One-day detection of PCR amplified *Chlamydia trachomatis* DNA in clinical samples: ELISA versus Southern blot hybridisation

R T J M Roymans, G Onland, B H Postma

Abstract

***Aims*—To compare ELISA and Southern blot hybridisation for the detection of PCR amplified *Chlamydia trachomatis* DNA extracted from clinical samples; to assess the value of the ELISA method in a clinical setting.**

***Methods*—DNA was extracted from urogenital samples of 508 patients, purified and amplified using *C trachomatis* specific primers, one of which was end-labelled with biotin. Amplification products were detected by Streptavidin biotin based ELISA and non-radioactive Southern blotting.**

***Results*—Of the 508 samples, 29 were positive and 479 negative by both methods. No discrepant results were observed.**

***Conclusion*—Streptavidin biotin based ELISA and Southern blotting were equally sensitive for detecting PCR amplified *C trachomatis* DNA. Using ELISA, test results could be generated within a single day.**

(*J Clin Pathol* 1996;49:581–583)

Keywords: *Chlamydia trachomatis*, PCR, ELISA, Southern blotting.

Chlamydia trachomatis is a major cause of sexually transmitted disease worldwide.[1 2] In women infection is frequently asymptomatic or may be associated with urethritis, cervicitis and proctitis. Because untreated infections may progress to endometritis, salpingitis and infertility,[3] early and rapid detection of these infections is essential. Cell culture is labour intensive and depends on the presence of live organisms, which limits time and conditions of transport. As a result, cell culture has largely been replaced by antigen detection by hybridisation with specific antibody and more recently by DNA amplification based assays such as PCR or the ligase chain reaction (LCR).

In an earlier study a non-isotopic hybridisation method for laboratory diagnosis of *C trachomatis* (Gen Probe) was compared with PCR amplification of plasmid DNA. Although the latter method was more sensitive and specific, unlike Gen Probe, PCR results could not be obtained within a single working day. As rapid turnaround is advantageous to both patient and clinician, a detection method permitting the production of same-day results (PCR ELISA detection kit, Boehringer Mannheim, Mannheim, Germany) was compared with non-radioactive Southern blot analysis. Our aims were to evaluate the sensitivity of the ELISA method compared with Southern blotting for the detection of PCR amplified *C trachomatis* DNA in patient samples and to assess its usefulness in a clinical setting.

Laboratory of Medical Microbiology, PO Box 2, 5500 AA Veldhoven, The Netherlands

Correspondence to:
Dr R T J M Roymans.

Accepted for publication
21 March 1996

Methods

SAMPLE COLLECTION AND TREATMENT

Urogenital samples were taken from 508 patients, visiting obstetrics/gynaecology and dermatology clinics of five local hospitals and a low threshold clinic (that is, no prior appointment required) for sexually transmitted diseases, in January and February 1995.

Samples were collected with dacron swabs and placed in Gen Probe transport medium. Upon arrival in the laboratory two drops of Fast Express (to decrease viscosity) were added and the samples were incubated in a water bath at 56°C for 10 minutes. Before removal, the swabs were pressed against the wall of the tube. Then 0.1 ml of sample was taken and stored at 4°C pending analysis.

ISOLATION AND PURIFICATION OF DNA

DNA extraction was preformed as described by Boom *et al.*[4] DNA was adsorbed onto purified silica particles (Celite, Janssen Chimica, Tilburg, The Netherlands). The Celite particles were washed three times with ethanol and once with acetone. DNA was released by addition of 0.1 ml TE buffer (10 mM Tris-HCl, pH 8.0) and incubated for 15 minutes at 56°C, during which the suspension was vortexed for a few seconds at five minute intervals. The Celite particles were sedimented by centrifugation for two minutes at 20 800 × *g* and the supernatant used as template. *C trachomatis* infected Hep-2 cells were used as a control.

POLYMERASE CHAIN REACTION

PCR was performed as described by Claas *et al.*[5 6] The primer set and probe (Isogen, Leiden, The Netherlands; table 1) were derived from sequences of the common endogenous plasmid of *C trachomatis*.[5 7]

Amplification of target DNA was performed in a 0.1 ml volume containing 10 µl 10× PCR solution (100 mM Tris-HCl, pH 9.0; 500 mM KCl; 25 mM $MgCl_2$; 0.1% gelatine; 1% Triton X-100 (all from Merck, Darmstadt, Germany); 20 µl 5× dNTP mix (1 mM each of dATP, dCTP, dGTP, and dUTP (Boehringer Mannheim)); 1 µl of each primer (500 µg/ml); 1 µl DNA polymerase (Sphaero-Q), final concentration 0.25 units per reaction mixture; 58 µl

Table 1 Sequences of oligonucleotides used as primers and probes

	Oligonucleotide sequence
Primer 1	5'-GGACAAATCGTATCTCGG-3'
Primer 2 (5'-biotin)	5'-GAAACCAACTCTACGCTG-3'
Probe (5'-digoxigenin)*	5'-CGCAGCGCTAGAGGCCGGTCTATTTATGAT-3'
Probe (3'-digoxigenin)*	5'-CGCAGCGCTAGAGGCCGGTCTATTTATGAT-3'

*Patent of Boehringer Mannheim, Germany.

aquadest (Brand). The reaction mixture, 90 µl, was pipetted into an Eppendorf tube and 10 µl purified sample DNA was added as well as two drops of glycerol (Merck) to prevent evaporation. Amplification was performed in a PCR processor (Biomed, Ditfurth, Germany) under the following conditions: 60 seconds at 94°C, 60 seconds at 42°C and 60 seconds at 72°C for 35 cycles.

PCR reactions were carried out using appropriate positive and negative controls. To prevent false positive results sterile procedures and guidelines for avoiding contamination were followed throughout. Reagents for sample processing and PCR were prepared in separate rooms.

ELISA DETECTION OF PCR PRODUCTS

All washing steps were performed with prewarmed buffers. Twenty microlitres of amplified product were diluted in 200 µl 1× SSC buffer with 0.5% Tween 20. Streptavidin coated wells were washed once, tapped dry and incubated with 200 µl of the diluted product for 30 minutes at 37°C.

Wells were washed four times, tapped dry and incubated for 10 minutes at room temperature with 200 µl 0.1 M NaOH, washed again and incubated for one hour at 37°C with 200 µl diluted 5'-digoxigenin labelled probe (final concentration 10 pmol/ml hybridisation buffer). Before diluting, the probe was incubated for three minutes at 96°C and put on ice. Wells were washed four times, tapped dry and incubated with 200 µl conjugate solution (anti-digoxigenin peroxidase) for 30 minutes at 37°C, washed again four times, tapped dry and incubated with 200 µl ABTS substrate (2,2 azino-di-[3-ethylbenzthiazoline sulphonate]) for 30 minutes at 37°C in the dark. After 15 and 30 minutes' incubation the wells were read in an Anthos ht II reader (Anthos Labtec Instruments, Salzburg, Austria) at 405 nm (reference filter 492 nm) The absorbency of a reagent blank (well A1, incubated with buffer and substrate only) was subtracted from the reading of each test sample.

SOUTHERN BLOT DETECTION OF PCR PRODUCTS

PCR products were analysed by electrophoresis in a 2% agarose gel (Sphaero-Q, 2 g agarose/100 ml TBE buffer (0.05 M Tris-HCl, 0.05 M boric acid, 1 mM EDTA)) at 100 mA for 30 minutes and stained with 75 µl ethidium bromide (1 mg/ml). The banding pattern generated was transferred to a nylon-Z-membrane (Hybond; Bio-Rad, Veenendaal, The Netherlands) in a Bio-Rad semi-dry transblot at 3.5 mA/cm^2 and maximally 25 V for 30 minutes. Membranes were dried after incubation for 10 minutes in 0.4 M NaOH, prehybridised for 60 minutes at 68°C in a Hybaid Micro-4 hybridisation oven (Hybaid Ltd, Middlesex, UK) in 10 ml hybridisation solution, containing 0.75 M NaCl, 0.075 sodium citrate, pH 7.0, 0.05 M maleic acid, 0.15 M NaCl, pH 7.5, 1% blocking agent (Bio-Rad), 0.02% SDS (Bio-Rad), 0.1% N-lauroylsarcosine (Bio-Rad), sealed in plastic bags and hybridised for 16 hours at 54°C in hybridisation solution with the 3'-digoxigenin labelled probe added (final probe concentration 10 pmol/ml). Before diluting, the probe was incubated for three minutes at 96°C and put on ice. The probe was labelled with digoxigenin using the 3'-α tailing kit from Boehringer Mannheim, according to the manufacturer's instructions. The following day, membranes were washed twice in washing solution 1 (2× SSC buffer, 0.1% SDS) and twice in washing solution 2 (0.1× SSC buffer, 0.1% SDS), each time for five minutes at 54°C in a 20 ml volume. Membranes were then incubated for 60 minutes at 37°C with anti-digoxigenin alkaline phosphatase (Boehringer Mannheim), washed three times for five minutes at 37°C with 10 ml washing solution 2 and once for five minutes at 37°C with 10 ml substrate buffer containing 0.1 M Tris-HCl, 0.1 M NaCl, 0.05 M $MgCl_2$, pH 9.5, and incubated with substrate solution (10 ml/50 cm^2 membrane) consisting of 9.920 ml substrate buffer, 45 µl NBT (100 mg nitroblue tetrazolium (Sigma) in 1.33 ml of a 70% solution of dimethylformamide (Merck) in aquadest), and 35 µl BCIP (100 mg 5-bromo-4-chloro-3-indolylphosphate (Sigma) in 2 ml dimethylformamide). When clearly defined bands appeared, the reaction was stopped by removing the substrate solution and washing the membranes in aquadest. Results were read visually and regarded as positive when there was a band at the same position as the positive control.

Results

Of the 508 samples, 29 were positive and 479 negative for *C trachomatis* DNA by ELISA and Southern blotting. No discrepant results were observed.

The mean extinction of the negative samples was 0.067 OD and an extinction of 0.150 OD was chosen as a cut off and samples with an extinction <0.150 OD were considered negative. All samples with an original extinction of ⩾0.150 OD had an extinction of ⩾0.150 OD on retesting and were duly considered positive. The mean extinction of the positive samples was 1.537 OD with a minimum of 0.192 OD and a maximum of 2.361 OD.

In this group of patients a prevalence of *C trachomatis* infection of 5.7% was recorded by both methods.

Discussion

Reliable same-day results in laboratory diagnosis of urogenital infections with *C trachomatis* are important to both patient and clinician, especially in the setting of a low threshold clinic. In this study we compared the sensitivity of an ELISA based one-day detection method for PCR amplified *C trachomatis* DNA with non-isotopic Southern blotting. Both methods

had equal sensitivity, no discrepant results were observed and the ELISA proved to be a rapid and reliable detection method.

Before actually starting our study, a panel of known positive and negative samples from an earlier study was used to optimise the ELISA detection method. A maximum difference between mean OD values of positive and negative samples was observed when the probe was incubated for one hour at 37°C in a concentration of 10 pmol/ml. Incubation time and concentration of conjugate were as recommended by the manufacturer. An initially occurring rather strong background colour could be eliminated when SSC buffer was replaced by incubation buffer such as used in Southern blotting.

A 3'-digoxigenin labelled probe was used in Southern blot hybridisation. As labelling was done enzymatically, the number of digoxigenin molecules per molecule of probe may vary but as a rule at least three digoxigenin molecules are bound. This has no consequences for probe attachment and may even be of advantage in Southern blotting, giving more intensely coloured bands. To get constant and reproducible results in ELISA the number of digoxigenin molecules attached to the probe should be constant. The chemically labelled probe provided by the manufacturer meets this requirement as one molecule of digoxigenin is attached consistently to each molecule of probe.

In this study we recorded a prevalence of *C trachomatis* infection of 5.7%. In an earlier study this was 10.5%. The latter prevalence was recorded for a series of 507 patients from the same clinics; there were no differences in male:female ratios or age distributions in each group. In the earlier study samples were mainly taken in August and September; in the present study in January and February, but whether the observed difference in prevalence is accidental or related to the period of sampling is unknown at present.

Genitourinary infection with *C trachomatis* is especially problematic in women because although it may pass unnoticed initially, its late sequelae can be very serious. Prevention of spread from male to female and rapid treatment can reduce this risk substantially. A rapid and reliable laboratory diagnosis is needed for this purpose. As same-day test results are of advantage in the setting of a low threshold clinic, patients at risk of *C trachomatis* infection visiting these clinics should benefit most.

PCR followed by ELISA detection can be automated easily. The possibility of including other causative agents of sexually transmitted diseases in this detection system is currently under investigation in our laboratory.

We conclude that ELISA detection of PCR amplified *C trachomatis* DNA in clinical samples can provide reliable test results on the day of sampling and may contribute substantially to rapid diagnosis in high risk groups.

The authors thank Dr W Quint, Diagnostic Centre, SSDZ, Delft, for help with the manuscript.

1 Centre for Disease Control. Chlamydia trachomatis infection. Policy guidelines for prevention and control. *MMWR* 1985;**35**:535–74.

2 Smith JW, Rogers RE, Katz BP, Brickler JF, Lineback PL, Van der Pol B, *et al.* Diagnosis of chlamydial infection in women attending antenatal and gynaecologic clinics. *J Clin Microbiol* 1987;**25**:868–72.

3 Brunham RC, Maclean IW, Binns B, Peeling R. Chlamydia trachomatis: its role in tubal infertility. *J Infect Dis* 1985;**152**:1275–82.

4 Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME, Van der Noordaa J. Rapid and simple method for purification of nucleic acid. *J Clin Microbiol* 1990;**28**:495–503.

5 Claas HCJ, Melchers WJG, Niesters HGM, Van Muyden R, Stolz E, Quint WGV. Infections of the cervix uteri with human papillomavirus and Chlamydia trachomatis. *J Med Virol* 1992;**37**:54–7.

6 Claas HCJ, Wagenvoort JHT, Niesters HGM, Tio TT, Rijsoort-Vos JH, Quint WGV. Diagnostic value of the polymerase chain reaction for chlamydia detection as determined in a follow-up study. *J Clin Microbiol* 1991;**29**:42–5.

7 Sriprakash KS, Macavoy ES. Characterization and sequence of a plasmid from the trachoma biovar of Chlamydia trachomatis. *Plasmid* 1987;**18**:205–14.