CtP1 + 5'-TAGTAAGTCC-CTTCATCA-3' and CtP2-5'-TTCCCCTTGTAATTCGTTGC-3'; a MOMP gene primer set generating a 129-bp fragment,⁵ CtM1 + 5'-GCCGCTTTGAGT-TCTGCTTCCTC-3' and CtM2-5'-CCAAGTGGTG-CAAGGATCGCA-3'; and primers derived from the *C. psittaci* rRNA gene,¹⁹ CtR1 + 5'-GTGGATAG-

TCTCAACCCTAT-3'⁸ and CtR2-5'-CCCTAAGT-GTTGGCAACTAA-3' generating a band of 310 bp. The internal oligonucleotide probes used were: for the plasmid primer set, pCtP1 + 5'-ATCTCATTACCA-TGCATTAGCAGCTATCCA-3'; for the MOMP primer set, pCtM1 + 5'-TCCTTGCAAGCTCTGCC-TGTGGGGAATCCT-3'; and for the rRNA primer set, pCtR1 + 5'-GACATGTATTTGACCGCGGCA-GAAATGTCG-3'. The three internal oligonucleotide probes, to analyse amplified bands from the different primer sets, were selected by applying a Micro Genie computer program analysis (Beckman, USA). As an internal reaction control in clinical specimens, primers specific for the β -globin gene were used.³ All primers and oligonucleotide probes were synthesised on a Gene Assembler Plus (Pharmacia LKB, Sweden) by the methoxy-phosphoramidite method.

Generally, PCR was performed in 50 μ l of PCR solution, containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1.5 mM MgCl₂, 200 μ M of each dNTP, 50 pmol of each primer, 1 unit of Taq DNA polymerase (Amplitaq, Cetus, USA) and 10 μ l of DNA sample. The mixture was overlaid with several drops of paraffin oil to prevent evaporation and incubated for 4 min at 95 °C for DNA denaturation. Forty cycles of amplification were performed with a PCR processor (Biomed, Germany). Each PCR cycle consisted of a denaturation step at 95 °C for 1 min, a primer-annealing step at 55 °C for 2 min and a chain-elongation step at 72 °C for 1.5 min. After 40 cycles a temperature-delay step of 4 min at 72 °C was done to complete elongation. Ten μ l of the amplified PCR products were analysed by agarose 1.5% w/v gel electrophoresis.

Southern blot analysis of PCR products

DNA was transferred from agarose gel to a Biotrace HP polysulfon-based membrane (Biotrace, Gelman Sciences, USA) by diffusion blotting in 0.4 N NaOH overnight. The plasmid, MOMP and rRNA gene-derived PCR products were analysed for specificity with target-specific internal oligonucleotide probes (see above). Membranes were pre-incubated at 55 °C in a hybridisation solution (0.5 M sodium phosphate, pH 7.2, SDS 7% w/v) for 2 h. Hybridisation occurred overnight at 55 °C with 20 pmol of T4 kinase 5'[³²P] end-labelled oligonucleotide probes according to standard procedures.¹⁸ The probes were labelled together in one reaction when hybridised as a cocktail. The filters were washed three times for 15 min with 3 $\times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) with SDS 0.5% w/v at 55 °C. Autoradiography was performed with Kodak Royal X-omat film and intensifying screens overnight at -80 °C.

Results

PCR with C. trachomatis L2 DNA

Initial experiments were performed on *C. trachomatis* L2 DNA (corresponding to 10 ifu in the PCR)

with the three PCR primer sets at annealing temperatures of 45°, 50° and 55 °C, to determine the optimal annealing temperature. The PCR results were not substantially influenced by the annealing temperature within the range tested. In further experiments, an annealing temperature of 55 °C was used. Relative sensitivity and specificity of PCR with different primer sets were determined by PCR on a 10-fold dilution series (10²–10⁷) of serovar L2 DNA in twice-distilled water.

Fig. 1 shows that the highest sensitivity was reached with the PCR that used primers directed against the endogenous plasmid (lanes 8–14). An equivalent of 0.1 ifu could be detected after ethidium bromide staining in agarose gel (lane 11). Amplification of a part of the MOMP gene (lanes 1–7) and of the 16S rRNA gene (lanes 15–21) was *c.* 10-fold less sensitive. For all three primer sets, the sensitivity of the PCR increased at least 10-fold after hybridisation, as compared to direct staining of the agarose gel. Human placental DNA (100 ng in the PCR reaction) affected the sensitivity of the PCR slightly. With the PCR directed against the MOMP gene and the endogenous plasmid, additional PCR products were found (fig. 2a, lanes 1–7 and 8–14). However, these products, did not hybridise with the respective specific probes (fig. 2b). With the plasmid primers, at least 0.1 ifu was detectable after hybridisation (lane 11).

PCR with culture-positive material

The relative value of the use of primer sets against different targets for the detection of *C. trachomatis* infection was determined by PCR with DNA isolated from cervical smears of 13 patients that showed various degrees of positivity in culture. All 13 patient samples contained amplifiable DNA as shown by the generation of the 110-bp band amplified from the β -globin gene. Fig. 3 shows typical PCR results from cervical smears of four patients; the greatest sensitivity was obtained with the plasmid primer set (patients a–d, lane 2). The intensity of PCR signals decreased with decreasing numbers of ifu in the corresponding culture. The overall results obtained for 13 cervical samples are summarised in table I. All 13 samples were positive by plasmid-directed PCR after hybridisation, but only 12 and 10 gave positive results with the MOMP and rRNA genes, respectively, as target. The PCR-negative samples were all weakly positive in culture (< 10 ifu).

Application of the PCR

The diagnostic implications of the PCR for detection of *C. trachomatis*, in comparison with DIF and culture, were investigated by monitoring four *C. trachomatis*-positive patients for several weeks after the start of their treatment with doxycycline. All four patients who were initially DIF-positive were also found to be positive by culture. In three of the four patients, the MOMP gene, plasmid and rDNA PCR

Fig. 1. PCR on a 10-fold dilution series in water (10^2 – 10^7 ; plus negative control) of *C. trachomatis* L2 DNA with CtM1,2, CtP1,2 and CtR1,2 primers. **a**, PCR products after electrophoresis on an agarose 1.5% w/v gel and ethidium bromide staining. lanes 1–7, MOMP; 8–14, plasmid; 15–21, rRNA primers; M, pBR322 digested with *Hinf*I. **b**, Southern blot analysis after hybridisation with target-specific oligonucleotide probes (pCtM1, pCtP1 and pCtR1).

Fig. 2. PCR as in fig. 1, with 100 ng of human placental DNA in the reaction mixture. **a**, PCR products after electrophoresis on an agarose 1.5% w/v gel and ethidium bromide staining. Lanes 1–7, MOMP; 8–14, plasmid; 15–21, rRNA primers; M, pBR322 digested with *Hinf*I. **b**, Southern blot analysis after hybridisation with target-specific oligonucleotide probes (pCtM1, pCtP1 and pCtR1).

Fig. 3. Detection of *C. trachomatis* in DNA isolated from cervical smears with different numbers of ifu. The positive DNA controls applied in this PCR are shown in fig. 1. **a**, PCR products after electrophoresis on agarose gel 1.5% w/v and ethidium bromide staining. Lanes 1–4, the negative controls for the MOMP, plasmid, rDNA and β -globin primers; M, pBR322 digested with *Hinf*I. Patients (**a–d**) with +, ++, +++ and ++++ culture results respectively are shown after PCR with MOMP (1), plasmid (2), rRNA (3) and β -globin primers (4). **b**, Southern blot analysis after hybridisation with “cocktail” probe of target-specific oligonucleotides (pCtM1, pCtP1 and pCtR1). Due to photographic reduction, no hybridisation signal is seen with the rRNA primers of patient b (lane 3).

Table 1. Detection of *C. trachomatis* in 13 culture-positive cervical smears, by PCR with primers directed against the MOMP gene, plasmid and 16S rRNA gene

Patient no.	PCR results with						Numbers of ifu/sample by culture
	MOMP gene CtM1,2		Plasmid CtP1,2		16S rRNA gene CtR1,2		
	Gel	Blot	Gel	Blot	Gel	Blot	
1	+	+	+	+	+	+	> 200
2	+	+	+	+	+	+	> 200
3	—	+	—	+	—	—	< 10
4	+	+	+	+	+	+	10–100
5	—	—	—	+	—	—	< 10
6	—	+	—	+	—	—	< 10
7	—	+	+	+	—	+	< 10
8	—	+	+	+	—	+	< 10
9	+	+	+	+	+	+	> 200
10	+	+	+	+	+	+	< 10
11	—	+	+	+	+	+	10–100
12	—	+	+	+	+	+	10–100
13	—	+	+	+	—	+	101–200

gave positive results; in one patient, only the plasmid PCR gave a positive result (table II, patient 4). One week after treatment, all DIF and culture results were negative, as were those for MOMP gene and rDNA PCR. However, in three of four patients, the plasmid PCR remained positive for 1–3 weeks after the start of treatment.

Discussion

Current routine methods for detection of *C. trachomatis* are cell culture, EIA and DIF. More recently, a hybridisation test with a non-isotopic DNA probe has become available commercially.²⁰ None of these tests shows optimal sensitivity and only cell culture is 100%

Table II. Results for four *C. trachomatis*-positive patients treated with doxycycline, in a follow-up study

Week after start of doxycycline treatment	Patient no. 1			Patient no. 2			Patient no. 3			Patient no. 4		
	DIF	Culture	PCR	DIF	Culture	PCR	DIF	Culture	PCR	DIF	Culture	PCR
0*	+	+	++†	+	+	++†	+	+	++†	+	+	++†
1	—	—	++‡	—	—	—	—	—	++‡	—	—	++‡
2	ND	ND	ND	—	—	—	—	—	++‡	—	—	—
3	—	—	++‡	—	—	—	—	—	—	—	—	—
4	ND	ND	ND	—	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—	—	—	—	—
8	—	—	—	—	—	—	ND	ND	ND	ND	ND	ND

ND, not determined.

*Week 0, samples taken immediately before start of doxycycline treatment.

†PCR positive for plasmid, MOMP and rRNA target.

‡PCR positive only for plasmid target.

specific.² It is hoped that PCR may overcome these shortcomings. Several authors have described a PCR method for the detection of *C. trachomatis* with primers against the MOMP gene,^{5, 9, 11, 12, 15–17} the one universally present (with one exception²¹) and highly conserved endogenous plasmid^{6, 8, 10, 13, 17} and the 16S rRNA gene.^{7, 8, 14} It is clearly important to know whether these three PCR methods differ in sensitivity. Therefore, we compared them for the detection of *C. trachomatis* by performing PCR with primers derived from these three targets, with regard to their sensitivity, and relationship to culture method. Primers were chosen that produce relatively short fragments which are amplified efficiently by PCR performed directly on clinical samples and on pre-treated paraffin-embedded tissues.

The results of PCR with DNA isolated from *C. trachomatis* serovar L2 grown on HeLa cells show that the plasmid PCR was the most sensitive. This can be explained by the presence of multiple copies of the plasmid in each chlamydia cell. Palmer *et al.*²² reported that, in serovar L2, there were 10 plasmid copies/chromosomal DNA equivalent. The relatively low sensitivity obtained with the 16S rDNA primers was unexpected, because other prokaryotic organisms, such as *Escherichia coli*, have multiple copies of the 16S rRNA gene.²³ A four-fold increase in the primer concentration did not result in an increased sensitivity of the rRNA gene-directed PCR. Therefore, a decrease in sensitivity due to annealing of primers to rRNA is less likely.

With the plasmid primers, an equivalent of about 0.1 ifu was detected in ethidium bromide-stained agarose gels. After hybridisation, a sensitivity of at least 0.01 ifu was reached (fig. 1). An explanation for the higher sensitivity of the PCR compared with culture may be that non-viable chlamydiae are present in the sample, or that chlamydiae which are in their reticulate-body state are detected by PCR and not by tissue culture, because they are not infectious. It is also possible that more than one chlamydia particle is necessary for the formation of one inclusion. Similar results in comparisons of the sensitivity of PCR with

the different primer sets and in relation to culture were obtained with serovars E and G, two of the eight serovars commonly detected in cervical smears.

Addition of excess human placental DNA (fig. 2) resulted in a slight decrease in the sensitivity of the PCR for all primers, concomitantly with the appearance of additional non-specific amplification products. Mainly, the MOMP primers gave many additional bands; however, the sensitivity obtained with these primers was only slightly decreased. This suggests that, despite the annealing to cellular sequences, the MOMP primer concentration was sufficient to provide a high sensitivity. With the plasmid and rDNA primers, almost no background was found indicating that these primers are highly specific. Under these conditions, the plasmid primers reach a sensitivity of at least 0.1 ifu (fig. 2, lane 11).

Several investigators have determined the sensitivity of the PCR in a model system. When purified elementary bodies (EBs) were used, one copy of target DNA could be detected by both a MOMP gene-directed¹² and plasmid-directed PCR.¹⁰ However, our data are based on the infectivity of *C. trachomatis* serovar L2 in HeLa cells, and are more closely related to the clinical situation. In terms of ifu, the sensitivity of the PCR applied to each of the different targets is in the same range as that found by other workers.^{7, 11, 13} When the 16S RNA gene,⁷ MOMP gene,¹¹ or plasmid-derived primers were used,¹³ sensitivities of c. 0.2, 0.25, and 0.01 ifu, respectively, were demonstrated.

Other authors have applied PCR to patient samples that were also tested by culture. Claas *et al.*⁸ found that, in a total of 156 samples, 26 were culture-positive. These 26 samples and two additional ones gave positive results in PCR with both plasmid and rDNA primers. Holland *et al.*⁹ tested 14 samples, of which two were weakly positive, five strongly positive and seven negative in culture. Forty cycles of PCR on the MOMP target were needed to give visible bands in gels with the weakly positive samples. Bobo *et al.*¹¹ cultured 104 samples and all of 46 that gave positive results were also found to be positive by PCR with MOMP primers. Østergaard *et al.*¹⁰ tested 223 clinical

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