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Solid-Phase C1q-Directed Bacterial Capture Followed by PCR for Detection of *Chlamydia trachomatis* in Clinical Specimens

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An antigen capture system based on the binding of bacteria to solid-phase immobilized complement C1q followed by PCR for detection of *Chlamydia trachomatis* in clinical samples was developed and clinically evaluated. Comparison of C1q-directed antigen capture PCR with cell culture and direct PCR on 71 consecutive clinical specimens revealed an identical sensitivity. In this group, all 11 cell culture-positive samples were positive by direct PCR and C1q-directed antigen capture PCR. In addition, two samples found negative by cell culture were found positive by both direct PCR and C1q-directed antigen capture PCR. To further assess the sensitivity of C1q-directed antigen capture PCR, 20 clinical samples with one to five inclusions in cell culture and 20 clinical samples with 6 to 20 inclusions in cell culture were tested. Results obtained showed sensitivities of 95 and 90% for clinical samples with 6 to 20 and 1 to 5 inclusions in cell culture, respectively. Using C1q-coated solid phases, C1q-binding *Chlamydia* particles can be concentrated from large volumes with concomitant removal of inhibitors of PCR, allowing the use of large volumes of clinical samples for clinical testing. Since C1q has been shown to bind to a range of gram-negative bacteria, the newly developed technique has utility for a broad range of bacteria.

Conventional laboratory techniques for the detection of pathogenic microorganisms are often laborious and time-consuming. In many cases, diagnosis can be accelerated by using short culture techniques, followed by identification with specific monoclonal antibodies or DNA-RNA probes (10, 23). Detection assays such as immunofluorescence, enzyme immunoassays, and DNA hybridization techniques directly on the clinical specimen have been shown to be highly specific. However, sensitivity is often low compared with culture techniques (17, 24, 28). PCR has led to the development of assays with high sensitivity and specificity for the detection of RNA or DNA sequences (19, 21, 22).

Analysis of crude extracts from clinical specimens is hampered by the limited sample volume that can be tested in PCR and the presence of inhibitors of the PCR assay (8, 27, 29, 30). To overcome these problems, assays that use solid-phase-coated, microbe-specific antibodies for concentration and purification of microbial particles from crude clinical specimens have been developed (11, 14, 20, 29, 31). In these assays, intact microorganisms are captured on a solid phase coated with antibodies specific for microbial membrane antigens. With such methodology, microorganisms present in large volumes of clinical specimens can be concentrated. In addition, inhibitors of the PCR are removed by a washing step.

Although antibody-directed antigen capture can be used for concentration of microorganisms from crude samples, each individual assay requires the coating of microbe-specific antibodies to the solid phase. A more universal capture system allowing the capture of different microorganisms would simplify the recovery from clinical specimens and reduce the workload. Microbial detection could then be performed by PCR, using microbe-specific primers.

It has been shown for some time that the classical complement pathway is important in the killing of gram-negative bacteria such as *Klebsiella* sp., *Escherichia coli*, *Shigella* sp., *Salmonella* sp. and *Mycoplasma* sp. (6). Serum-sensitive bacteria can bind high amounts of C1q from serum, independently of the presence of bacterium-specific antibodies. Direct binding of C1q to serum-sensitive bacteria has been reported by several laboratories (1, 5, 16, 26). It was shown that lipopolysaccharides and porins potentiate the binding of C1q in the absence of specific antibody (4, 15).

Although no reports concerning the direct binding of C1q to *Chlamydia trachomatis* have been published, it has been shown that chlamydiae can activate the complement cascade in fresh serum in vitro (7, 18).

Considering the C1q binding properties of gram-negative bacteria reported in the literature, we decided to evaluate the efficacy of solid-phase immobilized C1q for concentration and purification of C1q-binding bacteria. As a model system, *C. trachomatis* was used.

MATERIALS AND METHODS

Clinical specimens. Clinical specimens used in this study were samples submitted routinely to the microbiology laboratory for detection of *C. trachomatis*. The samples were taken with an ear, nose, and throat swab (Boehringer, Mannheim, Germany) and placed in 2 ml of transport medium (0.2 M sucrose in phosphate buffer). The specimens for chlamydia culture were stored at 4°C or, when not tested within 24 h after collection, –70°C. All specimens were processed within 7 days. The remainder of the samples was stored at –70°C until further testing.

Cell culture. *C. trachomatis* was cultured in cycloheximide-treated McCoy cells, grown in 96-well microtiter plates as described by Thewissen et al. (27). Briefly, two wells per plate were each inoculated with 0.2 ml of patient sample. After centrifugation for 60 min at 1,400 × g, the supernatant was replaced with 0.1 ml of complete growth medium (Eagle minimal essential medium; Flow) containing 10% fetal calf serum, 1% vitamins (Flow), 5 µg of gentamicin per ml, 1% 200 mM L-glutamine (Flow), 5 µg of amphotericin B per ml, 10 ml of 7.5% NaHCO₃ per liter, 25 µg of vancomycin per ml, 4.5 g of glucose per liter, and 0.5 µg of cycloheximide (Sigma) per ml. The plates were incubated at 37°C for 48 h. Thereafter, the monolayers were fixed, stained with a fluorescent monoclonal

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anti-*Chlamydia* antibody (MicroTrak), and examined for inclusions. Culture results were scored as described by Kluytmans et al. (12): 0, no inclusions per two wells; 1, 1 to 5 inclusions per two wells; 2, 6 to 20 inclusions per two wells; and 3, >20 inclusions per two wells.

Isolation of human C1q. Human C1q was isolated as described by Tenner et al. (25). Briefly, fresh human serum was adjusted to 5 mM EDTA and applied to a Biorex 70 column, equilibrated with starting buffer (82 mM NaCl, 2 mM EDTA, 50 mM sodium phosphate [pH 7.2]). After being washed with 1,000 ml of starting buffer, the column was eluted with an ionic-strength gradient composed of 600 ml of starting buffer and 600 ml of buffer (pH 7.2) containing 300 mM NaCl, 2 mM EDTA, and 50 mM sodium phosphate. Fractions containing C1q, as measured by immunodiffusion, were pooled and concentrated by 33% saturated ammonium sulfate precipitation. The precipitate was dissolved in 50 mM Tris-HCl (pH 7.2) containing 500 mM NaCl-1 mM EDTA and applied to a Bio-Gel A5m gel filtration column equilibrated with the same buffer. C1q-containing fractions were pooled and concentrated by 33% ammonium sulfate precipitation. The pellet was dissolved in 5 ml of 0.002 M NH_4CO_3 and dialyzed extensively against the same buffer. Following lyophilization, purified C1q was stored at -20°C . Purity was determined by sodium dodecyl sulfate (SDS)-gel electrophoresis.

C1q coating of magnetic beads. Coating of magnetic beads with C1q was performed according to the manufacturer's instructions. Tosyl-activated Dynabeads (500 μl) (6×10^8 to 7×10^8 beads per ml; M-280, Dynal AS, Oslo, Norway) were pelleted by placing the tube in the powerful magnetic field of a magnetic particle concentrator (Dynal MPC-6). Following removal of storage buffer, the beads were washed once with 1 ml of coating buffer (0.05 M borate, pH 9.5). After a final concentration, 250 μl of coating buffer was added, the beads were suspended, and 250 μl of human C1q (0.4 mg/ml of coating buffer) was added. Coupling of C1q was performed by gentle rotation for 24 h at 37°C . Thereafter, the beads were washed three times with phosphate-buffered saline (PBS; pH 7.2) containing 0.1% bovine serum albumin (BSA). Following an overnight wash at 4°C with the same buffer, the beads were suspended in 0.5 ml of PBS-0.1% BSA and stored at 4°C .

Antigen capture. For each assay, 10 μl of C1q-coated bead suspension was transferred to a 1.5-ml tube. All buffers were sterilized by filtration, using a 0.22- μm -pore-size filtration device. Thereafter, 940 μl of capture buffer (0.1 M Tris-HCl [pH 8.0], 20 mM EDTA, 20 mM dithiothreitol, 0.5% Triton X-100) and 50 μl of clinical sample were added. For samples with low inclusion numbers in cell culture, 740 μl of capture buffer and 250 μl of clinical sample were used. The suspension was incubated for 1.5 h at 37°C with gentle rotation. The beads were washed twice with PBS containing 0.5% Triton X-100 as described above.

PCR. (i) Proteinase K treatment of beads. After magnetic concentration, 50 μl of lysis buffer (50 μg of proteinase K per ml, 0.75% Triton X-100) was added. The beads were incubated for 60 min at 37°C , and the proteinase K was inactivated by a 15-min incubation at 100°C . The sample was cooled to room temperature for 5 min in air. Ten microliters was used for PCR. From samples with low inclusion numbers, 50 μl was used for PCR.

(ii) Amplification and detection of chlamydial DNA. PCR was used for the amplification of *C. trachomatis* target DNA (22). The primer sequences were selected from the common endogenous plasmid of *C. trachomatis* (2, 3) which generates a species-specific fragment of 517 bp with all known *C. trachomatis* serovars (P1, 5' GGACAAATCGTATCTCGG 3'; P2, 5' GAAACCAACTACGCTG 3'). This PCR product was positively identified by using an internal labelled oligonucleotide probe (5' CGCAGCGCTAGAGCCGGTCTATTATGAT 3'). The primers and probes were synthesized on an Applied Biosystems 381A DNA synthesizer, using the β -cyanoethyl phosphoramidite method. Specificity was determined as described before (2).

A spatial separation of the different steps of the technique was routinely used to prevent contamination of the samples.

(a) DNA amplification. The reaction was performed in a volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl_2 , 0.01% gelatin, 200 μM (each) deoxynucleoside triphosphates, 50 pmol of both primers, and 0.25 U of *Taq* DNA polymerase (Sphero Q). Ten microliters or 50 μl for samples with low inclusion numbers, of sample was routinely used. The reaction mixture was overlaid with 3 drops of mineral oil (Sigma M-3516) and subjected to 40 cycles of amplification in a thermocycler (Biomed, Dittfurth, Germany). Each cycle consisted of a 1-min DNA denaturation step at 94°C , a 1-min primer annealing step at 42°C , and a 1-min elongation step at 74°C . As a positive control for the PCR reaction, 1 ng of DNA from *C. trachomatis*-infected cells was used. Water was used as a negative control.

(b) Analysis of PCR product. The clinical specimens were analyzed by Southern blot hybridization. Hybridization was performed as described by Claas et al. (2, 3). Briefly, prehybridization was performed at 37°C for 15 min in a solution containing $5\times$ SSC (75 mM sodium citrate, 750 mM NaCl), $5\times$ Denhardt solution (0.1% BSA, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% SDS, 5 mM EDTA, and 0.1 mg of denatured, sonicated herring sperm DNA per ml. Hybridization was performed in the same mixture by adding the probe to the prehybridization mixture. Hybridization was routinely done for 16 h. After hybridization, the blots were washed twice for 15 min each time at 42°C in $2\times$ SSC containing 0.1% SDS. Autoradiography was performed for 4 h on a Kodak Royal X-Omat film, using two intensifying screens at -70°C .

TABLE 1. Comparison of cell culture, direct PCR, and C1q-directed antigen capture PCR for detection of *C. trachomatis* in 71 consecutive clinical samples

Test and result	Cell culture result (no. of samples)	
	Positive	Negative
Direct PCR		
Positive	11	2
Negative	0	58
C1q capture		
Positive	11	2
Negative	0	58

Direct PCR on clinical specimens. For direct PCR, 800 μl of sample was centrifuged at room temperature for 30 min at $14,000 \times g$. The supernatant was removed and the pellet was resuspended in 300 μl of PBS by vortexing. To 60 μl of the resuspended sample, 10 μl of 6% Triton X-100 and 10 μl of proteinase K (6.25 $\mu\text{g}/\text{ml}$) was added. After being vortexed briefly, the sample was incubated for 1 h at 37°C . The proteinase K was inactivated by a 15-min incubation at 100°C . After addition of 100 μl of distilled water to the sample, 10 μl , corresponding to 9 μl of the initial crude clinical sample, was used in the PCR. For samples with low inclusion numbers in cell culture, 400 μl of clinical sample was centrifuged at room temperature for 30 min at $14,000 \times g$. The pellet was resuspended in 40 μl of lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 1 mM EDTA, 400 μg of proteinase K per ml). For samples with low inclusion numbers in cell culture, 8 μl , corresponding to 80 μl of the initial crude clinical sample, was used for PCR.

RESULTS

To compare the sensitivities of C1q-directed antigen capture PCR, direct PCR, and cell culture for detection of chlamydiae, serial dilutions of a freshly prepared suspension of *C. trachomatis* LGV-2 were tested. The sensitivities of direct PCR and C1q-directed antigen capture PCR were identical to that of cell culture. In addition, the sensitivity of C1q-directed antigen capture PCR in relation to direct PCR was determined, using 34 patient specimens positive by direct PCR. All samples positive by direct PCR were also positive by C1q-directed antigen capture PCR.

To compare the sensitivity of C1q-directed antigen capture PCR with those of direct PCR and cell culture for clinical testing, 71 consecutive clinical specimens were tested. The results are shown in Table 1. Of these 71 clinical specimens, 11 samples were positive by cell culture, direct PCR, and C1q-directed antigen capture PCR. Of the 11 specimens with a positive cell culture, 9 showed a 3+ score (more than 20 inclusions) and 2 showed a 2+ score (6 to 20 inclusions). By direct PCR and C1q-directed antigen capture PCR, an additional two clinical specimens were positive. None of the samples negative by direct PCR or C1q-directed antigen capture PCR was found positive by cell culture.

Since the 11 culture-positive samples all showed a 2+ or 3+ score in cell culture, we further assessed the sensitivity of C1q-directed antigen capture PCR by testing 20 clinical specimens with a 1+ score (1 to 5 inclusions per two wells) and 20 clinical specimens with a 2+ score (6 to 20 inclusions per two wells) in cell culture. For optimal comparison with cell culture, a comparable amount (250 μl) of initial clinical specimen was used for C1q-directed antigen capture PCR. For direct PCR, 80 μl of initial clinical specimen was used. The results are shown in Table 2. Of 20 clinical samples yielding 6 to 20 inclusions in cell culture, 19 were found positive by C1q-directed antigen capture PCR, whereas 18 of 20 clinical samples were positive by direct PCR. For the clinical samples yielding one to five inclusions in cell culture, 18 of 20 were positive by C1q-directed antigen capture PCR, whereas direct PCR

TABLE 2. Comparison of cell culture, direct PCR, and C1q-directed antigen capture PCR for detection of *C. trachomatis* in 40 clinical samples with low inclusion numbers

Inclusion no. in culture	No. of samples			
	C1q capture		Direct PCR	
	Positive	Negative	Positive	Negative
1-5	18	2	15	5
6-20	19	1	18	2

yielded a positive result for 15 of the 20 clinical specimens. Although C1q-directed antigen capture PCR of clinical specimens with one to five inclusions in cell culture was more sensitive than direct PCR, the observed difference was statistically not significant ($P = 0.053$; chi-square test).

DISCUSSION

PCR (22) has found wide application for the detection of microbial target sequences in clinical specimens. Provided that proper precautions are taken to avoid contamination, PCR shows a high specificity and sensitivity. Strict separation of the different technical steps in different locations is essential to avoid contamination (13). One of the laborious steps in the analysis of clinical samples by PCR is sample preparation.

The process of sample preparation remains quite laborious, often requiring a DNA-RNA extraction step. Liang et al. (14) and Zeldis et al. (31) described the use of solid phases coated with hepatitis B-specific antibodies for capturing the virus from human serum, thus avoiding the need for nucleic acid extraction. Widjoatmodjo et al. (29) used magnetic beads coated with *Salmonella*-specific antibodies to concentrate and purify *Salmonella* bacteria from fecal samples. In addition, Niesters et al. (20) and Hedrun et al. (11) described a specific antibody-directed antigen capture followed by PCR based on the specific capture of chlamydial particles by solid phase coated with chlamydia-specific antibodies.

Since many gram-negative bacteria are able to bind complement C1q in the absence of specific antibodies, we were interested in developing a system capable of capturing a broad range of bacteria. Preliminary results have shown that a number of gram-negative bacteria (*Neisseria gonorrhoeae*, *Campylobacter jejuni*, *Salmonella typhimurium* D, *E. coli*) bind to C1q-coated beads (unpublished data).

To evaluate the efficacy of solid-phase-immobilized C1q for concentration and purification of C1q binding bacteria in a clinical setting, *C. trachomatis* was used as a model system.

An essential point in the procedure is the use of detergent-free microbial transport medium. Experiments have shown that addition of a detergent such as deoxycholate is deleterious to test results, probably due to capture of membrane-released chlamydial antigens instead of chlamydial particles containing the microbial genome (unpublished results).

By using serial dilutions of *C. trachomatis* LGV-2 and 34 well-defined clinical samples, C1q-directed antigen capture PCR revealed a sensitivity identical to that of direct PCR and cell culture. Comparison of cell culture, direct PCR, and C1q-directed antigen capture PCR in 71 consecutive clinical samples revealed 2 clinical samples negative by cell culture and positive by C1q-directed antigen capture PCR and direct PCR. These two additional positive samples probably reflect the presence of noninfectious chlamydial particles. Alternatively, the discrepancy between cell culture and C1q-directed antigen

capture might be explained by sampling error, due to low numbers of chlamydia particles in the sample.

Since the positive samples mainly consisted of samples with high inclusion numbers in cell culture, we decided to analyze the sensitivity of both direct PCR and C1q-directed antigen capture PCR with larger volumes of clinical specimens (80 and 250 μ l, respectively) with low inclusion numbers in cell culture. In this setting, sensitivities for C1q-directed antigen capture PCR of 90 and 95% were obtained for samples containing 1 to 5 and 6 to 20 inclusions, respectively, in cell culture, whereas direct PCR revealed sensitivities of 75 and 90%, respectively. Of the 40 clinical samples with low inclusion numbers, only 3 were negative by C1q-directed antigen capture PCR, probably due to sampling errors. This is further supported by the fact that the two samples with one to five inclusions in cell culture, which were negative in C1q-directed antigen capture PCR, were only positive in one of the two wells that had been inoculated in cell culture.

An important feature of C1q-directed antigen capture is its utility toward a broad range of bacteria. Following a single concentration step using C1q-coated solid phases, the nature of the captured bacteria can be determined by PCR with bacterium-specific primers.

Like antibody capture-directed antigen capture systems, C1q-directed antigen capture allows the concentration of microorganisms from large volumes of clinical specimens with concomitant removal of inhibitors of PCR. Several studies have shown that, by immunomagnetic concentration, inhibitors of PCR can be effectively removed from complex biological fluids such as blood (14, 31), urine (11), and feces (29).

In conclusion, using C1q-coated solid phases, C1q-binding *Chlamydia* particles can be concentrated from large volumes with concomitant removal of inhibitors of PCR, allowing the use of large volumes of clinical samples for clinical testing. Since C1q has been shown to bind to a range of gram-negative bacteria, the newly developed technique has utility toward a broad range of bacteria.

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