JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 1999, p. 3791–3799 Vol. 37, No. 12
0095-1137/99/$04.00+0


# MINIREVIEW

# Molecular Diagnosis of *Chlamydia pneumoniae* Infection

JENS BOMAN,[1]* CHARLOTTE A. GAYDOS,[2] AND THOMAS C. QUINN[3]

*Department of Virology, Umeå University, Umeå, Sweden[1]; Department of Medicine, Division of Infectious Diseases, The Johns Hopkins University, Baltimore, Maryland[2]; and National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland[3]*

*Chlamydia pneumoniae* is a common and important intracellular bacterium implicated in upper and lower respiratory tract infections in humans. Also, *C. pneumoniae* has been associated with chronic diseases such as atherosclerosis and asthma. Since *C. pneumoniae* can cause severe clinical disease, correct diagnosis and therapy are important issues. However, conventional assays for the detection of *C. pneumoniae* have limitations, and there is a need for more accurate diagnostic methods. Nucleic acid amplification (NAA) techniques have the potential to offer clinical laboratories a convenient means of detecting *C. pneumoniae* rapidly and reliably, ensuring optimal clinical decisions and patient care, including choice of appropriate antibiotic therapy. This minireview discusses the molecular biology-based amplification methods that are currently available for the detection of *C. pneumoniae* as well as potential new techniques. Topics that are discussed include specimen collection, preparation of nucleic acid from samples, choice of gene target and primer set selection, optimal amplification conditions, and detection of the amplification product. Also reviewed are methods for recognition and prevention of false-positive and false-negative results, evaluations of new and old tests, and clinical applications.

## METHODOLOGICAL ASPECTS

**General.** Routine implementation of NAA techniques requires that several methodological issues be considered. Issues that must be addressed include the selection of appropriate gene targets for amplification, choice of appropriate primers, specimen collection, nucleic acid preparation, detection and attenuation of inhibitors of the amplification reaction, appropriate detection of amplification products, and the use of controls for contamination and nonspecific reactions (33, 57, 67). Because of the inherent complex interactions of the PCR components, no single PCR protocol will be appropriate for all situations. Therefore, each new diagnostic PCR application will require careful optimization of procedures. Common problems include no detectable product or a low yield of the desired product, nonspecific background DNA products, primer-dimer formation, and mutations or heterogeneity due to misincorporation of nucleotides in the genome (34). Suboptimal reaction conditions may occur for a number of reasons, including the use of inappropriate primers, the use of improper cycling time and temperature conditions, variable polymerase enzyme quality, temperature inconsistencies across the block of the thermal cycler, and the use of suboptimal $Mg^{2+}$ concentrations (92). Reagent concentrations to be optimized by titration include those of the enzyme, deoxynucleoside triphosphates, magnesium, and primers. Additional considerations include the performance of enzymes from different suppliers, variations in PCR buffers, use of proteins for enzyme stabilization, and alteration of annealing, extension, and denaturation temperatures and times and cycle number (34, 67, 76). Higher-stringency annealing temperatures during the first cycles will help to increase specificity, and the touchdown PCR (19) with decreasing annealing temperatures is a technique that has been adopted for reduced background amplification and thus increased specificity (10, 85). Nested PCR with amplification in two steps with two different primer pairs may also be used to greatly increase both the sensitivity and the specificity of the PCR procedure (6, 10). The nested PCR technique requires careful control of product carryover contamination. However, in this technique the final and shorter PCR product generated during the second amplification cannot serve as a target of the primers of the first PCR step, providing additional specificity. This is an important advantage compared to reamplification with the same primer pair, e.g., booster PCR (75). Another technique that may be used for increased specificity is hot-start PCR, in which all the PCR components except one critical reagent are mixed together and kept at a temperature above the threshold of nonspecific binding of primer to template. The missing component is then added immediately before cycling to allow the cycling to begin at a high temperature, precluding nonspecific primer binding (57).

The use of an expanded "gold standard" with a second NAA test that targets a different gene (78, 79) is needed to accurately validate new nucleic acid-based techniques since culture may lack sufficient sensitivity. Presently, the lack of a consensus standard makes it difficult to evaluate new methods, and, thus far, few PCR tests for the detection of *C. pneumoniae* DNA have been adequately evaluated in comparison with culture, species-specific serology, and alternative NAA assays in studies with large numbers of clinical specimens. The fact that *C. pneumoniae* may be carried in the respiratory tract for prolonged periods (29) may make it difficult to evaluate the use of different diagnostic tests for the diagnosis of acute infections, especially in comparisons of serology and detection methods such as PCR and culture. A relative comparison of PCR with cell culture inclusion-forming units (IFU) per milliliter from stock solutions may not be very informative since different culture systems may vary considerably in sensitivity. If an insensitive culture system is used, the PCR may appear to be very sensitive, which may not be the case. Since PCR can detect the presence of *C. pneumoniae* DNA derived from noninfectious reticulate bodies and nonviable elementary bodies, a PCR sensitivity less than 1 IFU can be expected, even if a very sensitive culture system is used (93). Comparison with elementary body

* Corresponding author. Mailing address: Department of Clinical Virology, University Hospital of Umeå, Building 6G, Room 178, S-901 85 Umeå, Sweden. Phone: 46-90-7851304. Fax: 46-90-129905. E-mail: jens.boman@climi.umu.se.

count, e.g., by using direct fluorescent assay (DFA), can, in the hand of an experienced microscopist, be a better method for estimation of PCR sensitivity (42, 85). Since NAA techniques aimed at targeting DNA can detect both viable and nonviable organisms, detection of cDNA by reverse transcription-PCR of mRNA may be a useful complement to cell culture to establish if the *C. pneumoniae* infection is productive (41).

**False-positive results.** In order to minimize the risk of false-positive results due to contamination of specimens with previous amplification products (product carryover), positive controls or positive specimens (cross-contamination), or exogenous sources such as an infected technician, guidelines have been formulated. It is imperative to pay attention to these recommendations, which include the use of meticulous laboratory techniques, strict physical separation of work areas and containment devices, inactivation protocols, and environmental decontamination (47, 91). The use of strongly positive controls, i.e., highly concentrated solutions of extracted *C. pneumoniae* DNA, should be avoided. It is also recommended that several well-characterized negative controls and multiple reagent controls be included with each amplification (48). The negative controls, which should include all PCR components except the target DNA, should be run in parallel with the samples throughout the whole procedure, beginning with extraction of the nucleic acid from the clinical specimens to detection of amplification product. The positive controls should consist of low and very low DNA levels for *C. pneumoniae*. Confirmation of all positive findings by reanalysis with reextraction of the original sample and repeat PCR is recommended to minimize the risk of false-positive results due to sporadic contamination events, since there is a low risk that a sporadic contamination event occurs twice in the same way (11, 47). Nonreproducible PCR results can be due to contamination, low copy number, inhibition, primer mismatch due to strain variation at the primer recognition site, or technical or methodological errors.

**False-negative results.** False-negative results may be due to inhibitors, human error including volume sampling error, inadequate extraction of nucleic acid, equipment failure, reagent problems such as use of an inappropriate magnesium concentration or of an inappropriate primer synthesis procedure, low sensitivity of the detection system, low organism load, or poor specimen quality (91). False-negative results can often be detected by means of good quality control procedures, and it is recommended that a positive control that amplifies weakly but consistently be used (48). PCR inhibitors generally act at one or more of three essential points in the reaction: interference with lysis of the host cell in the clinical specimen, target nucleic acid degradation, and inhibition of polymerase activity for amplification of target DNA (92). Sophisticated and complicated DNA extraction methods for the removal of PCR inhibitors may be used in research laboratories but create work-flow problems in routine diagnostic laboratories. In order to identify inhibitors of the PCR, the negative samples can be spiked with a small amount of target DNA (36), but this procedure is labor-intensive. Another solution for direct monitoring of inhibitory activity is to include a competitive hybrid internal control for each sample that is coamplified (when inhibition does not occur) in the reaction mixture with the same primers as those used for the real target. A low concentration of internal control is added to each PCR mixture to confirm true-negative PCR results by ruling out the presence of detectable PCR inhibition and to identify those specimens that require specific measures in order to attenuate PCR inhibitors. A positive internal control amplification indicates the lack of consequential inhibitors in the clinical specimen (70, 86).

By adding the internal control before the DNA extraction procedure (43), the control not only detects inhibition of the PCR but also monitors the sample preparation procedure (33), with the exception of the release of nucleic acid from the cell. Nonamplifiable samples can be reanalyzed after dilution of the processed sample, storage at 4°C, freezing-thawing, heating, or treatment of the sample by an alternative extraction protocol (50, 70). Dilution of samples is a simple method that may improve amplification by reduction of inhibitors; however, the sensitivity may also be reduced as a result of dilution of target DNA, and consequently, samples with low copy number might be false negative due to this procedure. The use of a nested PCR reduces the problem with PCR inhibitors substantially, since present PCR inhibitors are diluted between the first and the second amplification reactions (6).

In theory, use of a larger volume of the sample in the amplification reaction may increase the sensitivity, but this strategy may be hampered by an unacceptable increase in the level of PCR inhibitors in a subset of the samples (2, 13, 33). Amplification of human genes such as the β-globin gene can be valuable for confirming the presence of human DNA in the sample (11), i.e., confirming that the sample contains cellular material, but is not recommended as a test of inhibition since the number of copies of this gene in each sample may be much higher than the number of copies of the *C. pneumoniae* target gene. In addition, the requirement for different amplification conditions may be another limitation when housekeeping genes are used for control of inhibition (79).

A large number of DNA polymerases originating from different sources are commercially available. These enzymes may exhibit differences in processivity and fidelity (1, 92), and some enzymes may be more suitable than others for specific tasks; it is therefore worthwhile to compare enzymes from different suppliers. A large number of endogenous and exogenous components that may inhibit or facilitate the amplification reaction are listed by Wilson (92). Compounds that may cause complete reaction failure or reduced sensitivity include phosphates when phosphate-buffered saline buffer is used to collect samples, hemoglobin, urea, heparin, constituents of bacterial cells, nontarget DNA, pollen, glove powder, dust, UV light-irradiated mineral oil, and laboratory plasticware including some microcentrifuge tubes.

**Specimen collection.** The specimen collection depends on the type of clinical condition. Suitable specimens for use in the detection of *C. pneumoniae* include sputum specimens (10, 85), bronchoalveolar lavage (BAL) specimens (17, 22), swabs of the nasopharynx and throat (6, 10, 20, 25, 29), nasopharyngeal (42, 86) and ear fluid (9, 82) aspirates, gargled water (71), and tissues from a biopsy or autopsy (45, 68). A few studies on the detection of *C. pneumoniae* DNA in blood specimens have been published (11, 61, 62), but the use of blood samples for direct detection of *C. pneumoniae* needs further evaluation. Deep-seated tissues are not easily accessible, and hence, detection of *C. pneumoniae* in blood specimens may be a valuable tool for the identification of individuals with systemic infection and for monitoring the response to antichlamydial therapy. Boman et al. (10) compared different respiratory sampling sites, and sputum samples seem to be superior to nasopharyngeal and throat swabs for the detection of *C. pneumoniae*. Nasopharyngeal and throat swab samples are of value for patients who cannot produce a sputum sample (10), such as young children. However, not all protocols seem to be useful for the detection of *C. pneumoniae* in sputum (20, 87). In a pneumonia treatment study with 260 previously healthy children aged 3 to 12 years, it was shown that the posterior nasopharynx may be superior to the throat as a source for isolation

of the organism (8, 30). Of 34 children from whom *C. pneumoniae* was isolated, nasopharyngeal samples were positive for all children, but throat samples were positive for only 50% of the same children (30).

For collection of nasopharyngeal specimens, a Dacron fiber-tipped swab with a plastic shaft is preferred over a calcium alginate fiber-tipped swab with an aluminum shaft since the latter may contain PCR inhibitors (88). For cell culture, some adhesives used to attach the Dacron to the swab can be toxic to the cells or inhibit the growth of *C. pneumoniae* (30). Sucrose-phosphate buffer containing heat-inactivated fetal calf serum and antibiotics is commonly used for collection and transport of samples for *Chlamydia* culture. However, *Chlamydia* transport media intended primarily for cell culture may not be optimal for the collection and transport of samples that should be analyzed by an NAA test (5). When only PCR is to be performed, simpler and more appropriate collection buffers that do not contain PCR inhibitors should be used (67). Black et al. (6) found that transport media commonly used for chlamydial swab specimens were inhibitory for PCR. Therefore, both sampling devices and transport media should be checked for the presence of PCR inhibitors. The samples should be transported to the laboratory as soon as possible and kept at 4°C or frozen at −70°C. For mRNA determinations it is important to freeze the samples at −70°C as soon as possible to prevent degradation (74).

**Processing of specimens.** For routine diagnostic purposes, the use of a simple method for processing of clinical samples is important. The main goal is development and evaluation of uncomplicated processing methods that efficiently release target DNA from the specimen without sacrificing sensitivity, in combination with successful removal of PCR inhibitors that may be present in the DNA preparation. The optimal technique used for extraction of nucleic acid depends on the type and amount of starting material, the amount of potential PCR inhibitors present in the sample, and the type of nucleic acid being extracted (65).

Gnarpe and Eriksson (25) compared use of the Amplicor sputum sample preparation kit (Roche Diagnostics) with proteinase K treatment with throat samples collected from 53 patients seeking medical attention for symptoms of long-standing respiratory infection. They found that the sensitivity of the *C. pneumoniae* PCR was increased by treatment involving more complete lysis of cells derived from the specimen by use of the Amplicor sputum sample preparation kit.

Daugharty et al. (18) evaluated seven procedures for extracting chlamydial DNA from buffy coats spiked with *Chlamydia* elementary bodies. The investigators concluded that the QIAamp kit (Qiagen) was the extraction method of choice due to its simplicity, rapidity, and sensitivity. Maass and Dalhoff (50) assessed the presence of PCR inhibitors in 75 BAL specimens after treatment by three common sample preparation methods. PCR inhibitors were present in 31% of the samples processed by heat treatment, in 12% of the samples for which single-step proteinase K digestion was used, and in 0% of the samples that were subjected to cetyltrimethylammonium bromide-based DNA purification. Unfortunately, the sample preparation method most effective in identifying *C. pneumoniae*-infected patients was the least convenient since it is labor-intensive.

Wilson et al. (93) compared four methods for DNA extraction from dilutions of cultured *Chlamydia*: (i) a guanidine isothiocyanate-silica method, (ii) an extraction method with Chelex, (iii) boiling of samples for 15 min followed by high-speed centrifugation for 10 min, and (iv) proteinase K treatment for 30 min followed by inactivation of the protease by boiling for 10 min. The proteinase K digestion-based method was 10 times more sensitive than the guanidine isothiocyanate-silica-based method. The Chelex-based method was between 10- and 100-fold less sensitive than the guanidine isothiocyanate-silica-based method. Finally, the boiling method was the least efficient one.

Meijer et al. (58) developed an extended procedure for isolation of DNA from vascular tissue using a commercial DNA extraction kit (Easy-DNA Kit; Invitrogen) followed by a silica-based method. PCR inhibitor-free chlamydial DNA was obtained from vascular tissue samples spiked with *C. pneumoniae* DNA. To increase the specificity of the reaction, anti-polymerase antibodies were used. The antibodies prevented primer-dimer formation. The investigators concluded that the method can serve as a reference method.

If a swab is used for collection, the tube should be vortexed with glass beads before removal of the swab from the collection medium (10). Sputum samples and other viscous samples should be treated with a mucolyzing agent (e.g., *N*-acetylcysteine), washed, and concentrated before the extraction of DNA (10, 80). Protein digestion with proteinase K followed by DNA extraction with phenol-chloroform and precipitation with alcohols could be used for high purity, but this extraction procedure is labor-intensive and evaluation of simpler methods should be made in clinical studies with different types of samples. Methods useful for extraction of DNA from swabs may not be appropriate for extraction of DNA from tissue (54).

**Target regions and choice of primers.** *C. pneumoniae* has a genome size of 1,230,230 bp, consisting of 1,073 likely protein-coding genes (39). For design of primers it is important to choose a gene sequence that is highly conserved within the species to avoid failure of the PCR due to mismatches between the primers and the target gene. Moreover, the sequence should differ significantly from other gene sequences, including sequences of other *Chlamydia* species, in order to avoid false-positive results due to amplification of non-*C. pneumoniae* sequences.

Several different targets, primers, and reaction protocols have been described; commonly used PCR methods and target regions are summarized in Table 1. Unfortunately, not many studies comparing the performance of PCR methods with different *C. pneumoniae* target regions and primers have been published (55). Important general and specific considerations for optimal primer design for the detection of *Borrelia burgdorferi* DNA have recently been described by Schmidt (79). These recommendations could also be adopted for use in choosing primers for amplification of *C. pneumoniae* DNA. The primer pair should (i) amplify all strains of the bacterium, (ii) amplify all strains with the same sensitivity, (iii) not amplify DNA from other microbes (i.e., it should be highly specific), (iv) form stable duplexes with the template, (v) be 15 to 30 nucleotides long, (vi) have a high G+C content (generally accepted value, 50%), (vii) not form 3′ end duplexes with themselves, (viii) not contain homo-oligomers or short repeated sequences, (ix) be stable at the 5′ termini but somewhat unstable at the 3′ ends, (x) be free of significant complementarity at their 3′ ends (which may promote primer-dimer formation), (xi) have melting temperatures that are very close to each other, and (xii) have a difference in melting temperatures with respect to the target that does not exceed 20°C (target self-annealing will otherwise predominate).

As mentioned above, when NAA-based methods are developed, attention must be paid to test specificity so that DNA from genetically related pathogens is not amplified by the primers. In general, the test specificity increases with elevated annealing temperature (long primers and primers with high

TABLE 1. In-house-developed PCR assays for detection of *C. pneumoniae*[a]

| Author(s) | Year of publication | Reference | Gene target | Product size (bp) | Type |
|---|---|---|---|---|---|
| Holland et al. | 1990 | 31 | MOMP | 144 | S+R |
| Watson et al. | 1991 | 89 | 60-kDa protein | 582 | S+R |
| Campbell et al. | 1992 | 13 | Cloned *Pst*I | 474 | S+H |
| Gaydos et al. | 1992 | 21 | 16S rRNA | 463 | S (+EIA) |
| Kaltenboeck et al. | 1992 | 40 | MOMP | 1,120, 930 | M+R (N) |
| Black et al. | 1992 | 5 | MOMP | 1,070 | S+R |
| Rasmussen et al. | 1992 | 73 | MOMP | 145 | S+R+H |
| Tong and Sillis | 1993 | 85 | MOMP | 333, 207 | N |
| Tjhie et al. | 1993 | 84 | MOMP | 360, 320, 237 | S+N+H |
| Black et al. | 1994 | 6 | 16S rRNA | 397, 858 | N |
| Kubota | 1996 | 44 | 53-kDa protein | 499 | S |
| Ong et al. | 1996 | 66 | MOMP | 498, 227 | N |
| Khan et al. | 1996 | 41 | DnaK | 415 | RT |
| Wilson et al. | 1996 | 93 | 16S rRNA | 317, 178 | N |
| Khan and Potter | 1996 | 42 | Cloned *Pst*I | 474, 204 | N+H |
| Maass et al. | 1997 | 51 | Cloned *Pst*I | 474, 128 | N+H |
| Messmer et al. | 1997 | 60 | 16S rRNA | 436, 221 | M |
| Nyström-Rosander et al. | 1997 | 64 | 16S rRNA | 463, 269 | N |
| Yoshida et al. | 1998 | 94 | MOMP | 258 | S+R |
| Jantos et al. | 1998 | 36 | 16S rRNA | 465 | S+EIA |
| Petitjean et al. | 1998 | 69 | 60-kDa protein | 183 | S+EIA |
| Madico et al. | Submitted | 56 | 16S rRNA | 195 | S or M |

[a] Abbreviations: S, single-step PCR; N, nested PCR; M, multiplex PCR; RT, reverse transcriptase; R, restriction enzyme digestion; H, hybridization.

G+C contents). It is necessary to use computer programs and sequence databases for selection of optimal primers. However, this procedure does not exclude subsequent careful practical and clinical experiments for testing of specificity since complete sequence information is not available for most organisms (57). Not only serial dilutions of cultured purified target DNA but also clinical specimens that may contain interfering extraneous DNA and inhibitory substances should be used to test for sensitivity (79).

**PCR methods.** Holland et al. (31) developed a major outer membrane protein (MOMP)-based PCR test that could identify three species of *Chlamydia* (*C. trachomatis*, *C. pneumoniae*, and *C. psittaci*) using three primer pairs and one restriction enzyme digestion.

Rasmussen et al. (73) described a protocol that amplifies a conserved genus-specific target of the chlamydial MOMP gene followed by restriction enzyme digestion for species identification.

Watson et al. (89) developed a PCR assay based on amplification of the 60-kDa cysteine-rich outer membrane protein genes of *C. psittaci*, *C. pneumoniae*, and *C. trachomatis*, followed by species differentiation with four restriction endonuclease digestion enzymes. Similarly, Tjhie et al. (84) developed a general PCR with a target within the MOMP gene. Subsequent species-specific differentiation of *C. trachomatis*, *C. pneumoniae*, and *C. psittaci* was performed by hybridization of the amplified PCR product with internal probes.

Several of the early methods described for the detection of *C. pneumoniae* are rather complicated and have been poorly evaluated with clinical specimens. The PCR protocol described by Tong and Sillis (85), in which a nested touchdown PCR with a target within the MOMP-coding gene is used, has proved to be both sensitive and specific compared with other diagnostic methods including cell culture, DFA, and microimmunofluorescence (MIF) serology (10, 36, 85). The outer primers amplify a 333-bp product from both *C. pneumoniae* and *C. psittaci* but do not amplify DNA from *C. trachomatis*. One of the internal primers is specific for *C. pneumoniae*; therefore, only the first-stage product from *C. pneumoniae* can be amplified in the nested PCR, yielding a 207-bp product.

16S rRNA genes (21) have also been widely used as the target, and a combined PCR and enzyme immunoassay (EIA) method developed by Gaydos et al. (22–24) has proved to be specific and fairly sensitive for detection of *C. pneumoniae* in respiratory specimens, although its sensitivity may be improved. The single primer pair product (463 bp) is detected in an EIA after hybridization to a biotinylated RNA probe complementary to a part of the amplified 16S rRNA gene. Thus, the method combines the sensitivity and specificity of PCR with a convenient and objective detection system based on the EIA technique after hybridization with an RNA probe.

Nyström-Rosander et al. (64) have developed a nested PCR using the 16S rRNA primers Cpn A and Cpn B described by Gaydos et al. (21) as the outer primers and Cpn 1 and Cpn 2 as the internal primers, generating a 269-bp product. The test was used to detect *C. pneumoniae* in sclerotic aortic valves of patients undergoing valve replacement.

Black et al. (6) used a nested primer strategy for direct species-specific detection of the 16S rRNA gene of *C. pneumoniae*. The test was applied to 58 swab specimens collected in two different studies with patients with respiratory illness and was found to be as sensitive as culture or serology for the detection of *C. pneumoniae* infection. Black and colleagues concluded 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 medium or in the specimen itself. The nested PCR overcame the inhibition problem with crude specimen preparations and was found to be a fast alternative to time-consuming and labor-intensive DNA purification and extraction methods. The satisfactory performance of a nested PCR has recently been confirmed (10).

Wilson et al. (93) have designed a one-tube nested PCR using two primer pairs with different melting points in one reaction mixture for amplification of a *C. pneumoniae*-specific

region of the 16S rRNA gene. The initial thermal cycles were selected to allow annealing and extension of only the outer primer pair, while in later cycles a temperature that allowed inner primer annealing was used. The inner primers were labeled with biotin (one primer) and fluorescein (the other primer) so that the dually labeled PCR product could be immobilized onto antibiotin-coated microtiter plates and then detected colorimetrically with an antifluorescein-enzyme conjugate.

Jantos et al. (36) have developed a simplified PCR-EIA based on the 16S rRNA primers described by Gaydos et al. (21). Biotin-labeled *C. pneumoniae* PCR products hybridize to a digoxigenin-labeled probe. The complex is captured in streptavidin-coated microtiter plates and then detected with an antidigoxigenin-peroxidase conjugate and a colorimetric substrate. The PCR-EIA was found to be much more sensitive than visualization of PCR products on an agarose gel and as sensitive as Southern blot hybridization.

Perhaps the most frequently used PCR system for the detection of *C. pneumoniae* DNA is the system developed by Campbell and colleagues (13), derived from a cloned 474-bp *C. pneumoniae*-specific *Pst*I fragment targeted by the primers HL and HR and the probe HM. A modification of this PCR test was made by Khan and Potter (42), who designed a fourth primer (primer HR-2) and developed a nested PCR with the internal primers HM-1 and HR-2, which generate a product of 204 bp. Also, Maass et al. (51) have developed a nested PCR method based on the method originally described by Campbell et al. (13). That PCR method yields a 128-bp amplification product. For additional confirmation, nonradioactive DNA hybridization was performed (51).

Kubota (44) has designed a PCR test that amplifies a sequence within the 53-kDa protein gene of *C. pneumoniae*. By this PCR, DNA was amplified from 24 *C. pneumoniae* strains collected from different geographical locations. DNAs from *C. trachomatis*, *C. psittaci*, *C. pecorum*, or several other bacteria commonly related to respiratory tract infections were not detected. Unfortunately, the test was not evaluated with clinical specimens.

Recently, Yoshida et al. (94) designed a primer pair for genus-specific detection of *Chlamydia* MOMP DNA. By restriction endonuclease analysis of the amplified products with *Alu*I and *Pvu*II, detection and differentiation of the four known *Chlamydia* species can be achieved.

**Multiplex PCR.** Multiplex PCR tests with primer sets included in the PCR for the amplification of more than one target sequence have also been developed. Kaltenboeck et al. (40) simultaneously used one genus-specific primer and three species-specific primers for amplification of the MOMP-coding gene of different *Chlamydia* groups (trachoma, pneumonia, and polyarthritis). The groups were differentiated by the length of the amplified DNA fragment. To discriminate the organisms at the species level, restriction endonuclease analyses are necessary after the second PCR step. Messmer et al. (60) developed a nested multiplex PCR for the simultaneous detection of *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* with the 16S rRNA gene as the target. Madico et al. (56) developed a multiplex PCR for the determination and differentiation of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* using primer sets from the 16S-23S rRNA genes, which provides excellent sensitivity and specificity.

Potential problems with multiplex PCR include sensitivity and specificity problems that might occur if the optimal annealing temperatures for the individual primers are not identical (33). Gröndahl et al. (28) developed a multiplex PCR method to allow in one test the detection of nine different microorganisms including *C. pneumoniae*. Nasopharyngeal aspirates were collected from 1,118 children with acute respiratory tract infections. Only one sample (<0.1%) was positive for *C. pneumoniae*, a surprisingly low detection rate.

**Detection of PCR product.** If a one-step-based PCR method is used, detection of the PCR product by a specific probe-based hybridization assay is recommended for increased sensitivity and specificity (79). Microplate-based colorometric methods with EIAs (PCR-EIAs) may give results comparable to those provided by Southern and dot blot hybridization and offer some advantages in terms of simplicity and speed (24, 36, 67, 93). For the detection of the products generated by nested PCR, agarose gel electrophoresis followed by ethidium bromide staining seems to be a reliable detection method (6, 10).

**Quantification of *C. pneumoniae* nucleic acid.** Direct, quantitative, real-time DNA and RNA detection is possible by using specifically designed automated equipment such as the Perkin-Elmer Applied Biosystem 7700 DNA sequence detector (65). Development of methods for quantitation of *C. pneumoniae* nucleic acid has begun, but the expense associated with the advanced instrumentation is a limitation that may make such systems prohibitive to many investigators.

**Standardization of different PCR protocols.** Presently, 14 laboratories in Europe ($n = 9$) and the United States ($n = 5$) are participating in a study comparing different PCR methods for the detection of *C. pneumoniae*. The results obtained in that study may be a guide in the choice of an appropriate PCR method, including choice of target gene, primers, PCR conditions, and detection system. Until then, it is too early to recommend a standard PCR procedure due to the lack of comparative studies.

**Alternative amplification techniques.** Besides PCR, alternative amplification techniques have been developed, including nucleic acid sequence-based amplification, transcription-mediated amplification, ligase chain reaction, Qβ replicase amplification, and strand displacement amplification (81, 90). These techniques may all be useful alternatives to PCR, but so far, no study describing the application of any of these methods for the detection of *C. pneumoniae* has been published.

## DIAGNOSTIC ASPECTS

**General clinical aspects.** Several clinical studies with PCR for the detection of *C. pneumoniae* in respiratory samples have been performed and their results have been published (6, 10, 17, 23, 26). Although the performance of many PCR methods seems to be very good, most of these studies have been conducted by skilled research personnel under carefully controlled conditions. These methods may not perform equally well in routine laboratories with crude specimen preparations. In addition, some of these methods are cumbersome and time-consuming and are not easily adapted or practical for use in routine clinical laboratories. Moreover, the risk of contamination is greatly increased when large numbers of specimens are analyzed in the laboratory (79). More studies need to be conducted to determine the sensitivity and specificity of PCR by testing large numbers of clinical specimens from symptomatic and asymptomatic individuals (30). The samples should be tested in parallel with a sensitive culture system (10, 23) and by one or several alternative amplification methods (e.g., a PCR that targets alternative gene sequences). Serology should also be performed to distinguish a carrier state from acute infection (10, 23, 87).

Introduction of highly sensitive NAA techniques such as PCR may increase our ability to identify *C. pneumoniae* carriers who are latently or persistently infected. Since the clinical

TABLE 2. Comparison of methods for diagnosis of respiratory *C. pneumoniae* infection

| Reference | Sample[a] | No. of samples | Subject age range (yr) | No. (%) of samples positive by the following detection test: | | | | Confirmation by MIF-serology (no. of samples positive/total no. of samples tested [%]) |
|---|---|---|---|---|---|---|---|---|
| | | | | PCR | Culture | EIA | DFA | |
| 6 | Np, Th | 58 | NS[b] | 10 (17) | 8 (14) | ND[c] | ND | 6/9 (67) |
| 10 | Np, Th, Sp | 116 | 3–79 | 30 (26) | 23 (20) | 26 (22) | ND | 26/27 (96) |
| 16 | Npa | 108 | 9–11 | 43 (45) | ND | ND | ND | ND |
| 17 | Ba | 104 | NS | 15 (14) | 4 (4) | ND | ND | ND |
| 22 | Ba | 108 | NS | 12 (11) | 0 (0) | ND | ND | 4/8 (50) |
| 23 | Np | 136 | NS | 27 (20) | 31 (23) | ND | 6/12[d] | 7/23 (30)[e] |
| 24 | Ba | 385 | NS | 13 (3) | 3 (1) | ND | ND | 3/13 (23) |
| 26 | Th | 54 | NS | 8 (15) | 7 (13) | ND | ND | 9/9 (100) |
| 28 | Npa | 1,118 | NS | 1 (0.1) | ND | ND | ND | ND |
| 32 | Np | 104 | 21–53 | 2 (2) | 1 (1) | ND | 1/1 | 0/2 (0) |
| 36[f] | Th | 368 | NS | 15 (4) | 1 (0.3) | ND | ND | ND |
| 42 | Npa | 152 | 0–2 | 2 (1) | ND | ND | ND | ND |
| 59[f] | Urs, Npa, Ba | 135 | NS | 19 (14) | ND | ND | ND | ND |
| 69 | Npa, Ba | 68 | 32–90 | 4 (6) | ND | ND | ND | 3/4 (75)[g] |
| 70 | Np | 237 | NS | 21 (9) | ND | ND | ND | ND |
| 71[f] | Gw | 193 | 5–16 | 3 (2) | ND | 0 (0) | ND | ND |
| 85 | Sp | 26 | <65 | 10 (38) | 1/10 | 9/10 | 10/10 | 7/10 (70) |
| 86 | Npa | 71 | NS | 2 (3) | ND | ND | ND | ND |
| 87 | Np, Th, Sp | 156 | 20–93 | 31 (20) | 1 (0.6) | ND | ND | 7/31 (23) |

[a] Np, nasopharyngeal swab; Th, throat swab; Sp, sputum; Npa, nasopharyngeal aspirate; Ba, BAL specimen or bronchial aspirate; Urs, upper respiratory samples; Gw, gargled water.
[b] NS, not stated.
[c] ND, not done.
[d] Results are number of samples positive/number of samples tested.
[e] Only a single serum was available.
[f] Confirmation was performed by an alternative PCR method.
[g] Immunoglobulin A titer, ≥1/32.

relevance of asymptomatic infection and prolonged carriage is unclear, NAA sensitivity may exceed clinical relevance (63, 87), and the interpretation of results may occasionally be problematic when there is no corresponding serologic response. In symptomatic patients, detection of a persistent and perhaps harmless *C. pneumoniae* coinfection may actually obscure the true etiology. A single PCR result, especially a qualitative one, negative or positive, must always be viewed with caution and interpreted in the context of the patient's symptomatology and together with clinical findings and the results of other procedures for the detection of *C. pneumoniae* and for other pathogens. Continuous education of clinicians in the proper use and interpretation of PCR-generated results, as well as in the limitations of the tests, is also of importance.

Comparison of the data from different clinical studies is complicated due to differences in sample collection, transportation, and extraction procedures, input sample volumes, target genes, primers, cycling parameters, and detection systems. More important, the lack of a consensus method for appropriate evaluation of the different methods precludes comparisons. The good test performance obtained in research studies with artificially prepared samples may not be achieved when the test is then used for amplification of targets from clinical samples (81). Nonetheless, various PCR procedures seem to be at least as sensitive as culture (10, 87), but specificity figures may be difficult to interpret due to the lack of an appropriate gold standard. Highly sensitive and specific, standardized, and commercially available amplified nucleic acid tests are sorely needed.

**Respiratory infection.** Since 1985 (77), *C. pneumoniae* has emerged as a common and important respiratory pathogen. More attention has recently been directed to diagnosis, since the acute infection is treatable with antibiotics. Appropriate therapy can be offered early in the course of the disease, provided that a rapid and reliable diagnostic test is available. This strategy may prevent the development of a chronic *C. pneumoniae* infection, although the opposite effect cannot be excluded (4). An outbreak of *C. pneumoniae* infections among University of Washington students was studied by Grayston and colleagues (26). Oropharyngeal swab specimens were collected and analyzed by culture and PCR; acute- and convalescent-phase serum samples were analyzed by MIF and complement fixation assays. A *C. pneumoniae* infection was diagnosed in 12 of 54 students with acute respiratory disease (pneumonia, $n = 7$; bronchitis, $n = 4$; sinusitis, $n = 1$) by MIF serology; 11 (92%) of these 12 MIF-positive students were also positive by the complement fixation test. *C. pneumoniae* was isolated from the oropharynges of 7 of the 12 MIF-positive students and was detected by PCR in the throat swab specimens of all 6 students with isolation-positive specimens examined and in 2 additional patients (8 of 11 patients tested). There was no evidence of *C. pneumoniae* organisms in the oropharynges by isolation or PCR in either patients ($n = 42$) or control persons ($n = 51$) without a serologic result that indicated acute *C. pneumoniae* infection. The investigators concluded that PCR is a promising technique for the quick demonstration of the organism in clinical specimens. Moreover, the findings provided evidence for an etiologic association of *C. pneumoniae* with pneumonia and bronchitis.

Dalhoff and Maass (17) used a PCR method based on published primers (12) and cell culture with HEp-2 cells to evaluate the incidence and clinical characteristics of *C. pneumoniae* in 57 hospitalized patients with pneumonia, 47 human immunodeficiency virus (HIV)-infected persons who were evaluated because of opportunistic lung disease or for staging of pulmonary involvement in systemic disease, and 100 control subjects

with noninfectious bronchopulmonary disorders. A BAL sample was collected from all 204 subjects included in the study. By using PCR, *C. pneumoniae* was detected in 16% of the patients with pneumonia, and 13% of HIV-infected persons but in none of the control persons. The sensitivity of cell culture was found to be much lower than that of PCR (4 versus 15 positive specimens), and no culture-positive but PCR-negative subjects were observed. That study (17) shows that *C. pneumoniae* may frequently be detected by PCR in samples from the lower respiratory tracts of hospitalized patients with pneumonia but is uncommon in immunocompetent patients without pneumonia, showing that the PCR results obtained with BAL samples are of clinical relevance in immunocompetent patients. Furthermore, *C. pneumoniae* seems to be common in BAL samples collected from HIV-infected persons. The finding of *C. pneumoniae* in BAL samples from immunocompromised patients has also been reported by Gaydos and colleagues (22), who demonstrated a prevalence of 11% by PCR of BAL samples from immunocompromised patients with pneumonia. *C. pneumoniae* may also be detected in the upper respiratory tracts of healthy subjects. Hyman et al. (32) addressed this question and conducted a prospective study with 104 subjectively healthy health care workers. Nasopharyngeal swab specimens were obtained and analyzed by culture and PCR-EIA. *C. pneumoniae* was detected by PCR-EIA in two (2%) individuals (one of whom was culture positive); none of them had serologic signs indicating acute infection. However, as the investigators concluded, these findings might not be generalized since health care workers can be expected to be exposed to patients with respiratory tract infections.

A comparison of different methods for the diagnosis of respiratory *C. pneumoniae* infection is presented in Table 2. It is obvious that the agreement between different methods is not always high or that confirmation of PCR findings by one or several alternative methods has not been performed.

**Cardiovascular infection.** PCR is commonly used, alone or in combination with other diagnostic methods, for the detection of *C. pneumoniae* in the cardiovascular system. *C. pneumoniae* DNA has been detected in atherosclerotic plaques in coronary arteries (14, 45, 52, 53), carotid arteries (15, 27, 35, 51), arteries of the lower extremities (46), abdominal aortic aneurysms (7, 38, 68), sclerotic heart valves (37, 64), and atrial tissue from donors of hearts for transplantation (83). The frequency of positive results varies considerably (from 0 to 100%) among different studies (3, 38, 49, 66, 90), which may be explained by the use of different PCR techniques and populations. However, even if samples from the same patients are investigated by different laboratories, the variation in the frequency of positive findings may be significant (72). Unfortunately, in a study by Ramirez et al. (72), the same tissue was not sent to the different laboratories since the samples were too small to divide; the samples submitted to each laboratory were therefore taken from different coronary arteries and from different sections of the atherosclerotic lesions. As *C. pneumoniae* may be randomly distributed in the plaques in the same patient (7, 35), it is difficult to compare the performance of the PCR methods used in that study. Only if the DNA extractions had been shared and analyzed by all the participating laboratories performing the PCR would it have been possible to compare the performances of the different PCR methods. In the future, such studies should be performed to elucidate the reported discrepancies in results among different laboratories.

**Other clinical applications.** Khan et al. (41) developed a reverse transcriptase (RT) PCR-based method to determine the antibiotic susceptibility of *Chlamydia* species. Those investigators found that the MICs for *C. pneumoniae* determined by RT-PCR were generally higher compared with those estimated by immunofluorescence staining. The authors concluded that the antibiotic concentrations which were sufficient to suppress detectable IFU were insufficient to cause complete inhibition of multiplication of *C. pneumoniae*, as measured by determination of RNA transcripts. Thus, the RT-PCR may provide a more sensitive and less subjective method than immunofluorescence staining for estimation of MICs and may even have applications for the diagnostic utility of NAA for detection of active multiplication of *C. pneumoniae* in patients with various disease states.

## CONCLUSIONS

Conventional methods for the detection of *C. pneumoniae* are complicated, time-consuming, and not always of high sensitivity and specificity. The application of NAA technology to *Chlamydia* research and routine diagnostics opens up new possibilities for the direct detection of *C. pneumoniae*. With further optimization, standardization, and simplification, NAA technology will certainly provide diagnostic laboratories with powerful and rapid means for the direct identification of *C. pneumoniae* in a variety of clinical specimens. Furthermore, researchers will be better equipped with tools to define the clinical spectrum of disease caused by this organism. However, until simplification and standardization of the NAA-based techniques have been achieved and/or commercial kits have been developed, diagnostic laboratories should implement these new techniques with caution and should pay attention to strict quality control procedures so that reliable results can be obtained.

### REFERENCES


1. **Abu Al-Soud, W., and P. Rådström.** 1998. Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting substances. Appl. Environ. Microbiol. **64:**3748–3753.
2. **Andersen, A. B., S. Thybo, P. Godfrey-Faussett, and N. G. Stoker.** 1993. Polymerase chain reaction for detection of *Mycobacterium tuberculosis* in sputum. Eur. J. Clin. Microbiol. Infect. Dis. **12:**922–927.
3. **Andreasen, J. J., S. Farholt, and J. S. Jensen.** 1998. Failure to detect *Chlamydia pneumoniae* in calcific and degenerative arteriosclerotic aortic valves excised during open heart surgery. APMIS **106:**717–720.
4. **Beatty, W. L., G. I. Byrne, and R. P. Morrison.** 1994. Repeated and persistent infection with *Chlamydia* and the development of chronic inflammation and disease. Trends Microbiol. **2:**94–98.
5. **Black, C. M., J. A. Tharpe, and H. Russell.** 1992. Distinguishing *Chlamydia* species by restriction analysis of the major outer membrane protein gene. Mol. Cell. Probes **6:**395–400.
6. **Black, C. M., P. I. Fields, T. O. Messmer, and B. P. Berdal.** 1994. Detection of *Chlamydia pneumoniae* in clinical specimens by polymerase chain reaction using nested primers. Eur. J. Clin. Microbiol. Infect. Dis. **13:**752–756.
7. **Blasi, F., F. Denti, M. Erba, R. Cosentini, R. Raccanelli, A. Rinaldi, L. Fagetti, G. Esposito, U. Ruberti, and L. Allegra.** 1996. Detection of *Chlamydia pneumoniae* but not *Helicobacter pylori* in atherosclerotic plaques of aortic aneurysms. J. Clin. Microbiol. **34:**2766–2769.
8. **Block, S., J. Hedrick, M. R. Hammerschlag, G. H. Cassell, and J. C. Craft.** 1995. *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in pediatric community-acquired pneumonia: comparative efficacy and safety of clarithromycin vs. erythromycin ethylsuccinate. Pediatr. Infect. Dis. J. **14:**471–477.
9. **Block, S. L., M. R. Hammerschlag, J. Hedrick, R. Tyler, A. Smith, P. Roblin, C. Gaydos, D. Pham, T. C. Quinn, R. Palmer, and J. McCarty.** 1997. *Chlamydia pneumoniae* in acute otitis media. Pediatr. Infect. Dis. J. **16:**858–862.
10. **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.
11. **Boman, J., S. Söderberg, J. Forsberg, L. S. Birgander, A. Allard, K. Persson, E. Jidell, U. Kumlin, P. Juto, A. Waldenström, and G. Wadell.** 1998. High prevalence of *Chlamydia pneumoniae* DNA in peripheral blood mononuclear cells in patients with cardiovascular disease and in middle-aged blood donors. J. Infect. Dis. **178:**274–277.
12. **Campbell, L. A., C. C. Kuo, and J. T. Grayston.** 1987. Characterization of the new *Chlamydia* agent, TWAR, as a unique organism by restriction endonuclease analysis and DNA-DNA hybridization. J. Clin. Microbiol. **25:**1911–1916.

13. **Campbell, L. A., M. Perez Melgosa, D. J. Hamilton, C. C. Kuo, and J. T. Grayston.** 1992. Detection of *Chlamydia pneumoniae* by polymerase chain reaction. J. Clin. Microbiol. **30:**434–439.
14. **Campbell, L. A., E. R. O'Brien, A. L. Cappuccio, C. C. Kuo, S. P. Wang, D. Stewart, D. L. Patton, P. K. Cummings, and J. T. Grayston.** 1995. Detection of *Chlamydia pneumoniae* TWAR in human coronary atherectomy tissues. J. Infect. Dis. **172:**585–588.
15. **Chiu, B., E. Viira, W. Tucker, and I. W. Fong.** 1997. *Chlamydia pneumoniae*, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. Circulation **96:**2144–2148.
16. **Cunningham, A. F., S. L. Johnston, S. A. Julious, F. C. Lampe, and M. E. Ward.** 1998 Chronic Chlamydia pneumoniae infection and asthma exacerbations in children. Eur. Respir. J. **11:**345–349.
17. **Dalhoff, K., and M. Maass.** 1996. *Chlamydia pneumoniae* pneumonia in hospitalized patients. Clinical characteristics and diagnostic value of polymerase chain reaction detection in BAL. Chest **110:**351–356.
18. **Daugharty, H., S. K. Skelton, and T. Messmer.** 1998. *Chlamydia* DNA extraction for use in PCR: stability and sensitivity in detection. J. Clin. Lab. Anal. **12:**47–53.
19. **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.
20. **Garnett, P., O. Brogan, C. Lafong, and C. Fox.** 1998. Comparison of throat swabs with sputum specimens for the detection of *Chlamydia pneumoniae* antigen by direct immunofluorescence. J. Clin. Pathol. **51:**309–311.
21. **Gaydos, C. A., T. C. Quinn, and J. J. Eiden.** 1992. Identification of *Chlamydia pneumoniae* by DNA amplification of the 16S rRNA gene. J. Clin. Microbiol. **30:**796–800.
22. **Gaydos, C. A., C. L. Fowler, V. J. Gill, J. J. Eiden, and T. C. Quinn.** 1993. Detection of *Chlamydia pneumoniae* by polymerase chain reaction-enzyme immunoassay in an immunocompromised population. Clin. Infect. Dis. **17:** 718–723.
23. **Gaydos, C. A., P. M. Roblin, M. R. Hammerschlag, C. L. Hyman, J. J. Eiden, J. Schachter, and T. C. Quinn.** 1994. Diagnostic utility of PCR-enzyme immunoassay, culture, and serology for detection of *Chlamydia pneumoniae* in symptomatic and asymptomatic patients. J. Clin. Microbiol. **32:**903–905.
24. **Gaydos, C. A., J. J. Eiden, D. Oldach, L. M. Mundy, P. Auwaerter, M. L. Warner, E. Vance, A. A. Burton, and T. C. Quinn.** 1994. Diagnosis of *Chlamydia pneumoniae* infection in patients with community-acquired pneumonia by polymerase chain reaction enzyme immunoassay. Clin. Infect. Dis. **19:**157–160.
25. **Gnarpe, J., and K. Eriksson.** 1995. Sample preparation for *Chlamydia pneumoniae* PCR. APMIS **103:**307–308.
26. **Grayston, J. T., M. B. Aldous, A. Easton, S. P. Wang, C. C. Kuo, L. A. Campbell, and J. Altman.** 1993. Evidence that *Chlamydia pneumoniae* causes pneumonia and bronchitis. J. Infect. Dis. **168:**1231–1235.
27. **Grayston, J. T., C. C. Kuo, A. S. Coulson, L. A. Campbell, R. D. Lawrence, M. J. Lee, E. D. Strandness, and S. P. Wang.** 1995. *Chlamydia pneumoniae* (TWAR) in atherosclerosis of the carotid artery. Circulation **92:**3397–3400.
28. **Gröndahl, B., W. Puppe, A. Hoppe, I. Kuhne, J. A. Weigl, and H. J. Schmitt.** 1999. Rapid identification of nine microorganisms causing acute respiratory tract infections by single-tube multiplex reverse transcription-PCR: feasibility study. J. Clin. Microbiol. **37:**1–7.
29. **Hammerschlag, M. R., K. Chirgwin, P. M. Roblin, M. Gelling, W. Dumornay, L. Mandel, P. Smith, and J. Schachter.** 1992. Persistent infection with *Chlamydia pneumoniae* following acute respiratory illness. Clin. Infect. Dis. **14:**178–182.
30. **Hammerschlag, M. R.** 1995. Diagnostic methods for intracellular pathogens. Clin. Microbiol. Infect. **1**(Suppl. 1)**:**S3–S8.
31. **Holland, S. M., C. A. Gaydos, and T. C. Quinn.** 1990. Detection and differentiation of *Chlamydia trachomatis*, *Chlamydia psittaci*, and *Chlamydia pneumoniae* by DNA amplification. J. Infect. Dis. **162:**984–987.
32. **Hyman, C. L., P. M. Roblin, C. A. Gaydos, T. C. Quinn, J. Schachter, and M. R. Hammerschlag.** 1995. Prevalence of asymptomatic nasopharyngeal carriage of *Chlamydia pneumoniae* in subjectively healthy adults: assessment by polymerase chain reaction-enzyme immunoassay and culture. Clin. Infect. Dis. **20:**1174–1178.
33. **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.
34. **Innis, M. A., and D. H. Gelfand.** 1990. Optimization of PCRs, p. 3–12. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols—a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
35. **Jackson, L. A., L. A. Campbell, C. C. Kuo, D. I. Rodriguez, A. Lee, and J. T. Grayston.** 1997. Isolation of *Chlamydia pneumoniae* from a carotid endarterectomy specimen. J. Infect. Dis. **176:**292–295.
36. **Jantos, C. A., R. Roggendorf, F. N. Wuppermann, and J. H. Hegemann.** 1998. Rapid detection of *Chlamydia pneumoniae* by PCR-enzyme immunoassay. J. Clin. Microbiol. **36:**1890–1894.
37. **Juvonen, J., A. Laurila, T. Juvonen, H. Alakarppa, H. M. Surcel, K. Lounatmaa, J. Kuusisto, and P. Saikku.** 1997. Detection of *Chlamydia pneumoniae* in human nonrheumatic stenotic aortic valves. J. Am. Coll. Cardiol. **29:**1054–1059.
38. **Juvonen, J., T. Juvonen, A. Laurila, H. Alakarppa, K. Lounatmaa, H. M. Surcel, M. Leinonen, M. I. Kairaluoma, and P. Saikku.** 1997. Demonstration of *Chlamydia pneumoniae* in the walls of abdominal aortic aneurysms. J. Vasc. Surg. **25:**499–505.
39. **Kalman, S., W. Mitchell, R. Marathe, C. Lammel, J. Fan, R. W. Hyman, L. Olinger, J. Grimwood, R. W. Davis, and R. S. Stephens.** 1999. Comparative genomes of *Chlamydia pneumoniae* and *C. trachomatis*. Nat. Gen. **21:**385–389.
40. **Kaltenboeck, B., K. G. Kousoulas, and J. Storz.** 1992. Two-step polymerase chain reactions and restriction endonuclease analyses detect and differentiate *ompA* DNA of *Chlamydia* spp. J. Clin. Microbiol. **30:**1098–1104.
41. **Khan, M. A., C. W. Potter, and R. M. Sharrard.** 1996. A reverse transcriptase-PCR based assay for in-vitro antibiotic susceptibility testing of *Chlamydia pneumoniae*. J. Antimicrob. Chemother. **37:**677–685.
42. **Khan, M. A., and C. W. Potter.** 1996. The nPCR detection of *Chlamydia pneumoniae* and *Chlamydia trachomatis* in children hospitalized for bronchiolitis. J. Infect. **33:**173–175.
43. **Kolk, A. H., G. T. Noordhoek, O. de Leeuw, S. Kuijper, and J. D. van Embden.** 1994. *Mycobacterium smegmatis* strain for detection of *Mycobacterium tuberculosis* by PCR used as internal control for inhibition of amplification and for quantification of bacteria. J. Clin. Microbiol. **32:**1354–1356.
44. **Kubota, Y.** 1996. A new primer pair for detection of *Chlamydia pneumoniae* by polymerase chain reaction. Microbiol. Immunol. **40:**27–32.
45. **Kuo, C. C., A. Shor, L. A. Campbell, H. Fukushi, D. L. Patton, and J. T. Grayston.** 1993. Demonstration of *Chlamydia pneumoniae* in atherosclerotic lesions of coronary arteries. J. Infect. Dis. **167:**841–849.
46. **Kuo, C. C., A. S. Coulson, L. A. Campbell, A. L. Cappuccio, R. D. Lawrence, S. P. Wang, and J. T. Grayston.** 1997. Detection of *Chlamydia pneumoniae* in atherosclerotic plaques in the walls of arteries of lower extremities from patients undergoing bypass operation for arterial obstruction. J. Vasc. Surg. **26:**29–31.
47. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature **339:**237–238.
48. **Kwok, S.** 1990. Procedures to minimize PCR-product carry-over, p. 142–145. *In* M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCR protocols—a guide to methods and applications. Academic Press, Inc., San Diego, Calif.
49. **Lindholt, J. S., L. Ostergard, E. W. Henneberg, H. Fasting, and P. Andersen.** 1998. Failure to demonstrate *Chlamydia pneumoniae* in symptomatic abdominal aortic aneurysms by a nested polymerase chain reaction (PCR). Eur. J. Vasc. Endovasc. Surg. **15:**161–164.
50. **Maass, M., and K. Dalhoff.** 1994. Comparison of sample preparation methods for detection of *Chlamydia pneumoniae* in bronchoalveolar lavage fluid by PCR. J. Clin. Microbiol. **32:**2616–2619.
51. **Maass, M., E. Krause, P. M. Engel, and S. Kruger.** 1997. Endovascular presence of *Chlamydia pneumoniae* in patients with hemodynamically effective carotid artery stenosis. Angiology **48:**699–706.
52. **Maass, M., C. Bartels, P. M. Engel, U. Mamat, and H. H. Sievers.** 1998. Endovascular presence of viable *Chlamydia pneumoniae* is a common phenomenon in coronary artery disease. J. Am. Coll. Cardiol. **31:**827–832.
53. **Maass, M., J. Gieffers, E. Krause, P. M. Engel, C. Bartels, and W. Solbach.** 1998. Poor correlation between microimmunofluorescence serology and polymerase chain reaction for detection of vascular *Chlamydia pneumoniae* infection in coronary artery disease patients. Med. Microbiol. Immunol. **187:**103–106.
54. **Madico, G.** Personal communication.
55. **Madico, G., C. A. Gaydos, J. Boman, and T. C. Quinn.** 1998. Enzyme time release-touchdown polymerase chain reaction (PCR) for improved detection of *Chlamydia pneumoniae* respiratory infection. Clin. Infect. Dis. **27:**1063.
56. **Madico, G., C. A. Gaydos, J. Boman, and T. C. Quinn.** Identification of *C. pneumoniae*, *C. trachomatis*, and *C. psittaci* by polymerase chain reaction using the 16S rRNA and 16S-23S rRNA spacer genes. Submitted for publication.
57. **Martin, G. P., and E. Timmers.** 1997. PCR and its modifications for the detection of infectious diseases, p. 79–99. *In* H. H. Lee, S. A. Morse, and O. Olsvik (ed.), Nucleic acid amplification technologies: application to disease diagnosis. BioTechniques Books, Division of Eaton Publishing, Cambridge, Mass.
58. **Meijer, A., J. A. van der Vliet, L. M. Schouls, A. de Vries, P. J. Roholl, and J. M. Ossewaarde.** 1998. Detection of microorganisms in vessel wall specimens of the abdominal aorta: development of a PCR assay in the absence of a gold standard. Res. Microbiol. **149:**577–583.
59. **Mengue Metogho, R., S. Deslandes, and E. H. Frost.** 1999. Comparison of PCR protocols including positive controls for detection of *Chlamydia pneumoniae* in respiratory specimens. Mol. Cell. Probes **13:**71–75.
60. **Messmer, T. O., S. K. Skelton, J. F. Moroney, H. Daugharty, and B. S. Fields.** 1997. Application of a nested, multiplex PCR to psittacosis outbreaks. J. Clin. Microbiol. **35:**2043–2046.
61. **Naidu, B. R., Y. F. Ngeow, and T. Pang.** 1997. MOMP-based PCR reveals presence of *Chlamydia pneumoniae* DNA in respiratory and serum samples

of patients with acute *C. pneumoniae*-associated infections. J. Microbiol. Methods **28:**1–9.
62. **Naidu, B. R., Y. F. Ngeow, P. Kannan, R. Jeyamalar, A. Khir, K. L Khoo, and T. Pang.** 1997. Evidence of *Chlamydia pneumoniae* infection obtained by the polymerase chain reaction (PCR) in patients with acute myocardial infarction and coronary heart disease. J. Infect. **35:**199–200.
63. **Normann, E., J. Gnarpe, H. Gnarpe, and B. Wettergren.** 1998. *Chlamydia pneumoniae* in children attending day-care centers in Gavle, Sweden. Pediatr. Infect. Dis. J. **17:**474–478.
64. **Nyström-Rosander, C., S. Thelin, E. Hjelm, O. Lindquist, C. Påhlson, and G. Friman.** 1997. High incidence of *Chlamydia pneumoniae* in sclerotic heart valves of patients undergoing aortic valve replacement. Scand. J. Infect. Dis. **29:**361–365.
65. **O'Leary, J. J., K. Engels, and M. A. Dada.** 1997. The polymerase chain reaction in pathology. J. Clin. Pathol. **50:**805–810.
66. **Ong, G., B. J. Thomas, A. O. Mansfield, B. R. Davidson, and D. Taylor-Robinson.** 1996. Detection and widespread distribution of *Chlamydia pneumoniae* in the vascular system and its possible implications. J. Clin. Pathol. **49:**102–106.
67. **Palmer, H. M.** 1994. The chlamydiales and mycoplasmatales, p. 493–510. *In* G. D. Ehrlich and S. J. Greenberg (ed.), PCR-based diagnostics in infectious diseases. Blackwell Scientific Publications, Boston, Mass.
68. **Petersen, E., J. Boman, K. Persson, C. Arnerlöv, G. Wadell, P. Juto, A. Eriksson, G. Dahlén, and K. A. Ängquist.** 1998. *Chlamydia pneumoniae* in human abdominal aortic aneurysms. Eur. J. Vasc. Endovasc. Surg. **15:**138–142.
69. **Petitjean, J., F. Vincent, M. Fretigny, A. Vabret, J. D. Poveda, J. Brun, and F. Freymuth.** 1998. Comparison of two serological methods and a polymerase chain reaction-enzyme immunoassay for the diagnosis of acute respiratory infections with *Chlamydia pneumoniae* in adults. J. Med. Microbiol. **47:**615–621.
70. **Pham, D. G., G. E. Madico, T. C. Quinn, M. J. Enzler, T. F. Smith, and C. A. Gaydos.** 1998. Use of lambda phage DNA as a hybrid internal control in a PCR-enzyme immunoassay to detect *Chlamydia pneumoniae*. J. Clin. Microbiol. **36:**1919–1922.
71. **Pruckl, P. M., C. Aspock, A. Makristathis, M. L. Rotter, H. Wank, B. Willinger, and A. M. Hirschl.** 1995. Polymerase chain reaction for detection of *Chlamydia pneumoniae* in gargled-water specimens of children. Eur. J. Clin. Microbiol. Infect. Dis. **14:**141–144.
72. **Ramirez, J. A., and the *Chlamydia pneumoniae*/Atherosclerosis Study Group.** 1996. Isolation of *Chlamydia pneumoniae* from the coronary artery of a patient with coronary atherosclerosis. Ann. Intern. Med. **125:**979–982.
73. **Rasmussen, S. J., F. P. Douglas, and P. Timms.** 1992. PCR detection and differentiation of *Chlamydia pneumoniae*, *Chlamydia psittaci* and *Chlamydia trachomatis*. Mol. Cell. Probes **6:**389–394.
74. **Rolfs, A., I. Schuller, U. Finckh, and I. Weber-Rolfs.** 1992. Biological material amenable to PCR, p. 68–78. *In* PCR: clinical diagnostics and research. Springer-Verlag, Berlin, Germany.
75. **Ruano, G., W. Fenton, and K. K. Kidd.** 1989. Biphasic amplification of very dilute DNA samples via 'booster' PCR. Nucleic Acids Res. **17:**5407.
76. **Saiki, R. K.** 1989. The design and optimization of the PCR, p. 7–16. *In* H. A. Erlich (ed.), PCR technology—principles and applications for DNA amplification. Stockton Press, New York, N.Y.
77. **Saikku, P., S. P. Wang, M. Kleemola, E. Brander, E. Rusanen, and J. T. Grayston.** 1985. An epidemic of mild pneumonia due to an unusual strain of *Chlamydia psittaci*. J. Infect. Dis. **15:**832–839.
78. **Schachter, J.** 1997. Evaluation of diagnostic tests—special problems introduced by DNA amplification procedures, p. 165–169. *In* H. H. Lee, S. A. Morse, and O. Olsvik (ed.), Nucleic acid amplification technologies: application to disease diagnosis. BioTechniques Books, Division of Eaton Publishing, Cambridge, Mass.
79. **Schmidt, B. L.** 1997. PCR in laboratory diagnosis of human *Borrelia burgdorferi* infection. Clin. Microbiol. Rev. **10:**185–201.
80. **Sillis, M., P. White, E. O. Caul, I. D. Paul, and J. D. Treharne.** 1992. The differentiation of *Chlamydia* species by antigen detection in sputum specimens from patients with community-acquired acute respiratory infections. J. Infect. **25**(Suppl. 1)**:**77–86.
81. **Soini, H., and M. K. Viljanen.** 1997. Gene amplification in the diagnosis of mycobacterial infections. APMIS **105:**345–353.
82. **Storgaard, M., L. Ostergaard, J. S. Jensen, S. Farholt, K. Larsen, T. Ovesen, H. Nodgaard, and P. L. Andersen.** 1997. *Chlamydia pneumoniae* in children with otitis media. Clin. Infect. Dis. **25:**1090–1093.
83. **Taylor-Robinson, D., G. Ong, B. J. Thomas, M. L. Rose, and M. H. Yacoub.** 1998. *Chlamydia pneumoniae* in vascular tissues from heart-transplant donors. Lancet **351:**1255.
84. **Tjhie, H. T. J., R. Roosendaal, J. M. M. Walboomers, J. J. H. Theunissen, R. R. M. Tjon Lim Sang, C. J. L. M. Meijer, D. M. MacLaren, and A. J. C. van den Brule.** 1993. Detection of *Chlamydia pneumoniae* using a general *Chlamydia* polymerase chain reaction with species differentiation after hybridisation. J. Microbiol. Methods **18:**137–150.
85. **Tong, C. Y., and M. Sillis.** 1993. Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. J. Clin. Pathol. **46:**313–317.
86. **Ursi, D., M. Leven, H. P. Van Bever, and H. Goossens.** 1998. Construction of an internal control for the detection of *Chlamydia pneumoniae* by PCR. Mol. Cell. Probes **12:**235–238.
87. **Verkooyen, R. P., D. Willemse, S. C. Casteren, S. A. Joulandan, R. J. Snijder, J. M. van den Bosch, H. P. van Helden, M. F. Peeters, and H. A. Verbrugh.** 1998. Evaluation of PCR, culture, and serology for diagnosis of *Chlamydia pneumoniae* respiratory infections. J. Clin. Microbiol. **36:**2301–2307.
88. **Wadowsky, R. M., S. Laus, T. Libert, S. J. States, and G. D. Ehrlich.** 1994. Inhibition of PCR-based assay for *Bordetella pertussis* by using calcium alginate fiber and aluminum shaft components of a nasopharyngeal swab. J. Clin. Microbiol. **32:**1054–1057.
89. **Watson, M. W., P. R. Lambden, and I. N. Clarke.** 1991. Genetic diversity and identification of human infection by amplification by amplification of the chlamydial 60-kilodalton cysteine-rich outer membrane protein gene. J. Clin. Microbiol. **29:**1188–1193.
90. **Weiss, S. M., P. M. Roblin, C. A. Gaydos, P. Cummings, D. L. Patton, N. Schulhoff, J. Shani, R. Frankel, K. Penney, T. C. Quinn, M. R. Hammerschlag, and J. Schachter.** 1996. Failure to detect *Chlamydia pneumoniae* in coronary atheromas of patients undergoing atherectomy. J. Infect. Dis. **173:**957–962.
91. **Whelen, A. C., and D. H. Persing.** 1996. The role of nucleic acid amplification and detection in the clinical microbiology laboratory. Annu. Rev. Microbiol. **50:**349–373.
92. **Wilson, I. G.** 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. **63:**3741–3751.
93. **Wilson, P. A., J. Phipps, D. Samuel, and N. A. Saunders.** 1996. Development of a simplified polymerase chain reaction-enzyme immunoassay for the detection of *Chlamydia pneumoniae*. J. Appl. Bacteriol. **80:**431–438.
94. **Yoshida, H., Y. Kishi, S. Shiga, and T. Hagiwara.** 1998. Differentiation of *Chlamydia* species by combined use of polymerase chain reaction and restriction endonuclease analysis. Microbiol. Immunol. **42:**411–414.