Chlamydia DNA Extraction for Use in PCR: Stability and Sensitivity in Detection

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We evaluated multiple procedures for extracting chlamydial DNA from specimens for detection in PCR tests. Commercial kits and an in-house method were tested for their sensitivity and utility. Quantifiable chlamydial elementary bodies (EB) were used for spiking buffy coats from EDTA-collected blood. EBs of *Chlamydia pneumoniae* at 2,500 and 25 EB/ml were used as specimens for DNA extraction using seven different procedures. These included either columns (3 procedures), centrifugation (1), glass (1), or patented extraction matrices (2), coupled with either alcohol precipitation (6) or heat-detergent treatment (1). Five procedures required 10–40 minutes manipulation; two required 2–5 hours. PCR results for DNA extracts using chlamydial 16S genus primers were gen-

erally more intensely positive with denser bands on electrophoresis gels for the higher concentrations of EB (up to 4+ for stained product on gels) than was PCR with lower EB concentrations (up to 2+). Further, the incidence of procedures with positive results was: 5 of 7 for chlamydial genus primers with 5 EB vs. 6 of 7 with 500 EB. Maximal sensitivity for one of the extractions was in the range of 2.5–5.0 EB/ml of test specimen with 4 of 5 replicates being positive with EB controls or extracts. Extracts were stable up to 2+ weeks at 4°C and were effective in multiplexing with fluorescent-tagged primers. Taking into consideration the time factor and sensitivities, the two procedures with extraction matrices are favored for routine laboratory use. J. Clin. Lab. Anal. 12:47–53, 1998. © 1998 Wiley-Liss, Inc

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INTRODUCTION

There are three major species of *Chlamydia*: *C. trachomatis*, *C. psittaci*, and *C. pneumoniae*. Most adult have serological evidence of past Chlamydial infection (1). The seroprevalence may be as high as 60% in some clinics where highest rates exist (2). *Chlamydial pneumoniae* alone is thought to be responsible for 6–10% of cases of community-acquired pneumonia (1), or in combination (3) with other respiratory pathogens. Even then, almost half of the cases go undiagnosed as to etiology (4), which may implicate even higher incidence rates of infections. More recent focus has been on the cardiovascular pathology (5) and arthritis (6,7) with deposition and persistence of live organisms in the latter (8).

High prevalence emphasizes the importance of detection of Chlamydial organisms. The complement fixation assay (CF) (1) has heretofore been the accepted and most standardized means for testing for infection. Its lack of sensitivity and inability to distinguish species of infection has led to the more recent applications of microimmunofluorescence (MIF) technique with enhanced, although still lacking, specificity (9). MIF demonstrates only 63% maximum specificity in detection (10). Other methods include the enzyme-linked

immunosorbent detection of single-cell antibody-producing antigen-specific lymphocytes (ELISPOT) (11), which may be advantageous for early detection but requires yet more standardization of its many variables.

Problematic serological techniques have pointed to the need for molecular biological assays sensitive enough for detecting the infective organism at minimal concentrations. Hybridization probes have been used in the past similar to ones used for HIV detection by in situ methods (12). Chlamydial detection has been possible by PCR after hybridization (13). There has been evidence of a lack of homology for genomic DNA for *C. psittaci* and *C. Trachomatis* (14). Thus distinguishing differences must exist such that restriction fragment length polymorphisms (RFLPs) of mRNA genes have provided suitable genetic markers for differentiation of Chlamy-

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dial species (15). It has been proven the *C. pneumoniae* has little 16s ribosomal DNA homology with *C. trachomatis* or *C. psittaci*, but is more closely related to the latter (16). Amplification by polymerase (PCR) and ligase chain reaction (LCR) have been used successfully for detecting *C. trachomatis* (17). The two-step PCR with restriction endonuclease analyses can differentiate the groups of *C. sp.* (18).

The emphasis of detection needed approaches the range of a single one to a few or fractional part of the infectious organisms. This task has led us to focus on DNA extraction procedures and delineation of usable PCR primers. Efficient Chlamydial elementary body (EB) production (19) and enumeration (20) are essential for ascertaining the level of sensitivity for detection of the infectious organisms. We have used Chlamydial organisms (EBs) as unextracted and extracted DNA standards for comparison in sensitivity determinations and stability evaluations. The unextracted EBs, although quantitated as infectious particles (IFU=inclusion forming units) most likely include some degradative particles or incomplete EBs. These incomplete EBs, although noninfectious, may be represented in PCR testing by amplifiable DNA signals.

MATERIALS AND METHODS

Chlamydia Elementary Bodies

Production. Chlamydia were cultured on HEP2 tissue culture monolayer cells established in 6-well plates (19), and 100 µl of inoculum from -70°C storage was used in a 2 ml Heppes-Sucrose-Citrate (HSC) diluent added per well of the plate. Plates were centrifuged 1 hour at 700g to enhance cellular attachment. The supernatant was aspirated and discarded, and 2.0 ml of Chlamydial Growth Media (=MEM, with Van, Glut, Glu, Amph, Cyclohexamide) was added per well. Plates were incubated at 36°C with CO₂ for 3 days, then infected cells were harvested.

Purification. The cell harvest was sonicated 10–15 seconds, then clarified by centrifugation at 700g for 15 minutes, then pelleted at 18,000g at 4°C for 30 minutes to 1 hour. The pellet was resuspended in HSC solution (~1 ml/plate of cell growth harvested). The suspension (3–5 ml) was layered over a 20 ml cushion (13 ml HSC + 7 ml Renografin) and centrifuged 18,000g at 4°C for 30 minutes to 1 hour. This was followed by two washings by pelleting and resuspending the Chlamydial organisms. The final suspension was to the original volume in 0.01 M phosphate-buffered saline without preservative.

Target DNA

Target DNA consisted of *Chlamydia sp.* In the unextracted form (positive controls), or extracted form obtained from seeding buffy coat material obtained from EDTA-blood. Dilutions of the unextracted (primarily intact) DNA from Chlamydial EBs were made from a known concentration for use as target

TABLE 1. DNA Extraction Kits

Product	Cat. #	Manufacturer
QIAamp, Blood Kit	29104	Quiagen Inc. Chatsworth, CA 91311
ELU-QUIK, DNA Purification Kit	74450	Schleicher & Schuell, Keene, NH 03431
IsoQuick, Nucleic Acid Extraction Kit	MXT020100	Microprobe Corp., Garden Grove, CA 92641
CDC/RDL	(in house)	Center for Disease Control, Atlanta, GA 30333
DNA/RNA Isolation Kit	73750	United States Biochemical Corp., Cleveland, OH 44128
Rapid Prep Micro Genomic	27-5225-01	Pharmacia Biotech Inc., Piscataway, NJ 08854
ASAP Genomic DNA Isolation Kit	1218620	Boehringer Mannheim Corp., Indianapolis, IN 46150

specimen in the PCR assay. Buffy coats with aliquots of known EB concentrations were used in the various extraction procedures. EB concentrations in the extracted products were calculated by extrapolation from end point dilutions of inclusions from growth of chlamydial EBs in tissue culture (19). IFU were measured by counting the number of inclusions in shell vial cultures from 10-fold serial dilutions of purified EBs. Measurements were made in triplicates for a more accurate estimate of EB concentrations. IFU quantitated in this manner were used as the positive control DNA. DNA extraction procedures were done using the extraction kits given in Table 1.

Primers

Genus-specific 16s rRNA primers of the following configuration of nucleotide sequences were used:

Sense 5'-3' ACG GAA TAA TGA CTT CGG

Antisense 5'-3' TAC CTG GTA CGC TCA ATT

Primer concentration in the PCR procedure was optimized with the Opti-Prime PCR Optimization Kit (Stratagene, La Jolla, CA). These primers at a working concentration of 0.2 microles each were used in a volume of 2.5 µl per specimen in the PCR mastermix solution.

TABLE 2. Master Mix Constituents

	µl/specimen
Boehringer-Mannheim reaction buffer, 10×	5.0
Bovine serum albumin (0.1%)	5.0
Distilled demineralized water	20.75
(less for specimens >5 µl)	
dNTPs, 5.3 µM, (Sigma Chemical Co., St. Louis, MO)	8.0
MgCl ₂ (25 mM)	1.0
Primers (2.5 µl each @ 0.2 µM)	5.0
Taq DNA polymerase (5 uts/ml) (Boehringer Mannheim)	0.25
Total (pH 8.3)	45.0

Fig.1. *Chlamydia pneumoniae* (TWAR) Elementary Bodies (EBs) under fluorescent microscopy at 400×. EBs were suspended in egg yolk sac protein.

TABLE 3. DNA Extraction Procedures

Procedures	Column	Centrif	Methodology				Time required
			Glass	Matrix	Alc ppt	Heat/deterg	
QUAamp	X				X		20 min.
Elu-Quik			X		X		30 min.
IsoQuick				X	X		25 min.
CDC/RDL		X				X	40 min.
U S B				X	X		20 min.
Rapid Prep	X	X			X		1+ min.
ASAP	X				X		2–5 min.

TABLE 4. PCR Amplification Intensity^a Using Genus-specific Primers and Extracted DNA

	500 EBs	5 EBs
QIAamp (blood)	+5	+2
Elu-Quik	+3	+1
IsoQuick	+2	+1
CDC-RDL	0	(+1)
U S B	+1	+2
Rapid Prep	+0	+0
A S A P	+4	+1
Controls ^b	+5	+3

^aIntensity of product on gel electrophoresis (with a scale of +1 to +5, +5 being the highest intensity of gel bands present). Replicate reproducibility indicated in Figure 4.

^bEBs (chlamydial elementary bodies) without the extraction process.

PCR Amplification

Samples for the PCR were prepared in a class II laminar flow hood according to the following procedure. Amplification and analysis of PCR products were each performed in separate location.

Master mix solution consisted of items listed in Table 2; 45 µl of Master Mix was added to each specimen tube. The specimen (target DNA) dilution was added to the reaction tube also. After overlaying with a drop of oil, the mixture was run in an

Omnigene Thermalcycler at 95°C for 2 minutes, then 35 cycles of 1 minute at each: 94°C, 55°C, and 72°C.

Product Detection

Amplification of products were separated by electrophoresis in the following: 2.5% agarose gels (1.5% Nusieve GTG agarose, FMC Bioproducts, Rockland, ME, and 1.0% Agarose, BioRad, Richmond, CA) in Tris-borate-EDTA #15581-044, GibcoBRL, Grand Island, NY). Product (8–10 µl) was mixed with an 80% solution brom phenol tracking dye and ethidium bromide (3 µl). Visualization of product in the electrophoresed gel was with ethidium bromide fluorescence and incident UV radiation.

RESULTS

The characteristically large inclusion body within the cytoplasm of the HEP2 host cells were visualized by light microscopy. These structures develop from the infective EBs that attach, enter, grow, and become dispersed after 18–25 hours throughout the cytoplasm. The new EBs that eventually develop from the inclusion body have a diameter of 0.25–0.35 µ (22) and are visualized by fluorosecent microscopy (Fig. 1).

The seven methods used in this study for extraction of DNA had variable methodology and time factors incorporated in

PCR Results for Extracted and Intact (Control) *C. sp.* DNA

Fig. 2. PCR Results for Extracted and Intact (Control) *C. sp.* DNA.

Minimal Sensitivity of PCR for Extracted *C. sp.* DNA

Fig. 3. Minimal sensitivity of PCR for extracted *C. sp.* DNA.

their procedures (Table 3). They include either columns (3 procedures), centrifugation (2), glass (1), or patented extraction matrices (2), coupled with either alcohol precipitation (6) or heat-detergent treatment (1). Five procedures required 10–40 minutes manipulation; two required 1–5 hours.

These methods were systematically evaluated for their efficiency in extracting DNA from buffy coats that were spiked (seeded) with 200 μ l working dilution of either Chlamydial EBs at 2,500 or 25 EB/ml (=500 or 5 EBs used in the test). The PCR amplification resulted in genus products similar for all three chlamydial species. Products detected from amplification of extracted DNA were 436 bp relative to the calibrated ladder. The position of these products was indicated by the intensity of bands on gel electrophoresis (Table 4). The 500 EBs resulted in bands that ranged from 0 (for two extraction methods) up to +4 (for two extraction methods that were nearly equivalent to the unextracted EB controls=+5). Bands obtained from extraction of 5 EBs were usually not so intense and were up to +2.

PCR results for intact (unextracted controls) and extracted DNA from *C. sp.* Indicate the sensitivity of detection of the amplification products. As noted in Figure 2, PCR result are positive in both replicates for extracted and unextracted EBs of *C. pneumoniae* and for *C. psittaci*. The level of sensitivity for

C. trachomatis was more variable and less demarcated with the number of organisms (EB) at concentrations of 0.5–5.0 in the 200 μ l used in the test (=2.5–25 EBs/ml of test specimen).

Minimal sensitivity of PCR was determined from PCR assays on end-point dilutions of the DNA extracts from *C. pneumoniae* and *C. psittaci*. The number of chlamydial organisms or fragments thereof in the maximal dilutions tested extrapolated to be in the range of 0.06–0.12 for the volume of extract used in the test (Fig. 3) (or $\times 5$ for 200 μ l = 0.3–0.6 EBs/ml of test specimens).

Sensitivity of the PCR for positivity of replicates was determined for QUAamp-extracted and unextracted EBs from *C. pneumoniae*. The unextracted, intact EBs had DNA that was positive for four out of four replicates (Fig. 4), whereas the extracted EBs had DNA that was positive for four out of five replicates. These replicates represented chlamydial organisms (EB) at concentrations of 0.5 in the test (or 2.5 EBs/ml test specimen) as was used for target specimen in the PCR reaction tube. Similar sensitivity was demonstrated for *C. psittaci*.

Stability of extracted Chlamydial DNA was tested for 200 μ l extracts of 0.5 EBs (=2.5 EBs/ml). Two replicates were positive for up to 1 week and 6 days at 22°C storage for *C. pneumoniae*, and up to 2 weeks and 2 days at 4°C for both *C. pneumoniae* and *C. psittaci*.

Fig. 4. QUAamp PCR results for extracted and intact (control) *C. pn.* DNA.

DISCUSSION

The PCR assay for detection of Chlamydial organisms has been proven to be a very sensitive and useful method. It requires that DNA extraction be efficient as related to the variability of procedures from which to select, those included in this study (Tables 3 and 4) along with others (17,18). The two procedures in our investigation with extraction matrices were fast and sensitive. The 20-minute column method was perhaps the best all-around procedure due to its simplicity, rapidity, and sensitivity. Multiple variables in the procedures still require standardization for adapting the test to a diagnostic setting. These factors may include specimen (such as buffy coat) preservation and optimization in handling for best extraction of DNA.

The PCR procedure used in this study detected EBs in the range of 0.5 ± 0.5 as used in the test (or 2.5 EB/ml of working dilution of specimen). This level of detection indicates a high sensitivity (Fig. 4) for the procedure as well as an efficient extraction process used for preparation of target DNA for testing. Probably both. Sensitivity of the PCR procedure detected 100%

of the unextracted positive controls (presumably intact, although probably with some fragmented DNA, which contributes to amplifiable DNA signals), and in the range of 80–100% (probably closer to the latter) of extracted buffy coat specimens. The level of amplification of other assays for detecting *C. trachomatis* has been demonstrated to be as low as three EBs using the PCR or the LCR (16). This sensitivity did not apply to other strains of Chlamydia, viz. *C. psittaci* or *C. pneumoniae*. This same level of specificity was also shown with DNA probe kits with an even less sensitivity level of 10^3 organisms.

Two factors play a role in improved Chlamydial detection and diagnosis. They include refined methods for performing the assay and options for preparation of target DNA for testing. Further improvement of diagnostic procedures using PCR will lend itself to understanding chlamydial pathogenesis. This will include elucidation of the role that abnormal or persistent chlamydiae plays in repeated or superimposed infection (8). Sensitive and standardized PCR assays with improved methods of DNA purification and more selective primer choices will permit species differentiation and diagnoses in Chlamydial infections.

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