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# Comparison of Sample Preparation Methods for Detection of *Chlamydia pneumoniae* in Bronchoalveolar Lavage Fluid by PCR

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**Amplification inhibitors can lead to false-negative results for PCR. In order to evaluate the reliability of PCR for the detection of *Chlamydia pneumoniae*, the presence of PCR inhibitors in 75 bronchoalveolar lavage specimens was assessed after treatment by various sample preparation methods. Specimens were collected from patients with acute respiratory infections, including four cases of proven *C. pneumoniae* infection. Substances inhibitory to the amplification of chlamydial DNA continued to be present in 12% of the samples treated according to the commonly used single-step proteinase K digestion and in 31% of the samples processed by heat treatment. However, the complexing of DNA-contaminating proteins and polysaccharides from digested specimens to cetyltrimethylammonium bromide (CTAB) followed by DNA extraction efficiently removed inhibitors from all experimental samples and provided subsequent identification of all positive clinical samples by PCR. The CTAB method and proteinase K treatment had comparable detection limits of approximately 0.01 inclusion-forming units. CTAB-based DNA purification of respiratory specimens is recommended to increase the diagnostic sensitivity of PCR and confidence in negative results.**

*Chlamydia pneumoniae* is a frequent cause of community-acquired pneumonia (7) and is possibly associated with coronary atheroma (13, 17). The recently described pathogen is far more difficult to recover from clinical specimens and to permanently grow in cell cultures than the other chlamydial species (7, 11, 12). There is seroepidemiologic evidence that, independent of geographic regions, nearly everybody will be infected either once or repeatedly by *C. pneumoniae* (7), though the majority of infections apparently will remain subclinical. The serodiagnosis of acute infection is sometimes possible by the microimmunofluorescence test but also problematic because of the late onset of antibody production, the common absence of detectable amounts of immunoglobulin M in reinfection (4, 7, 15), and the lack of defined species-specific antigens. For a pathogen otherwise difficult to diagnose, the use of molecular biological detection methods such as PCR is efficient and reasonable, provided the results are thoroughly controlled. Avoiding false-positive results for PCR is a much-discussed topic while the prevention of false negatives is commonly less considered, though the inhibition of the DNA amplification process by substances present in clinical samples may seriously impede PCR-based diagnosis. For a commonly encountered pathogen such as *C. pneumoniae*, the presence of PCR inhibitors in a substantial number of specimens results in an unacceptably high proportion of false negatives. Therefore, three common sample preparation protocols were applied to clinical and experimental specimens in order to define the one most reliable for subsequent detection of *C. pneumoniae* by enzymatic DNA amplification. The clinical application of PCR on throat swabs has been previously reported (2, 3), but the optimal sample sites for diagnosing *C. pneumoniae* infection by molecular biological or cultural detection methods have not yet been defined. In our experience, bronchoalveolar lavage (BAL) fluid, which contains cellular and soluble materials from the surfaces of the respiratory tract, is another valuable specimen and was used in this investigation.

**Culture of *C. pneumoniae*.** HEp-2 cells served as the host cell line for *C. pneumoniae* MUL-1, a regional respiratory isolate from our laboratory. The cell growth medium was Eagle's minimal essential medium with nonessential amino acids and 2 mM glutamine (EMEM) (GIBCO/BRL GmbH, Eggenstein, Germany), supplemented with 10% fetal calf serum (Biochrom KG, Berlin, Germany). Chlamydiae were continuously cultured as previously described (12, 14, 16, 19) by centrifugation (2,000 × *g* at 35°C for 45 min) of infectious inocula onto host cell monolayers in multiwell tissue culture plates. Supernatants were replaced by the chlamydial isolation medium consisting of EMEM with 1 μg of cycloheximide (Sigma Chemical Co., St. Louis, Mo.) per ml without fetal calf serum. Inoculated host cells were incubated at 35°C and 5% $CO_2$ for 3 days, and chlamydial growth was monitored by staining monolayers for inclusions with a *C. pneumoniae*-specific mouse monoclonal antibody and fluorescein isothiocyanate-coupled secondary antibody (Cellabs Diagnostics Pty. Ltd., Sydney, Australia). Infected monolayers were homogenized, the released elementary bodies were partially purified by differential centrifugation, and a stock suspension containing $10^5$ inclusion-forming units (IFU) per ml in EMEM was prepared. Tenfold dilutions containing $10^2$ to $10^{-4}$ IFU in 10-μl aliquots were subjected to the three sample preparation methods in order to determine subsequent sensitivity to detect *C. pneumoniae* by PCR.

**Patient samples.** BAL fluid with a volume of approximately 15 ml from 75 patients with suspected respiratory infection was collected at 1:2 in cell growth medium with antibiotics. Four of these samples were from patients with proven *C. pneumoniae* infections. Cultured by essentially the same procedure as that described above, the sample of patient 029 yielded four *C. pneumoniae* inclusions in a first passage, that of 045 yielded seven inclusions in a first passage, and that of 078 yielded four

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inclusions in a second cell culture passage; because of a microimmunofluorescence immunoglobulin M titer of 64 and an immunoglobulin G titer of 512, patient 084 met the serological criteria for acute *C. pneumoniae* infection as defined for this test (7). The remaining samples were culture negative, and the patients were serologically unsuspicious for recent chlamydial infection. BAL samples were examined for *C. pneumoniae* DNA and used to determine the possible presence of amplification inhibitors after treatment by three sample preparation methods. While a 4-ml portion of each BAL sample was experimentally made positive by the addition of 60 IFU of strain MUL-1, another 4-ml portion was examined without alteration. All samples were centrifuged at 20,000 × *g* and 4°C for 30 min. The pellets were suspended in 60 μl of water; therefore, the samples with experimentally added chlamydiae contained 1 IFU/μl. Then, 10-μl portions of the experimentally positive samples as well as the samples without added chlamydiae were subjected to the different DNA preparation methods described below before PCR was performed.

**Sample preparation methods.** Samples in 10-μl portions were obtained as described above and subjected to three common methods of preparing material for DNA amplification.

**(i) Heat treatment.** Samples were boiled in 50 μl of water for 10 min and then used for PCR.

**(ii) Single-step proteinase K treatment.** According to the method of Campbell et al. (2), samples were subjected to a 1-h proteinase K treatment in 50 μl of proteinase K lysis solution (100 μg of proteinase K per ml [Boehringer GmbH, Mannheim, Germany], 0.5% Tween 20 [Sigma], 0.5% Nonidet P-40 [Sigma]) at 60°C. Proteinase K was then inactivated by boiling the samples for 10 min before they were examined by PCR.

**(iii) CTAB treatment.** The third procedure was adapted from a standard cetyltrimethylammonium bromide (CTAB) DNA purification method (18). Briefly, samples were incubated in 600 μl of 10 mM Tris-EDTA (pH 7.5)–0.5% sodium dodecyl sulfate (Sigma)–100 μg of proteinase K per ml for 1 h at 60°C and then 100 μl of 5 M NaCl and 80 μl of 10% CTAB (Sigma), dissolved in 0.7 M NaCl, were added. Suspensions were incubated for 10 min at 65°C. DNA was then obtained from aqueous supernatants by standard phenol-chloroform-isoamyl alcohol extraction and precipitated overnight at 4°C in isopropanol. DNA pellets were washed in 70% ethanol, resuspended in 50 μl of water, and used for PCR. All preparations were made in duplicate.

**DNA amplification.** PCR was performed with the previously described HL-1 and HR-1 oligonucleotide primer pair, which amplifies a 438-bp target sequence of the *C. pneumoniae* genome of unknown function but proven species specificity (2, 3). The amplification mixture contained 100 μl of 1 μM each primer, 200 μM each deoxynucleotide triphosphate, 67 mM Tris-hydrochloride (pH 8.8), 16 mM $(NH_4)_2SO_4$, 3.5 mM $MgCl_2$, 10 mM β-mercaptoethanol (Sigma), 1 U of *Taq* DNA polymerase (Boehringer GmbH), and 50 μl of each sample. Prior to amplification, each sample was heated at 96°C for 5 min. The amplification conditions for 32 cycles consisted of denaturation at 96°C for 1 min 30 s, annealing at 55°C for 1 min 45 s, and extension at 72°C for 1 min 45 s. The final extension step was prolonged to 7 min. The mobility of each amplification product was determined on an ethidium bromide-stained 4% agarose gel under UV light. For confirmation and enhanced sensitivity, a DNA hybridization was performed with the probe oligonucleotide HM-1 (2, 3) 3′ end labeled with digoxigenin-ddUTP according to the instructions of the supplier (Boehringer GmbH). After the amplification was performed, 50 μl of the reaction mixture was vacuum blotted (Minifold I; Schleicher & Schuell, Dassel, Germany) onto a positively charged nylon membrane (Boehringer GmbH), fixed at 120°C for 30 min, hybridized for 6 h to the digoxigenin-labeled probe at 42°C, and stained according to the instructions of the manufacturer (Boehringer GmbH).

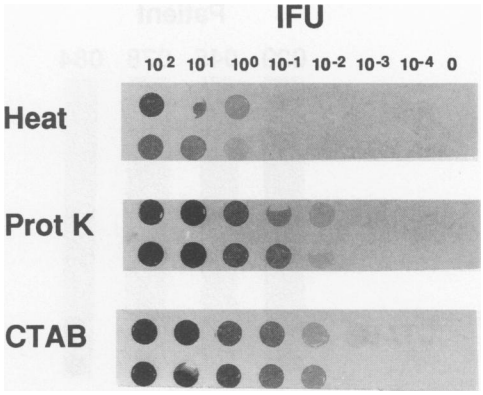


FIG. 1. Sensitivity limits for PCR detection of *C. pneumoniae* in relation to sample pretreatment. Preceding PCR, 10-fold dilutions of *C. pneumoniae* IFU were boiled for 10 min (Heat), processed by a single-step proteinase K treatment protocol (Prot K), or subjected to CTAB DNA purification in duplicate reactions. PCR-amplified DNA was dot blotted and then identified by the hybridization signal obtained with the digoxigenin-labeled HM-1 probe. Reproducible detection of ≥0.01 IFU was achieved after CTAB and proteinase K sample treatment. Heat treatment was approximately 100-fold less sensitive as a sample preparation technique.

Figure 1 shows the *C. pneumoniae* detection limits of PCR and nonradioactive hybridization in relation to the sample preparation method as determined with 10-fold IFU dilutions. By using the single-step proteinase K treatment protocol (2) or CTAB precipitation of DNA-contaminating substances, ≥0.01 IFU yielded a positive hybridization result. CTAB purification appeared to yield a somewhat stronger reaction, but the detection of 0.001 IFU was not reliably reproducible. Boiling was approximately 100-fold less efficient for sample preparation: a positive hybridization resulted only from ≥1 IFU.

In spite of the high PCR sensitivity for suspended chlamydiae, a failure to amplify the DNA of the experimentally added 10 IFUs of *C. pneumoniae* in BAL fluid occurred with 31% of the clinical samples that were only boiled for pretreatment. After single-step proteinase K digestion, PCR inhibitors were still present in 12% of samples. However, none of the samples that were subjected to CTAB-based DNA purification inhibited enzymatic DNA amplification from the experimentally added chlamydiae. Thus, CTAB treatment was the most efficient in removing the otherwise frequent inhibitory activity from BAL samples. When CTAB-based DNA purification was applied to the separately examined original BAL samples without experimentally added *C. pneumoniae*, positive PCR results were obtained for all four samples from known *C. pneumoniae*-infected patients. The application of the single-step proteinase K treatment led to correct amplification from three of the samples, but one was still missed because of the presence of inhibitors. Only two positive samples were recognized by PCR when simple boiling was used as the pretreatment. The hybridization results for the positive patients are shown in Fig. 2. The samples which were not consistently recognized as positive were among those previously shown to contain amplification inhibitors.

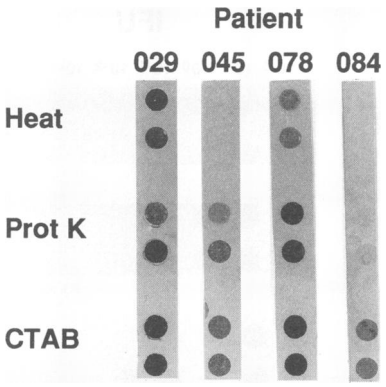


FIG. 2. Influence of sample preparation methods on the sensitivity of PCR-mediated detection of *C. pneumoniae* in clinical specimens. BAL samples which had yielded *C. pneumoniae* in cultures (patients 029, 045, and 078) and BAL material from a serologically proven case of acute *C. pneumoniae* infection (patient 084) were subjected to three sample preparation techniques, heat, proteinase K (Prot K), and CTAB pretreatment, before PCR was performed. Experiments were carried out in duplicate, and positive PCR results were detected by hybridization of the digoxigenin-labeled HM-1 probe to dot-blotted amplified DNA. Only CTAB-based sample preparation permitted the consistent identification of all positive samples as a result of efficient removal of PCR inhibitors. Because of the persistence of amplification inhibitors, two samples appeared to be false negative after heat treatment and only three of the positive samples were reproducibly detected following proteinase K treatment.

The detection limit of 0.01 IFU obtained in this study was approximately in the range of that found by Campbell et al., i.e., 0.05 IFU (3). In another study with different oligonucleotide primers, 0.4 IFU of *C. pneumoniae* was the detection limit when proteinase K treatment followed by phenol-chloroform DNA extraction was used for sample preparation (6). As less than 1 IFU could be distinctly detected, the number of chlamydiae present in suspension apparently exceeded the number retrievable by cell culture as represented by the IFU count. DNA amplification does not differentiate nonviable or nonreplicating organisms from cultivable organisms. Additionally, the biological meaning of the target sequence, which was obtained after *Pst*I restriction analysis of cloned *C. pneumoniae* DNA (3), is uncertain, and the sequence may be repetitive within the chlamydial genome, thus contributing to the high sensitivity of PCR.

This investigation shows that depending on the sample preparation protocol applied, a substantial number of BAL specimens contain potent inhibitors of enzymatic DNA amplification, with the consequence of false-negative reactions. In a report on PCR detection of *Chlamydia trachomatis* on endocervical swabs, boiling was used for sample preparation and was found to be sufficient for the detection of 500 elementary bodies in experimental samples, but the detection limit was not further tested and clinical samples were not examined for the presence of inhibitors (10). In this examination, boiling, obviously the most convenient sample preparation, was the least efficient method and was unacceptable for use with clinical BAL specimens subjected to *C. pneumoniae* PCR. The lysis buffer used here for the single-step proteinase K pretreatment was reported to contain an optimized combination of detergents (2) and is commonly used for PCR detection of *C. pneumoniae*. However, only the application of the more complex CTAB-based DNA purification protocol efficiently succeeded in removing PCR inhibitors from all BAL samples tested and permitted subsequent identification of all positive patients. The obvious disadvantage of the CTAB method is that additional steps are necessary to separate DNA and CTAB by the extraction and precipitation of DNA. A loss of target DNA in the course of extraction might be of concern, but use of the CTAB protocol on IFU dilutions without inhibitory activity yielded a sensitivity for the detection of *C. pneumoniae* at least equal to that of the single-step proteinase K sample treatment. Commercial kits to facilitate DNA purification without the loss of efficiency are urgently required, and their development also depends on better definitions of the PCR inhibitors present in clinical samples.

With the exception of the heme molecule and its derivatives (9), the inhibitors of enzymatic DNA amplification present in clinical samples are not well defined, and systematic studies of the inhibition of PCR are lacking. Acidic polysaccharides, which are present in the glycoproteins of respiratory secretions, are known to impede polymerases (8). A sample preparation protocol consisting solely of the proteinase K treatment leads to the degradation of proteins and polypeptides but does not eliminate those inhibitory polysaccharide components from specimens. On the other hand, additional application of CTAB to heterogeneous mixtures of macromolecules results in the precipitation of proteins as well as polysaccharides and DNA can be recovered from the aqueous supernatant (18). A possible copurification of PCR inhibitors with DNA has been described previously (5) but did not occur with the samples tested here. The effective removal of inhibitory polysaccharides from all specimens derived from the respiratory tract should be considered.

Several proposals for eliminating or monitoring the inhibition of DNA amplification have been made. With regard to the amplification of *C. pneumoniae* DNA from throat swabs, the control of possible inhibitors by testing various sample dilutions in parallel has been suggested (2) but this approach, apart from adding to the workload, simultaneously reduces the amount of PCR targets such that decreased diagnostic sensitivity may result. For BAL fluid, a highly diluted material, the largest possible sample volume appears to be appropriate. The routine addition of elementary bodies to a part of each clinical specimen as a control and screening method for PCR inhibitors carries a certain risk of sample contamination and again reduces the amount of each specimen available for diagnosis. The addition of an internal control to each PCR tube provides direct monitoring of inhibitory activity. For example, in a study testing BAL fluid for mycobacterial DNA, an internal control which contained the primer template sequences was constructed. Amplification of this internal control resulted in a PCR product larger than that obtained from the natural target sequence (1). Though this approach checks for PCR inhibitors in the actual reaction mixture. thus avoiding parallel tests from a single specimen, it does not circumvent the need to further purify DNA in case inhibitors are present, at least if samples are not to be entirely excluded from further processing. If a substantial number of specimens can be expected to contain PCR inhibitors, as observed for 12% of the solely proteinase K-treated BAL samples in this examination, it may be more convenient to start sample processing by routinely purifying DNA from all specimens to be subjected to PCR by use of an apparently more efficient method like the CTAB protocol, even though it is more complex.

Culture methods for *C. pneumoniae* have been improved recently (14, 16, 19), but since isolation is still time-consuming and labor-intensive and usually available only in specialized laboratories, further progress toward culture conditions more satisfactory to serve as the "gold standard" is mandatory.

Though not ideal for routine application either, PCR is a diagnostic alternative. Unfortunately, the sample preparation most effective in identifying positive patients was the least convenient, thus adding to the difficulties PCR already poses to the diagnostic routine. In summary, the risk of false negatives with suboptimal sample preparation protocols is unacceptably high for a frequently encountered pathogen. While this study focused only on optimizing PCR detection of *C. pneumoniae* by ensuring the accuracy of negative results, future studies will have to provide a correlation among PCR results, culture, and the clinical picture for a final evaluation of detection methods and further verification of the etiological role of *C. pneumoniae* in human disease.

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