JOURNAL OF CLINICAL MICROBIOLOGY, June 2002, p. 2273–2274 Vol. 40, No. 6
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.6.2273–2274.2002

Quantitative Detection of Respiratory *Chlamydia pneumoniae* Infection by Real-Time PCR

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Received 16 July 2001/Returned for modification 11 January 2002/Accepted 27 March 2002

Real-time PCR was evaluated as a quantitative diagnostic method for *Chlamydia pneumoniae* infection using different respiratory samples. Real-time PCR had efficiency equal to or better than that of nested touchdown PCR. This study confirmed sputum as the best sampling material to detect an ongoing *C. pneumoniae* infection.

Chlamydia pneumoniae is an obligate intracellular pathogen that causes upper and lower respiratory tract infections in humans (6) and is responsible for an average of 10% of cases of community-acquired pneumonia (8). Diagnosis of *C. pneumoniae* infection is usually based on serology, but other conventional methods with limited sensitivity, such as culturing and antigen detection, are also used. Nucleic acid amplification techniques have the potential to improve *C. pneumoniae* detection (2, 7), but there is no sufficiently standardized method available yet. A recent gene amplification technique is real-time PCR. It is an automated method that quantitatively monitors PCR products as they accumulate during thermal cycling (9). In the present study, real-time PCR was evaluated as a quantitative detection method for *C. pneumoniae* infection by analyzing respiratory samples previously tested by nested touchdown PCR, culture, and antigen enzyme immunoassay (1).

Samples. During an outbreak of pneumonia 1994 in Byske, Sweden, nasopharyngeal, sputum, and throat specimens were collected from patients with clinically suspected acute *C. pneumoniae* infection (1) and frozen. In this study, 105 of these respiratory samples were analyzed and consisted of three companion respiratory specimens from 35 patients. The sampling techniques and sample treatment with *N*-acetyl-L-cysteine (Mucomyst; Draco, Lund, Sweden) were described previously (1, 10). Sample DNA was extracted by using the Amplicor respiratory specimen preparation kit (Roche Diagnostic Systems, Branchburg, N.J.).

***Chlamydia* strains.** The strains were as follows: *C. pneumoniae* strain T45, isolated during the 1994 epidemic in Byske, Sweden; *C. pneumoniae* strain IOL 207; *C. psittaci* strains GP3, 6BC, and TT; and patient specimens positive for *C. trachomatis.*

Culture of *C. pneumoniae*. *C. pneumoniae* strain T45 was inoculated on HEp-2 cells in a 24-well culture plate and cultured for 7 days in RPMI 1640 medium (Sigma, St. Louis, Mo.) with 10% fetal bovine serum (Gibco BRL, Paisley, United Kingdom), 4.4% $NaHCO_3$ (pH 8.1), 20 mM HEPES (pH 8.0), 0.5% glucose, 8-μg/ml garamycin, 1-μg/ml amphotericin B (Fungizone), 25-μg/ml vancocin, and 1-μg/ml cycloheximide. On culture days 3, 4, and 5 the culture plate was centrifuged (2,500 × *g* for 1 h); on culture day 3, the culture medium was exchanged. Cultured *C. pneumoniae* organisms were stained with the Phadebact Chlamydia culture confirmation test (Boule Diagnostics AB, Huddinge, Sweden) and counted with a fluorescent microscope at a magnification of ×400. DNA of cultured *C. pneumoniae* was extracted with the QIAamp DNA Mini kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's instructions.

PCR. Real-time PCR was performed with the ABI Prism 7700 sequence detector system (Applied Biosystems, Foster City, Calif.). The gene coding for the major outer membrane protein (MOMP) was used as the target for amplification. DNA of cultured *C. pneumoniae* was used as the standard in dilutions between 1 copy and 6×10^4 copies. A pT7Blue-3 vector (Novagen, Madison, Wis.) with an inserted T45 MOMP gene (1,100 bp) of known concentration was compared to the standard with agreement. Either 5 or 10 μl of extracted DNA was analyzed with the PCR mixture in a total volume of 25 μl. The PCR mixture consisted of 10 mM Tris (pH 8.3), 50 mM KCl, 10 μM EDTA, 5 mM $MgCl_2$, 200 μM dATP, 200 μM dCTP, 200 μM dGTP, 300 μM dUTP, 1 U of Ampli*Taq* Gold DNA polymerase, and 0.25 U of AmpErase UNG (all obtained from Applied Biosystems); 200 nM probe (5′-FAM-TCC CCT TGC CAA CAG ACG CTG G-TAMRA-3′); and 300 nM forward (5′-AAG GGC TAT AAA GGC GTT GCT-3′) and reverse (5′-TGG TCG CAG ACT TTG TTC CA-3′) primers. The real-time PCR run was 2 min at 50°C, 10 min at 95°C, and 40 repeats of 15 s at 95°C and 1 min at 60°C. Nested touchdown PCR was performed with primers from the MOMP genes of *C. pneumoniae* and *C. psittaci* as described previously (4, 10). Five microliters of extracted DNA was analyzed in a total volume of 50 μl.

Twenty-three of 105 respiratory samples were positive by real-time PCR (Table 1). All samples were analyzed in triplicate, and samples with positive or indistinct results were reanalyzed at least once. A sample was considered positive if three of three assay results were positive in the triplicate test and if the average value for the PCR run was greater than or equal to 1.0.

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TABLE 1. Detection of *C. pneumoniae* in each of three locations by real-time PCR analysis of 35 patient samples

Origin of sample	Result of real-time PCR			
	No. negative/ total	No. positive/ total	Avg quantity[a]	Range[a]
Nasopharynx	29/35	6/35	15,000	600–50,000
Throat	31/35	4/35	3,100	1,400–6,100
Sputum	22/35	13/35	860,000	940–6,700,000

[a] Copies per milliliter of acetylcysteine-treated sample.

C. pneumoniae DNA copies varied from 6.0×10^2 to 6.7×10^6 per ml of the acetylcysteine-treated samples. The majority of all samples (70%) had copy numbers below 1×10^4 per ml, but the greater part of the sputum samples contained large amounts of *C. pneumoniae* DNA, with an average of 8.6×10^5 copies per ml. A parallel study with real-time PCR and nested touchdown PCR was performed with acetylcysteine-treated samples. Sixty-two samples, from where there was material left from previous sampling, were prepared and run directly on real-time PCR and nested PCR assays. Five microliters of extracted DNA was used with both methods. The real-time PCR method revealed a higher number of positive samples than nested PCR and showed no inhibitor tendency (Table 2). Sixteen of 23 real-time PCR-positive samples were confirmed by nested PCR and ranged between 6.0×10^2 and 2.5×10^6 copies per ml. The seven unconfirmed positive samples had all copy numbers below 4.4×10^3 per ml. With nested PCR as the "gold standard," real-time PCR had a sensitivity of 80% (16 of 20 samples) and a specificity of 83% (35 of 42 samples). The positive predictive value was 70% (16 of 23 samples) and the negative predictive value was 90% (35 of 39 samples). For both studies, patient samples had a mean coefficient of variation value of 26% in the triplicate assays. A titration of full-length bacterial DNA with plasmid DNA, both corresponding to 0.5 to 50,000 copies, was also analyzed by real-time PCR and nested PCR in parallel. The detection limit was around one to five copies for both methods, independent of template DNA origin. The real-time PCR system was tested against different strains of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci*; only the *C. pneumoniae* strains were amplified. A theoretical comparison with all GenBank, EMBL, and DDBJ sequences was also performed.

The conventional methods that are used to detect respiratory *C. pneumoniae* infections all have limitations, and there is a need for new, automated, and better-validated diagnostic methods. Recently, real-time PCR has been used as a quantitative gene amplification technique for the detection of both bacteria and viruses associated with respiratory tract diseases (3, 5, 11). In this study, real-time PCR detected *C. pneumoniae* DNA of a wide quantitative range. Twenty-three of 105 analyzed samples were real-time PCR positive and originated from 13 patients, all with positive results from sputum specimens. The sputum specimens, with an average of 8.6×10^5 copies per ml, had large amounts of *C. pneumoniae* DNA in comparison to nasopharyngeal and throat specimens. This can be explained by a higher number of cells and larger sampling volume for sputum than for the other two sampling types. This result could also be due to a higher rate of bacterial secretion in sputum. Sputum sampling is easy but limited, since a *C. pneumoniae* infection does not always generate sputum production. Therefore, we conclude that sputum samples should be collected when possible from patients with a suspected respiratory *C. pneumoniae* infection. The sensitivity of real-time PCR was investigated by a parallel comparison with nested touchdown PCR. Nested PCR has previously been validated as a sensitive and specific detection method for *C. pneumoniae* when compared to other methods such as cell culture (1, 10). Sixteen of 20 samples positive by nested PCR were confirmed by real-time PCR. An additional seven samples were detected as positives by real-time PCR; five of these samples originated from four patients previously not diagnosed as having a *C. pneumoniae* infection. It is possible that real-time PCR is a more sensitive detection method than nested PCR when samples from patients are used. There are several advantages of real-time PCR as a diagnostic method, including rapidity, automation, quantitative measurement, and minimized risk for carryover contamination. Nested touchdown PCR has shown high sensitivity for *C. pneumoniae* detection, but it is labor-intensive and has no capacity for quantitative measurement. We conclude that real-time PCR is a promising future diagnostic method for the quantitative detection of respiratory, and perhaps other, *C. pneumoniae* infections.

TABLE 2. Comparison of real-time and nested touchdown PCR testing of 20 nasopharyngeal, 22 throat, and 20 sputum samples from 22 patients

PCR assay	No. of positive samples/total from:		
	Nasopharynx	Throat	Sputum
Real time	4/20	6/22	13/20
Nested	4/20	6/22	10/20
Congruent samples	2/6	5/7	9/14

REFERENCES

1. **Boman, J., A. Allard, K. Persson, M. Lundborg, P. Juto, and G. Wadell.** 1997. Rapid diagnosis of respiratory *Chlamydia pneumoniae* infection by nested touchdown polymerase chain reaction compared with culture and antigen detection by EIA. J. Infect. Dis. **175:**1523–1526.
2. **Boman, J., C. A. Gaydos, and T. C. Quinn.** 1999. Molecular diagnosis of *Chlamydia pneumoniae* infection. J. Clin. Microbiol. **37:**3791–3799.
3. **Desjardin, L. E., Y. Chen, M. D. Perkins, L. Teixeira, M. D. Cave, and K. D. Eisenach.** 1998. Comparison of the ABI 7700 system (TaqMan) and competitive PCR for quantification of IS*6110* DNA in sputum during treatment of tuberculosis. J. Clin. Microbiol. **36:**1964–1968.
4. **Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick.** 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19:**4008.
5. **Gault, E., Y. Michel, A. Dehée, C. Belabani, J.-C. Nicolas, and A. Garbarg-Chenon.** 2001. Quantification of human cytomegalovirus DNA by real-time PCR. J. Clin. Microbiol. **39:**772–775.
6. **Grayston, J. T., L. A. Campbell, C.-C. Kuo, C. H. Mordhorst, P. Saikku, D. H. Thom, and S.-P. Wang.** 1990. A new respiratory tract pathogen: *Chlamydia pneumoniae* strain TWAR. J. Infect. Dis. **161:**618–625.
7. **Ieven, M., and H. Goossens.** 1997. Relevance of nucleic acid amplification techniques for diagnosis of respiratory tract infections in the clinical laboratory. Clin. Microbiol. Rev. **10:**242–256.
8. **Kuo, C.-C., L. A. Jackson, L. A. Campbell, and J. T. Grayston.** 1995. *Chlamydia pneumoniae* (TWAR). Clin. Microbiol. Rev. **8:**451–461.
9. **Livak, K. J., S. J. Flood, J. Marmaro, W. Giusti, and K. Deetz.** 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. **4:**357–362.
10. **Tong, C. Y. W., and M. Sillis.** 1993. Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. J. Clin. Pathol. **46:**313–317.
11. **van Elden, L. J. R., M. Nijhuis, P. Schipper, R. Schuurman, and A. M. van Loon.** 2001. Simultaneous detection of influenza viruses A and B using real-time quantitative PCR. J. Clin. Microbiol. **39:**196–200.