Detection of *Chlamydia pneumoniae* in Clinical Specimens by Polymerase Chain Reaction Using Nested Primers

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A nested primers strategy was used to develop a two-step PCR test for the direct species-specific detection of the 16s rRNA gene of *Chlamydia pneumoniae*. This test was applied to 58 nasopharyngeal or oropharyngeal swab specimens collected from patients in studies of community-acquired pneumonia and in a local outbreak of respiratory disease. Twelve patients (21 %) showed evidence of *Chlamydia pneumoniae* infection in serological tests (7/56; 13 %), culture (8/58; 14 %) or PCR (10/58; 17 %). Nested PCR but not single-step PCR was found to be as sensitive as culture or serology for detection of infection with this organism. In summary, nested PCR can be useful in direct testing of clinical specimens for *Chlamydia pneumoniae*, making additional DNA purification steps unnecessary.

Chlamydia pneumoniae is a frequently encountered pathogen responsible for 10 % to 20 % of community-acquired upper and lower respiratory tract infections seen in common practice (1). At present, definitive diagnosis is made by isolation of the organism in cell culture followed by identification with a *Chlamydia pneumoniae* specific monoclonal antibody, or serologically by the microimmunofluorescence test. Recently, the PCR was used for species-specific identification of *Chlamydia pneumoniae* (2, 3). The sensitivity of a single-step PCR applied directly to clinical specimens is often much less than the sensitivity observed with purified DNA samples, due to the presence of thermostable inhibitors of the polymerase reaction in the specimens (4, 5). The addition of DNA purification steps to the sample preparation protocol can overcome this limitation to some extent, but this procedure is unsuitable for routine diagnostic application because of the technical difficulty and time required for DNA extraction. Use of nested primers has been shown to increase both the specificity and sensitivity of PCR tests (6). We present the results of a study using a species-specific PCR with nested primers for detection of *Chlamydia pneumoniae* in clinical specimens.

Materials and Methods. Throat swabs and acute and convalescent sera were collected from patients with community-acquired pneumonia at a hospital in Atlanta, GA, USA (7). Nasal swabs and acute and convalescent sera were collected from patients during an outbreak of respiratory infection in a Norwegian military camp (8). Swab specimens were kept in Scott's transport medium (Chlamydiaport; Adams Scientific, USA) or 2-sucrose phosphate transport medium (9). The culture of specimens for *Chlamydia pneumoniae* was performed as previously described (7, 8). Serum specimens were assayed by the microimmunofluorescent test (10) for specific IgG and IgM antibodies using strain TW-183 as antigen (7, 8). *Chlamydia psittaci* strains Guinea Pig Inclusion Conjunctivitis and 6BC; two clinical isolates of *Chlamydia psittaci*; *Chlamydia trachomatis* serovars A-K, L_{1-3} and Mouse pneumonitis; and *Chlamydia pneumoniae* strains TW-183, CWL-011, CWL-029, CWL-050, CM-1, FML-16, 2023 and 2043 were used to test the specificity of the nested PCR. *Chlamydia pneumoniae* strains were grown and purified as described (11, 12).

Chlamydial elementary bodies (EBs) were quantitated by flow cytometry (13). Five hundred µl aliquots of purified EBs at varying dilutions in 0.15 M phosphate buffered saline, pH 7.2, were mixed with 10 µl DNA intercalating fluorescent dye (100 µg/ml in dimethyl sulfoxide) (Hoechst 33248; Molecular Probes, USA). Five µl of a blue fluorescent microsphere suspension (1.5×10^5 beads/µl) (0.7 µm diameter; Duke Scientific, USA) was added to each sample for alignment and quality control of the cytometer. Accuracy of counts was verified by visual counts under dark-field microscopy with a linear correlation of 0.991. The samples were immediately analyzed on a mercury arc lamp-based, computer-controlled flow cytometer (ACR 1000; Bruker Instruments, USA) using a 360-nm band pass excitation filter, a 440-nm long pass emission filter and a flow rate of 300–500 cells/sec. Acquisition and analysis of data were done with the computer software package FLOWER (Bruker Instru-

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ments). Data processed included delivery and volume analyzed, total number of particles counted, peak fluorescence value, average fluorescence value and percent coefficient of variation.

Specimens and quantitated EBs were sonicated, and 500 µl aliquots removed and processed for PCR as previously described (14). The end-product represented a 4- to 8-fold concentration over the starting volume, and 5 µl were used in the PCR. DNA was prepared from bacterial strains other than chlamydiae as previously described (15). Nested sets of primers were designed to detect a fragment of the 16s rRNA gene of *Chlamydia pneumoniae* (16) by computer analysis using the EMBL/GenBank library for minimal homology to nucleotide sequences of this gene from other *Chlamydia* spp. (17). Primers were synthesized on a DNA synthesizer (Model 381A, Applied Biosystems, USA) using β-cyanoethyl phosphoramidite. For single-step PCR, only the outer set of primers was used. DNA was amplified in 50-µl volumes containing 200 µM of each deoxynucleoside triphosphate, 2 µM of each primer, 1.0 unit AmpliTaq polymerase (Perkin-Elmer Cetus, USA), 10 mM Tris-HCl, pH 8.3, 2.0 mM $MgCl_2$, 50 mM KCl and 0.01 % gelatin. Amplifications were performed in an automated thermocycler (Perkin-Elmer) for 35 cycles at 94°C for 1 min (3 min for the first cycle), 45°C for 1 min, and 72°C for 1 min (7 min for the last cycle). Single step PCR was performed with external primers (sense 71–91: 5' ATAATGACTTCGGTTG TTAT 3'; antisense 1448–1468: 5' TATAAATAGGTTGAGTCAAC 3'), and the nested PCR was performed with nested primers (sense 177–199: 5' AGTGTAATTAG GCATCTAATAT 3'; antisense 1018–1035: 5' GCTGTATTTCTACAGTTG 3'). The nested PCR was performed using 1:1000 dilutions of the first PCR product. Amplification products were made visible in 1.5 % NuSieve GTG agarose – 1.0 % agarose gels with ethidium bromide. Samples for PCR were prepared in a class II laminar flow hood, and amplification and analysis of PCR products were each performed in physically separate locations. PCR was performed at least twice on all samples to ensure reproducibility. To verify that amplified DNA represented the target sequence, the PCR products were hybridized with a *Chlamydia pneumoniae*-specific 16s rRNA chemiluminescent probe (Gen-Probe, USA) with an analytical sensitivity of 10–20 pg of rRNA (E. Mather et al., 92nd Annual Meeting of the American Society for Microbiology, 1992, Abstract C425). Hybridizations were performed according to the manufacturer's instructions (PACE-2) (18).

Results of serological tests, culture and PCR were compared using McNemar's test (19).

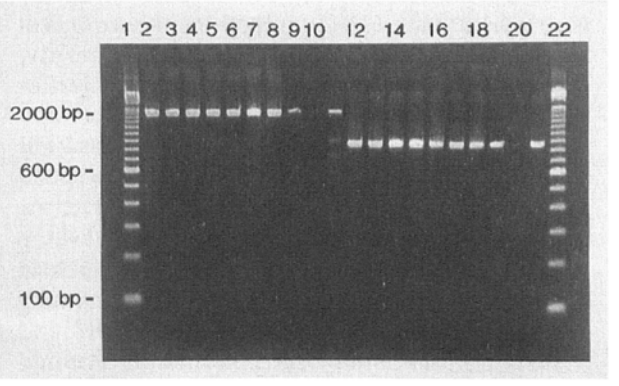

Figure 1: Amplification products with the single-step PCR (1397 bp in size; lanes 2–11) and nested PCR (858 bp in size; lanes 13–23) using DNA from strains of *Chlamydia pneumoniae* as template. Lanes 1, 22: 100 bp ladder (BRL, USA). Lanes 10, 20: no template controls.

Table 1: Results of single-step and nested PCR amplification of quantitated elementary bodies (EBs) of *Chlamydia pneumoniae* suspended in buffer, transport media and clinical specimens.

Sample diluent	No. of EBs added	Single step PCR	Nested PCR
Tris buffer	100	+	+
	10	+	+
	1	+	+
Transport media[a]	100	+	+
	10	–	+
	1	–	+
Clinical specimen[b]	100	+	+
	10	–	+
	1	–	+

[a] Scott's transport medium (Adams Scientific, USA) or 2-sucrose phosphate medium (18).
[b] Chlamydial EBs added to a specimen from a patient with no evidence of infection with *Chlamydia pneumoniae* on serological testing, culture or PCR test.

Results and Discussion. Sensitivity of the single-step PCR compared to the nested PCR was tested using dilutions of quantitated chlamydial EBs suspended in Tris buffer. Limits of detection were determined for EBs added to transport medium alone without specimen and to nasopharyngeal swab specimens in transport medium obtained from patients previously found to be negative for

Chlamydia pneumoniae infection in serological tests, PCR and cell culture (Table 1). The sensitivity of the single-step PCR was 1 EB in Tris buffer and 10 EBs in swab specimens or transport medium. The sensitivity of the nested PCR was 1 EB in Tris buffer, transport medium or swab specimens. Thus, the nested PCR was found to be about ten-fold more sensitive than single-step PCR for direct amplification of target DNA in clinical specimens. Transport media commonly used for chlamydial swab specimens were found to be inhibitory in the PCR. This inhibition could be overcome by use of nested PCR (Table 1).

Templates from all 18 strains of *Chlamydia pneumoniae* resulted in amplification products that were visible in agarose gels at similar limits of detection (Figure 1). When template DNA prepared from 20 other bacterial species commonly encountered in the respiratory tract including strains of *Chlamydia trachomatis* and *Chlamydia psittaci* was tested, no visible DNA products were amplified with either single-step or nested PCR. To verify that amplified products represented the target sequence, PCR products were hybridized to a *Chlamydia pneumoniae*-specific chemiluminescent probe. Based on a positive cut-off value defined as 300 relative light units by use of reference standards, hybridization of the probe with the products amplified from strains TW-183 and CWL-029 produced strongly positive results of 17,447 and 17,699 relative light units, respectively. Negative control values of 120–135 relative light units were observed following hybridization of the probe with unrelated PCR products obtained by amplification of templates from the same strains with primers targeting a sequence from the *omp*1 gene (14).

Twelve patients showed evidence of infection with *Chlamydia pneumoniae* in two clinical studies. In the Norwegian study, 8 of 30 patients (27 %) showed evidence of infection in serological tests, culture or PCR (Table 2). All patients (8/8) were positive in the nested PCR, 5 of 8 (62 %) were culture positive, and 3 of 8 (37 %) were serologically positive. Two of the 8 patients (25 %) had elevated titers of IgM antibodies specific for *Chlamydia pneumoniae*. The serologic status of two patients could not be determined because convalescent serum specimens were not available. In the Atlanta study, 4 of 28 patients (14 %) showed evidence of infection with *Chlamydia pneumoniae* in serological tests, culture or PCR.

Table 2: Test results in clinical specimens from patients with evidence of *Chlamydia pneumoniae* infection.

Patient no.[a]	MIF antibody titer[b]		Culture results	PCR results[c]
	Acute	Convalescent		
Norwegian study				
N1	64	64	–	+
N2	64	256	+	+
N3	16[d]	16	+	+
N4	< 8	< 8[d]	+	+
N5	< 8	< 8	–	+
N6	< 8	ND	–	+
N7	16	16	+	+
N8	< 8	ND	+	+
Atlanta study				
A1	128	256[d]	+	+
A2	1024	1024	–	–
A3	16	1024[d]	+	–
A4	64[d]	ND	+	+

[a] Specimens were collected from a Norwegian study of an outbreak of respiratory infection in a military camp (8) and from an Atlanta study of community-acquired pneumonia (7). Positive patients had serological, culture or PCR test evidence of acute *Chlamydia pneumoniae* infection.

[b] Acute and convalescent serum anti-*Chlamydia pneumoniae* IgG titers in the microimmunofluoresent test (MIF). Serological evidence of acute infection was defined as a fourfold increase in IgG or IgM titer, IgG titer ≥ 512, or IgM titer ≥ 16. ND, no convalescent serum collected.

[c] Positive PCR was defined as visible bands after electrophoresis of nested PCR products in agarose gels and staining by ethidium bromide fluorescence.

[d] Serum specimens with an IgM titer of ≥ 16 in the MIF test.

Of these, 2 of 4 patients (50 %) were positive in the nested PCR, 3 of 4 (75 %) were culture positive, and all (4/4) were serologically positive. Three of 4 patients (75 %) had elevated titers of IgM antibodies specific for *Chlamydia pneumoniae*.

All but one clinical specimen from both studies that were culture positive were also positive in the nested PCR (Table 2). In addition, specimens from three Norwegian patients that were culture negative were positive in the nested PCR. With single-step PCR, only 2 (A1, A4) of 12 (17 %) specimens produced visible products compared to 10 of 12 (83 %) that were positive with nested PCR.

Overall for both clinical studies, 12 of 58 patients (21%) showed evidence of infection with *Chlamydia pneumoniae* in one or more test (serological test, culture and PCR). Taking the tests individually, nested PCR appeared to be the most sensitive, and serology the least sensitive, 10 of 58 specimens (17 %) being PCR positive, 8 of 58 (14 %) culture positive, and 7 of 56 (13 %) serologically positive. However, these values were not statistically different ($p > 0.05$).

While exquisitely sensitive and reproducible in the research laboratory under carefully controlled conditions applied to purified template DNA preparations, the PCR test often lacks sensitivity and reproducibility, and is prone to both false-positive and false-negative results when applied to direct testing of clinical specimens. Such unsatisfactory performance of the PCR has mainly been due to contamination with previously amplified DNA (false-positive) or to the presence of potent inhibitors of the polymerase enzyme in various types of clinical material (false-negative) (4, 5). The nested primer PCR strategy, originally developed to increase the specificity of target detection (6), also increases the sensitivity of detection in crude samples (20).

We have not attempted to make direct comparisons between our nested PCR and the single-step PCR tests described by Campbell et al. (2) and Gaydos et al. (3) for *Chlamydia pneumoniae*. Campbell et al. (2) reported positive PCR tests in all culture-positive specimens (8/8) and 50 % of seropositive/culture-negative specimens (4/8). Gaydos et al. (3) reported positive PCR tests in 8 of 9 culture-positive specimens and 2 of 3 culture-negative specimens from experimentally inoculated monkeys (serologic status not defined). In these studies, sensitivity of target detection was in the same range as the nested PCR reported here, but tests were confined to purified EBs or purified DNA. In neither study was the sensitivity of the PCR test determined with artificially inoculated clinical specimens, as was done in the present study. In addition, both of these studies used laborious DNA purification methods in their clinical specimens that were not used in our study. In the 58 clinical specimens tested, we found positive PCR test results in all culture positive specimens but one, and in three seronegative/culture negative specimens. One seropositive/culture negative specimen was PCR negative. Although this could represent a false-negative PCR result, it is unlikely since the patient probably did not have a recent or acute infection with *Chlamydia pneumoniae*, as indicated by antibody titers that were already high at the time of the first collection of serum (Table 2).

In summary, we have reported data demonstrating that a nested PCR was as sensitive as serology or culture in detecting *Chlamydia pneumoniae* infection in two different studies of patients with respiratory illness. Further, we have found that single step PCR may be unreliable for direct testing of clinical specimens regardless of the size of the product amplified unless a nested PCR is performed, sensitive PCR product detection methods are used, or steps are taken to eliminate inhibitors in the transport media or in the specimen itself. The nested PCR test reported here overcame the inhibition present in crude specimen preparations and is a faster alternative to time- and labor-intensive DNA purification and extraction techniques. Thus, nested PCR may be more practical for early detection of *Chlamydia pneumoniae* infections in clinical laboratories.

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References

1. **Grayston JT, Campbell LA, Kuo C-C, Mordhorst CH, Saikku P, Thom DH, Wang SP:** A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. Journal of Infectious Diseases 1990, 161: 618–625.
2. **Campbell LA, Melgosa MP, Hamilton DJ, Kuo CC, Grayston JT:** Detection of *Chlamydia pneumoniae* by polymerase chain reaction. Journal of Clinical Microbiology 1992, 30: 434–439.

3. **Gaydos CA, Quinn TC, Eiden JJ:** Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. Journal of Clinical Microbiology 1992, 30: 796–800.
4. **White TJ, Madej R, Persing DH:** The polymerase chain reaction: clinical applications. Advances in Clinical Chemistry 1992, 29: 161–196.
5. **Holodiny M, Kim S, Katzenstein D, Konrad M, Groves E, Merigan TC:** Inhibition of human immunodeficiency virus gene amplification by heparin. Journal of Clinical Microbiology 1991, 29: 676–679.
6. **Erlich HA, Gelfand D, Sninsky JJ:** Recent advances in the polymerase chain reaction. Science 1991, 252: 1643–1650.
7. **Campbell JF, Barnes RC, Kozarsky PE, Spika JS:** Culture-confirmed pneumonia due to *Chlamydia pneumoniae*. Journal of Infectious Diseases 1991, 164: 411–413.
8. **Berdal BP, Fields PI, Mitchell SH, Hoddevik G:** Isolation of *Chlamydia pneumoniae* during an adenovirus outbreak. In: Bowie, WR, Caldwell HD, Jones RP, Mårdh P-A, Ridgway, GL, Schachter, J, Stamm WE, Ward ME (ed): Chlamydial Infections. Cambridge University Press, Cambridge, 1990, p. 445.
9. **Barnes RC:** Laboratory diagnosis of human chlamydial infections. Clinical Microbiology Reviews 1989, 2: 119–136.
10. **Wang SP, Grayston JT:** Human serology in *Chlamydia trachomatis* infection with microimmunofluorescence. Journal of Infectious Diseases 1974, 130: 388–397.
11. **Black CM, Johnson JE, Farshy CE, Brown TM, and Berdal BP:** Antigenic variation among strains of *Chlamydia pneumoniae*. Journal of Clinical Microbiology 1991, 29: 1312–1316.
12. **Howard L, Orenstein NS, King NW:** Purification on Renografin density gradients of *Chlamydia trachomatis* grown in the yolk sac of eggs. Applied Microbiology 1974, 27: 102–106.
13. **Skarstad K, Steen HB, Boye E:** Cell cycle parameters of slowly growing *Escherichia coli* B/r studied by flow cytometry. Journal of Bacteriology 1983, 154: 656–662.
14. **Black CM, Tharpe JA, Russell H:** Distinguishing *Chlamydia* species by restriction analysis of the major outer membrane protein gene. Molecular Cellular Probes 1992, 6: 395–400.
15. **Black CM, Talkington DF, Messmer TO, Facklam RR, Hornes E, Olsvik O:** Detection of streptococcal pyrogenic exotoxin genes by a nested PCR and colorimetric reaction. Molecular Cellular Probes 1993, 7: 255–259.
16. **Gaydos CA, Palmer L, Quinn TC, Falkow S, Eiden JJ:** Phylogenetic relationship of *Chlamydia pneumoniae* to *Chlamydia psittaci* and *Chlamydia trachomatis* as determined by analysis of 16S ribosomal sequences. International Journal of Systematic Bacteriology 1993, 43: 610–612.
17. **Weisburg WG, Hatch TP, Woese CR:** Eubacterial origin of chlamydiae. Journal of Bacteriology 1986, 167: 570–574.
18. **Limberger RJ, Biega R, Evancoe A, McCarthy L, Slivienski L, Kirkwood M:** Evaluation of culture and the Gen-Probe PACE-2 assay for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in endocervical specimens transported to a state health lab. Journal of Clinical Microbiology 1992, 30: 1162–1166.
19. **Sleiss JL:** Statistical methods for rates and proportions. John Wiley & Sons, New York, 1981, p. 114.
20. **Evander M, Edlund K, Bodén E, Gustafsson Å, Jonsson M, Karlsson R, Rylander E, Wadell G:** Comparison of a one-step and a two-step polymerase chain reaction with degenerate general primers in a population based study of human papillomavirus infection in young Swedish women. Journal of Clinical Microbiology 1992, 30: 987–992.

Value of Antigen and Antibody Detection in the Serological Diagnosis of Invasive Aspergillosis in Patients with Hematological Malignancies

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In a study to determine the value of antigen and antibody detection in the serological diagnosis of invasive aspergillosis in patients with hematological malignancies, 436 sequential serum samples from 79 neutropenic patients were tested. Circulating galactomannan antigen was detected with a latex agglutination method (Pastorex Aspergillus) and antibody to *Aspergillus fumigatus* with an immunodiffusion test on agar gel. Among the 79 patients 18 cases of invasive aspergillosis were detected (4 proven, 6 highly probable, 3 probable, 5 suspected). Antigen was detected in 7 of these 18 patients. The sensitivity of the antigen test was 38.8 % and the specificity 95 %. The antibody test showed 55.5 % sensitivity and 100 % specificity. In this study the antigen test was less sensitive than previously reported but highly specific and might serve as a supplementary diagnostic test.

Invasive aspergillosis is one of the major causes of morbidity and mortality in patients with acute leukemia, lymphoma or organ transplants. The mortality due to invasive aspergillosis is very high and can be substantially reduced if an early diagnosis is made and the proper therapy given. This

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