Rapid Detection of *Chlamydia pneumoniae* by PCR-Enzyme Immunoassay

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*Chlamydia pneumoniae* is an important human respiratory pathogen. Laboratory diagnosis of infection with this organism is difficult. To facilitate the detection of *C. pneumoniae* by PCR, an enzyme immunoassay (EIA) for analysis of PCR products was developed. Biotin-labeled PCR products generated from the 16S rRNA gene of *C. pneumoniae* were hybridized to a digoxigenin-labeled probe and then immobilized to streptavidin-coated microtiter plates. Bound PCR product-probe hybrids were detected with antidigoxigenin peroxidase conjugate and a colorimetric substrate. This EIA was as sensitive as Southern blot hybridization for the detection of PCR products and 100 times more sensitive than visualization of PCR products on agarose gels. The diagnostic value of the PCR-EIA in comparison to cell culture was assessed in throat swab specimens from children with respiratory tract infections. *C. pneumoniae* was isolated from only 1 of 368 specimens tested. In contrast, 15 patient specimens were repeatedly positive for *C. pneumoniae* by PCR and Southern analysis. All of these 15 specimens were also identified by PCR-EIA. Of the 15 specimens positive by 16S rRNA-based PCR, 13 specimens could be confirmed by *omp1*-based PCR or direct fluorescent-antibody assay. Results of this study demonstrate that PCR is more sensitive than cell culture for the detection of *C. pneumoniae*. The EIA described here is a rapid, sensitive, and simple method for detection of amplified *C. pneumoniae* DNA.

**MATERIALS AND METHODS**

*Patients and specimens.* Throat swab specimens were collected from hospitalized children with acute lower respiratory tract infections. Specimens were placed into 1.5 ml of sucrose-phosphate-glutamate buffer (pH 7.4) supplemented with 10% fetal calf serum, gentamicin (50 μg/ml), vancomycin (50 μg/ml), and amphotericin B (2.5 μg/ml). Prior to storage at −75°C a 300-μl aliquot of the patient specimen was withdrawn for PCR analysis.

*Cell culture.* Patient specimens were thawed, vortexed, and sonicated briefly. Aliquots (100 μl) of each sample were inoculated in duplicate onto HEP-2 cells (American Type Culture Collection, Manassas, Va.) grown in two 96-well culture plates (Corning Costar, Bodenheim, Germany). Plates were centrifuged at 1,340 × g at 30°C for 1 h. After incubation at 37°C for 1 h, the inoculum was replaced by 200 μl of Eagle’s minimal essential medium (MEM) supplemented with 10% fetal calf serum, 25 mM HEPES, 56 mM glucose, 2 mM L-glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) vitamins, gentamicin (50 μg/ml), amphotericin B (2.5 μg/ml), and cycloheximide (1.5 μg/ml). Cultures were incubated for 72 h at 36°C in a humidified atmosphere of 5% CO₂. The monolayers of one plate were fixed with methanol and stained for chlamydial inclusions with a fluorescein-conjugated genus-specific antibody to the *Chlamydia* lipopolysaccharide (Sanofi diagnostics Pasteur, Freiburg, Germany). On subsequent passages isolates were identified as *C. pneumoniae* by staining with a fluorescein-conjugated species-specific antibody (catalog no. K 6601; DAKO, Hamburg, Germany). Inclusion-negative cultures were passaged once. After freezing at −75°C, cultures were thawed and the cells were scraped off. Cell suspensions were transferred to microcentrifuge tubes, sonicated, and then in-oculated onto new HEP-2 cells as described above.

*C. pneumoniae* TW-183 (Washington Research Foundation, Seattle, Wash.) was grown to high titers in cycloheximide-treated HEP-2 cells (21). Titrations of

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freshly harvested organisms were done in triplicate in shell vials as described previously (15).

**Primers and probes.** The 16S rRNA gene and the major outer membrane protein (omp1) were used as targets for amplification of *C. pneumoniae* DNA. Oligonucleotide primers were synthesized and purified as described previously in detail (14). Patient specimens were routinely screened for *C. pneumoniae* DNA. Oligonucleotide primers were synthesized and purified as described previously (24). The external primers CP1 and CP2 amplified a 333 bp-fragment from the *omp1* gene of *C. pneumoniae*. A 207 bp sequence of this PCR product was amplified in a second PCR with the internal primers CPC and CPD.

**PCR.** A 300-μl aliquot of the patient specimen or 100 μl of serial 10-fold dilutions of *C. pneumoniae* TW-183 was centrifuged at 13,000 × g for 30 min. The resulting pellet was treated with 100 μl of proteinase K-detergent buffer (PCR buffer with proteinase K [200 μg/ml], 0.5% Tween 20, and 0.5% Nonidet P-40) for 1 h at 58°C (15). After inactivation of proteinase K for 10 min at 98°C, the samples were placed on ice.

A 10-μl aliquot of proteinase K-treated clinical specimen or chlamydial suspension was processed in a 100-μl reaction volume containing PCR buffer (10 mM Tris [pH 8.3], 50 mM KCl); 200 μM dATP, dCTP, and dGTP; 400 μM dUTP; 2.5 mM MgCl₂; a 0.5 μM concentration of each primer; 1.5 U of AmpliTaq DNA polymerase (Perkin-Elmer, Weiterstadt, Germany); and 1 U of a heat-labile uracil-N-glycosylase (UNG) (Boehringer) (23). Amplifications were carried out in a GeneAmp 9600 DNA thermal cycler (Perkin-Elmer). To detect contaminating amplification products from previous PCRs with UNG, reaction volumes were first incubated at 25°C for 10 min and then heated for 2 min at 93°C to inactivate the UNG. Forty amplification cycles of 15 s at 94°C, 15 s at 55°C, and a 15-s extension at 72°C were performed. After the last cycle, samples were incubated for 10 min at 72°C. If samples were not analyzed immediately, they were stored at −20°C. In the first round of amplification, patient specimens were tested for the presence of inhibitors. A 10-μl sample was withdrawn from the proteinase K-treated patient specimens, spiked with the DNA from approximately 1 inclusion-forming unit (IFU) of *C. pneumoniae*, and amplified by PCR. Specimens that did not yield a visible band after PCR on an ethidium bromide (EtBr)-stained agarose gel were subjected to phenol-chloroform extraction. Two positive controls containing the DNA from *C. pneumoniae* and four negative controls (water) were included in each assay. The A₅₉₀ of each specimen was determined with an enzyme-linked immunosorbent assay reader as a net value after subtracting the A₅₉₀ of the blank.

**RESULTS**

**Detection of amplified *C. pneumoniae* DNA.** The target for detection of *C. pneumoniae* in clinical specimens was the 16S rRNA gene (8). We used a biotinylated and a nonbiotinylated primer for the amplification of a 465-bp fragment from the 16S rRNA gene. After amplification biotinylated PCR products were hybridized in solution to a DIG-labeled 446-bp internal probe and then were immobilized to streptavidin-coated microtiter wells and detected with anti-DIG peroxidase conjugate and a colorimetric substrate. All incubation steps and reaction components of this EIA were optimized prior to use with clinical specimens. To compare the level of detection of this assay with the sensitivities of EtBr staining of agarose gels and Southern blot hybridization, serial 10-fold dilutions of PCR products were analyzed in parallel by all three methods. In repeated experiments the EIA was as sensitive as Southern blot hybridization for the detection of PCR products from *C. pneumoniae*. Both methods were approximately 100 times more sensitive than EtBr staining of agarose gels (Fig. 1).

**Control of carryover contamination with UNG.** A heat-labile UNG was used to degrade contaminating amplification products from previous PCRs in the reaction mixtures (23). To determine whether the use of dUTP instead of dTTP had influenced the sensitivity of the PCR, amplifications were carried out with either dTTP or dUTP. The use of dUTP did not lead to a decrease of the sensitivity of the PCR (data not shown). To assess the efficiency of UNG inactivation, 10-μl aliquots of serial 10-fold dilutions of PCR products from the amplification of 4 IFU of *C. pneumoniae* were treated with 1, 0.5, 0.1, or 0.05 U of heat-labile UNG before reamplification. When reaction mixtures were incubated with 1 or 0.5 U of UNG, reamplification products were not detected by Southern analysis in all specimens tested. Incubation of reaction mixtures with 0.1 or 0.05 U of UNG did not prevent the reamplification of PCR products. In addition, UNG treatment did not influence the sensitivity of the PCR (data not shown).

**Detection of *C. pneumoniae* in clinical specimens.** (i) Cell culture. A total of 368 throat swab specimens from children with lower respiratory tract infections were inoculated onto...
cycloheximide-treated HEp-2 cells. C. pneumoniae was isolated from only one of these specimens at the first passage.

(ii) PCR-EIA. In addition to being examined by cell culture, specimens were examined for the presence of C. pneumoniae by 16S rRNA-based PCR. All specimens were routinely tested in two PCR assays. C. pneumoniae sequences amplified from the 16S rRNA gene were detected by EtBr staining of agarose gels, Southern blot hybridization, and EIA. Analysis of specimens by EIA was done blinded. A total of 15 specimens, including the culture-positive sample, were positive by 16S rRNA-based PCR and Southern blot hybridization (Table 2). When a cutoff value of 0.100 was used, we found complete agreement between the results of Southern analysis and the EIA. The distribution of the A_{450} of Southern blot-positive specimens is shown in Fig. 2. The lowest A_{450} of a positive specimen was 0.171. The highest A_{450} of a Southern-blot negative specimen was 0.067. Thus, Southern blot-positive and -negative specimens were clearly distinguishable by the EIA. A band of the expected size was visible on the gel in only 8 (53%) of the Southern blot- and EIA-positive specimens (Table 2). Of 15 specimens that tested positive by 16S rRNA-based PCR, 11 were positive in two consecutive runs. The remaining four samples were positive in only one of two assays. For these samples one or two additional PCR runs with primers specific for the 16S rRNA gene were needed to obtain a second positive result.

Altogether, 14 specimens were culture negative but positive by 16S rRNA-based PCR. These specimens were analyzed further by DFA and a nested PCR with primers specific for omp1. Of the 14 specimens, 12 could be confirmed as positive by omp1-based PCR and 10 were positive by DFA. The remaining 2 PCR-positive, culture-negative samples were negative by both omp1-based PCR and DFA. Therefore, these specimens were taken to be false positives (Table 3). The sensitivity of the PCR-EIA was 100%, and the specificity was 99.4%. The positive and negative predictive values of the PCR-EIA were 86.6 and 100%, respectively.

![FIG. 1. Comparison of EtBr staining of agarose gel, Southern blot hybridization, and EIA for the detection of PCR products generated from C. pneumoniae DNA. A 465-bp sequence was amplified from the 16S rRNA gene of C. pneumoniae. PCR products were serially diluted 10-fold (lanes 1 to 7 [lane 1, undiluted; lane 2, 10-fold diluted, etc.]) and analyzed in parallel by all three detection methods. (A) EtBr-stained agarose gel (leftmost lane, DNA molecular weight marker); (B) Southern blot; (C) A_{450} of PCR products from panel B, obtained by EIA. Cutoff = 0.100.](http://jcm.asm.org/)

![FIG. 2. Distribution of the A_{450} obtained by EIA of 15 clinical specimens which were positive by Southern blot hybridization. Cutoff = 0.100.](http://jcm.asm.org/)

**TABLE 2. Comparison of methods for detection of amplified C. pneumoniae DNA from 368 clinical specimens**

<table>
<thead>
<tr>
<th>Method</th>
<th>No. (%) of clinical specimens with resulta</th>
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<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Southern analysis</td>
<td>15 (4)</td>
</tr>
<tr>
<td>EIA</td>
<td>15 (4)</td>
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<tr>
<td>EtBr staining</td>
<td>8 (2)</td>
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</table>

a The results are from two runs.

**TABLE 3. Test results of clinical specimens positive by 16S rRNA-based PCR-EIA**

<table>
<thead>
<tr>
<th>Specimen no.</th>
<th>Cell culture</th>
<th>16S rRNA-based PCRb</th>
<th>omp1-based PCR</th>
<th>DFA</th>
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<tbody>
<tr>
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<td>15</td>
<td>+</td>
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b Numbers in parentheses are the number of assays needed to obtain two positive results.

c False-positive result, because confirmatory omp1-based PCR and DFA were negative.

d, positive in assay; −, negative in assay.
DISCUSSION

In the present study, *C. pneumoniae* was detected in throat swab specimens from pediatric patients with lower respiratory tract infections by PCR-EIA and by isolation in cell culture. The organism was recovered in culture from only 1 of 368 specimens tested. The reasons for the low sensitivity of culture in this study are unknown. Sensitivity of cell culture depends on a number of factors, such as sufficient numbers of viable chlamydiae, collection method, transport and storage conditions of specimens, and choice of cell lines. We examined throat swab specimens, and it is possible that these samples did not include enough cells which harbored the organism. We cannot exclude the possibility that the isolation rate of *C. pneumoniae* might have been higher with other types of specimens. Comparative studies on the relative efficacy of throat swab, nasopharyngeal swab, and sputum samples for recovery of *C. pneumoniae* from culture revealed that positive results were most frequently obtained with sputum specimens (3). Furthermore, prolonged storage of specimens at −75°C might have influenced the viability of organisms. It has been previously demonstrated that freezing results in the loss of a significant proportion of *C. pneumoniae* (17). While this work was in progress it was reported that pretreatment of clinical specimens with trypsin leads to an increase in the isolation rate of *C. pneumoniae* (16). It remains to be determined in further studies whether this method will prove to be an efficient technique for recovery of *C. pneumoniae* from clinical specimens.

In contrast to the cell culture results, *C. pneumoniae* was detected by 16S rRNA-based PCR in 15 out of 368 specimens (Tables 2 and 3). Eleven of these samples were positive in two consecutive assays. For the remaining 4 samples, either three or four runs were necessary to obtain a second positive test result, which might have been due to a low number and/or unequal distribution of organisms in these samples. Of 15 specimens positive by 16S rRNA-based PCR, 13 could be confirmed as true positives by *omp1*-based PCR and 11 were positive by DFA. Two 16S rRNA-based PCR-positive specimens were negative by either *omp1*-based PCR or DFA and were taken to be false positives. In our study, patient specimens containing inhibitors were identified by monitoring the amplification of DNA from *C. pneumoniae* TW-183, which was added to a duplicate test sample. Therefore, it is possible that despite strict precautions the false-positive samples might have been contaminated with natural *C. pneumoniae* DNA during the setup of the reaction mixtures. To eliminate this possible source of contamination, we have constructed an internal control for monitoring PCR inhibition (our unpublished observations).

In the present study the 16S rRNA gene was used as the target for detection of *C. pneumoniae* (7, 8). Other targets commonly used for identification of *C. pneumoniae* are the *omp1* gene and a specific DNA fragment (5, 24). Various PCR procedures for detection of *C. pneumoniae* by different detection systems are under investigation. However, these assays have not yet been compared with each other. Nested PCRs with *omp1*- or 16S rRNA-based primers have been reported to be more sensitive than single-step PCR (1, 3). At least part of this difference in sensitivity may be due to the use of agarose gel analysis for detection of PCR products, a method which lacks sensitivity. A serious disadvantage of nested PCRs is the high risk of carryover contamination. In our study *omp1*-based nested PCR was found to be useful only as a confirmatory test but not for routine testing of specimens. Furthermore, UNG, which destroys products from previous amplifications, can be used in a nested PCR only in the second round of amplification. This is the first PCR assay for detection of *C. pneumoniae* which includes a dUTP-UNG system for carryover prevention. We used a new heat-labile UNG. This UNG from a marine bacterium is inactivated more rapidly by heat and shows much less residual activity than the corresponding enzyme from *Escherichia coli* (23). The dUTP-UNG protocol was found to be highly efficient and had no negative effect on the sensitivity of the PCR.

A major objective of our study was the development and evaluation of a rapid, simple, and sensitive detection system for amplified *C. pneumoniae* DNA. Our previous studies with primers derived from a specific DNA fragment of *C. pneumoniae* suggested, when throat swab specimens were examined, that a single-step PCR followed by agarose gel analysis of PCR products lacks sensitivity (15). Since Southern analysis is too labor-intensive for routine use in diagnostic laboratories, an EIA for detection of PCR products was established. Detection of amplified *C. pneumoniae* sequences by EIA has been reported previously by Gaydos and coworkers (6). Compared to our assay, the EIA performed by Gaydos is a more complicated detection method, requiring the labor-intensive production and purification of an RNA probe. One of the advantages of our system is its simplicity. For example the DIG-labeled probe used for detection of PCR products can be easily generated by PCR. Results obtained with our EIA were compared to those obtained by EtBr staining of PCR products on agarose gels and Southern blot hybridization and demonstrated the high sensitivity of this detection system. When serial dilutions of PCR products from *C. pneumoniae* were analyzed, Southern analysis and EIA were equally sensitive and both were at least 100 times more sensitive than agarose gel electrophoresis and EtBr staining (Fig. 1). When clinical specimens were examined for the presence of *C. pneumoniae*, there was complete agreement between the results of Southern analysis and EIA. A total of 15 specimens were positive both by Southern hybridization and by EIA. In contrast, in only 8 of these specimens was a band of the expected size visible on EtBr-stained agarose gels (Table 2). These findings may be attributable to low numbers of chlamydiae in the specimens and/or inefficient amplification due to the presence of PCR inhibitors. Nevertheless, these observations clearly demonstrate that detection of PCR products on agarose gels is not sensitive enough for detection of *C. pneumoniae* sequences which were amplified by single-step PCR from throat swab specimens.

In conclusion, our study confirms and extends findings of previous studies which have demonstrated that PCR is more sensitive than cell culture for the detection of *C. pneumoniae* in clinical specimens. We developed and evaluated a new EIA for the analysis of amplified *C. pneumoniae* DNA. The advantages of this EIA are its simplicity, the use of a carryover prevention system, and its high sensitivity. This assay is comparable in sensitivity to Southern analysis but is less labor-intensive and much faster. Results of specimens which do not contain inhibitors can be obtained within 1 day. We hope that this assay will help to facilitate the diagnosis of *C. pneumoniae* infections.

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