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Diagnostic Value of the Polymerase Chain Reaction for *Chlamydia* Detection as Determined in a Follow-Up Study

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The diagnostic value of the polymerase chain reaction (PCR) for detection of *Chlamydia trachomatis* in comparison with that of the culture technique was established in a follow-up study of 32 patients (81 samples) who were treated for a *C. trachomatis* infection. The PCR was performed with two different sets of primers, a genus-specific primer set directed against the rRNA genes and a *C. trachomatis*-specific set directed against the common endogenous plasmid. After treatment with doxycycline, all patients became culture negative after 1 week. Results for the detection of *C. trachomatis* by the PCR were in complete agreement with the results by the culture method of detection, except for one culture-negative sample, which was found to be positive by the PCR. The results indicated that 1 week after treatment, no residual chlamydial DNA was found in the samples. Therefore, the PCR can be used for monitoring infections by chlamydiae.

In developed countries, *Chlamydia trachomatis* is a major cause of sexually transmitted diseases. Although safe sexual behavior is an important issue in the strategy of AIDS prevention, this did not markedly decrease the prevalence of reported *Chlamydia* infections (2). As a result of improved detection methods and the large number of asymptomatic carriers of the microorganism, the prevalence of these infections has even increased. Prevalences up to 20% have been reported in asymptomatic, sexually active adolescents (7, 16, 17). *C. trachomatis* infections may lead to severe complications and may eventually lead to infertility in women. Endometritis and salpingitis are associated with these infections (9, 10). Epididymitis has not been proven to lead to infertility in men (1, 4).

Sensitive screening methods are necessary for early detection of *Chlamydia* infections. Rapid treatment could control and decrease the spread of the disease. In the past year, a polymerase chain reaction (PCR) was described for detection of chlamydiae (5, 14). The technique appeared to be suited to clinical specimens as well (3). In the previous study, the specificity of the PCR was 100% and the sensitivity was 108%. To evaluate the use of the PCR for diagnostic purposes and the determination of the effectiveness of treatment, a follow-up study was performed. Inactivated *Chlamydia* particles, which are possibly present in the posttreatment control swabs, give a negative culture but positive PCR results. Patients positive for chlamydiae and treated with doxycycline for 1 week were monitored until they were negative by the culture technique and the PCR. Complete agreement was obtained between the results of the culture technique and those of the PCR in this follow-up study, indicating that no detectable chlamydial DNA was in the samples after treatment.

MATERIALS AND METHODS

Study group. A total of 986 samples were collected from women and men attending the outpatient clinic for sexually transmitted diseases of the University Hospital Dijkzigt in Rotterdam between 17 July and 25 October 1989. From this group, follow-up samples were selected from 23 women and 9 men (i) with at least one sample that was positive for *chlamydia* on culture and (ii) who returned for a checkup after treatment (200 mg of doxycycline for 7 days) until they were negative for chlamydiae in culture. The first checkup had to be within 10 days after the cessation of therapy. From the selected group of patients, two culture-positive results were found for six patients. They were primarily treated with metronidazole for infection with *Gardnerella vaginalis*. Because of the positive culture result for chlamydiae, specimens were collected again before initiation of the treatment with doxycycline. After treatment, three of these six patients had one negative culture result, two patients had two negative culture results, and one patient had four negative culture results. Twenty-six patients in the selected group had one positive culture for chlamydiae. After treatment, 22 of the patients, including the 9 males, had one negative culture results, two patients had two negative culture results, one patient had three negative culture results, and one patient had four negative culture results. All clinical specimens (cervical and urethral swabs) were immediately placed into 2 ml of transport medium (2-sucrose phosphate buffer [pH 7.4] supplemented with 10% fetal bovine serum, 25 μg of gentamicin per ml, and 2.5 μg of amphotericin B per ml) and stored at 4°C. If they were not tested within 24 h, they were stored at −70°C and tested within 1 week.

Isolation technique. The isolation of chlamydiae in cultured cells was performed as described by Thewessen et al. (19). In brief, after agitation, 0.1 ml of a sample was added to each of two wells of a microtiter plate containing monolayers of HeLa 229 cells, which were rinsed in 15 μg of DEAE-dextran per ml in Hanks balanced salt solution (Flow Laboratories, Irvine, Scotland). After centrifugation at 5,000 × *g* for 60 min, the supernatant was replaced with 0.1 ml of complete growth medium with glucose and 0.5 μg of cycloheximide per ml. The plates were incubated at 37°C for 48 h. The monolayers were fixed, stained with a fluorescent

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monoclonal anti-*C. trachomatis* antibody (MicroTrak; Syva Co., Palo Alto, Calif.), and examined for inclusions.

DNA isolation. DNA was extracted from 0.5 ml of each sample by standard isolation methods (15). The samples were treated with pronase (15 mg/ml) in 0.5% sodium dodecyl sulfate. Nucleic acids were extracted with phenol, phenol-chloroform-isoamyl alcohol, and chloroform-isoamyl alcohol and collected by ethanol precipitation. For PCR analysis, 100 ng of sample DNA was used.

Amplification of chlamydial DNA. The PCR was performed as described previously (3). Two sets of oligonucleotide primers were used. The first set was derived from 16S rRNA gene sequences of *Chlamydia psittaci* (20) and generated 208-bp amplified products with all three *Chlamydia* species, i.e., *C. trachomatis*, *C. psittaci*, and *C. pneumoniae* (R1 = GTGGATAGTCTCAACCCTAT, R2 = TATCTGTCCTTG CGGAAAAC, probe = ACTCAAAAGAATTGACGGGGG CCCGCACAA). The second primer set was derived from sequences of the common endogenous plasmid of *C. trachomatis* (18) and generated species-specific, 517-bp amplified products with all known *C. trachomatis* serovars (T1 = GG ACAAATCGTATCTCGG, T2 = GAAACCAACTCTACG CTG, probe = CGCAGCGCTAGAGGCCGGTCTATTTAT GAT). Oligonucleotide primers and probes were synthesized on an Applied Biosystems 381A DNA synthesizer by using the β-cyanoethyl phosphoramidite method. Besides negative patient samples, water and human spleen DNA were used as negative controls in every PCR experiment.

The amplification reaction was performed in a volume of 100 μl containing 100 ng of sample DNA; 10 mM Tris hydrochloride (pH 8.3); 50 mM KCl; 2.5 mM $MgCl_2$; 0.01% gelatin; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 100 pmol of each primer; and 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Three drops of mineral oil (Sigma, St. Louis, Mo.) was added to prevent evaporation. The amplification was performed in a PCR processor (Biomed, Ditfurth, Federal Republic of Germany); and each cycle contained a denaturation step at 94°C for 1 min, a primer annealing step at 42°C for 2 min, and an elongation step at 74°C for 3 min. After 40 cycles, 40 μl of the reaction mixture was analyzed by electrophoresis on a 2% agarose gel (15).

Southern blot and dot spot hybridization. For Southern blot hybridization, the amplified products were transferred from the gel to a nylon membrane (Hybond; Amersham, Buckinghamsire, United Kingdom) by diffusion blotting in 0.4 M NaOH. For dot spot hybridization, 40 μl of the PCR products was used. After denaturation of the samples in 0.5 N NaOH at 65°C for 10 min, they were neutralized with 10 M ammonium acetate (pH 5.6) and spotted onto a nylon membrane. The blots were baked at 80°C for 2 h. Prehybridization was performed at 37°C in a solution containing 5× SSC (75 mM sodium citrate plus 750 mM NaCl), 5× Denhardt solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate, 75 mM EDTA, and 0.1 mg of denatured sonicated herring sperm DNA per ml. Hybridization was performed in the same solution at 37°C for at least 3 h with a $\gamma^{32}P$-labeled oligonucleotide probe directed against an internal sequence of the amplified products. The blots were washed three times in 2× SSC–0.1% sodium dodecyl sulfate, the first two times at 37°C and the last time at 55°C. Autoradiography was performed at −80°C for 3.5 h on Kodak Royal X-Omat film by using intensifying screens.

RESULTS

Analysis of the PCR products showed that all 38 culture-positive samples (29 cervical scrapes and 9 urethral swabs) were found to be positive in the PCR with the rRNA primers as well as the *C. trachomatis*-specific endogenous plasmid primers. All these samples were collected before the initiation of treatment, i.e., administration of doxycycline for 1 week. Of the 44 negative culture results (30 cervical scrapes and 14 urethral swabs), 43 were found to be negative in the PCR. Typical PCR results are shown in Fig. 1. One culture-negative cervical swab, however, was found to be positive in the PCR with both primer sets. Subsequent analysis of the patient data showed that this culture-negative sample was a pretreatment sample.

In summary, all 39 pretreatment samples were PCR positive. In culture, one of these was found to be negative. One week after treatment, all samples were culture and PCR negative. The culture and PCR results were in complete agreement, except for one false-negative sample by the culture technique.

DISCUSSION

In recent years, diagnostic methods for the detection of chlamydiae were extensively studied. Different immunoassays for antigen detection were designed and compared with each other and with the culture technique (6, 12, 13). Although no complete agreement was obtained, the results were acceptable, and several immunoassays for chlamydiae are commercially available. The sensitivity of *Chlamydia* detection could be increased by using the PCR. In theory, one molecule of target DNA can be detected. *C. trachomatis*-specific PCR assays have been developed with primers directed against chromosomal genes (5), rRNA genes (3, 14), and the endogenous plasmid (3). Because of the sensitivity of the PCR, the diagnostic value of these assays must be established. Treatment with antibiotics may kill or inactivate the target microorganism, but if DNA remains present, the PCR may give positive results. To study this possibility, a group of 32 patients was selected who were initially culture positive for chlamydiae and had at least one posttreatment negative culture. All culture-positive results were confirmed by the PCR with the *C. trachomatis*-specific primers directed against the endogenous plasmid as well as with primers directed against the rRNA genes. The PCR with this genus-specific rRNA primer set not only confirms the results obtained with the endogenous plasmid primers but also detects *C. psittaci*, *C. pneumoniae*, or unknown *C. trachomatis* strains. In most cases, the amplified products are visible on the agarose gel. However, with specimens from some patients, the amplified products were masked by a smear of DNA. For this reason, confirmation of the results by oligonucleotide hybridization is an essential part of the PCR technique.

The results of all positive and negative samples from the 32 patients showed complete agreement by the culture and PCR techniques, except for one sample from a patient with a previous positive culture result. This sample was negative in the culture assay, but it was found to be positive in the PCR with both primer sets. The patient data showed that this sample was taken before treatment and therefore was a false-negative culture result because it was obtained before treatment. It is known that some *Chlamydia* samples are toxic for the cell culture monolayers and that correct transportation and storage of the *Chlamydia* samples are impor-

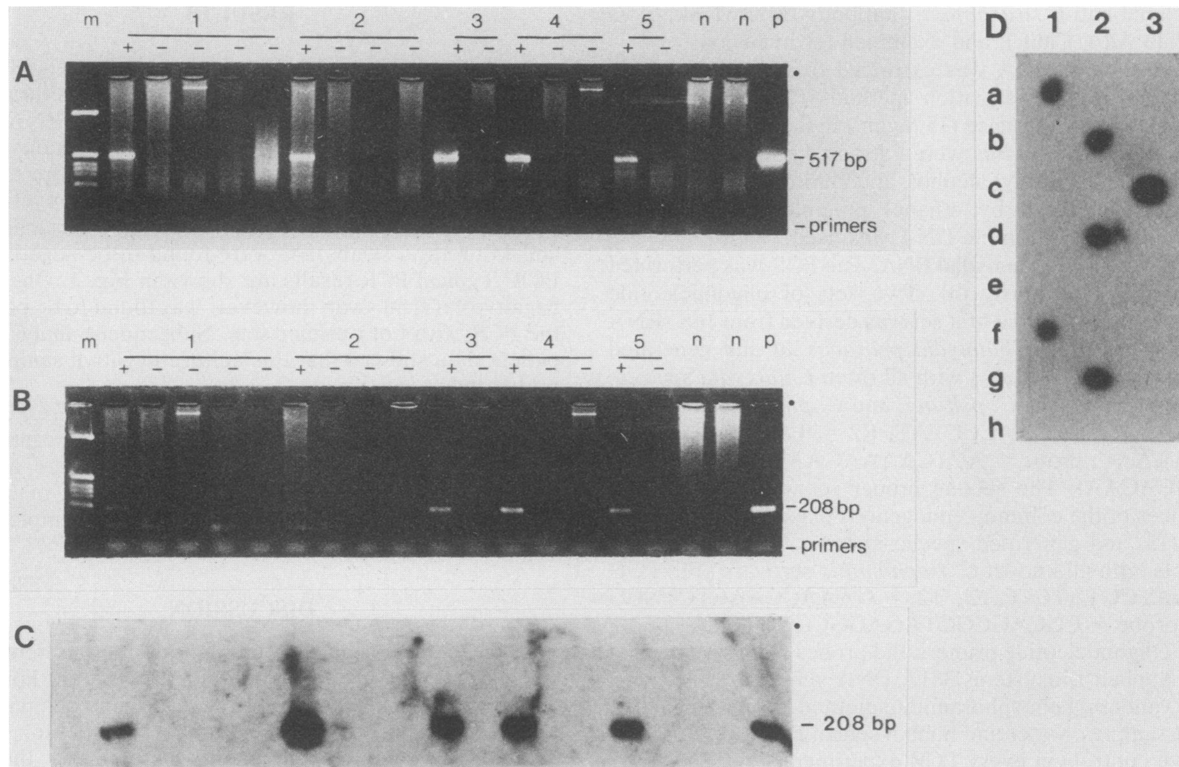

FIG. 1. PCR of samples from patients (lanes 1 through 5) with a positive culture result before treatment (lanes +) and negative culture results after treatment (lanes −). The products of the PCR with the species-specific primers of the common endogenous plasmid of *C. trachomatis* (A) and the genus-specific rRNA primers (B) were electrophoresed on a 2% agarose gel. Plasmid pBR322 digested with *Hin*fI was used as a marker (lanes m). Two negative controls (lanes n) were included, and a cultured laboratory strain of *C. trachomatis*, LGVII, was used as a positive control (lanes p). A Southern blot of the gel in panel B was hybridized with an oligonucleotide probe directed against the 208-bp amplified products of the genus-specific rRNA primers (C). A dot spot hybridization with a specific probe against the 517-bp amplified products of the *C. trachomatis*-specific plasmid primers (D) shows positive results for patients 1 (a1), 2 (f1), 3 (b2), 4 (d2), and 5 (g2). The other patient samples (patient 1, b1 to e1; patient 2, g1 to a2; patient 3, c2; patient 4, e2, f2; and patient 5, h2) and the negative controls, spotted on 3a and 3b, respectively, are negative in this hybridization. The positive control is spotted on 3c. The spotted samples correspond to the samples on the gel shown in panel A.

tant for the viability of the microorganism (8). In immunoassays or DNA assays, the toxicities of the samples and the instability of chlamydiae do not influence detection. Until April 1990, 258 samples from the routine microbiology laboratory were examined for *C. trachomatis*, and 37 were found to be positive by the culture technique. By using the PCR, 40 positive samples, including all culture-positive samples, were detected (unpublished data).

The most important aspect of the follow-up study was that detection of chlamydiae by the PCR after treatment of the patients was in complete agreement with the results obtained by the cell culture technique. Chlamydial DNA was absent at the sampling site 1 week after treatment with doxycycline. As a method for the detection of *C. trachomatis* infections, the PCR is superior to the culture technique. Use of the PCR overcomes specific culture problems such as toxicity of clinical specimens or the viability of the microorganisms during transportation and storage. Besides the higher sensitivity, an additional advantage is that clinical samples can be examined for different microorganisms simultaneously by the same technique. Simultaneous infections with *C. trachomatis* and other sexually transmitted microorganisms are quite common. The follow-up patient group was also examined for other microorganisms. Besides six patients positive for *Gardnerella vaginalis*, *Candida albicans* was detected in seven patients, *Trichomonas vaginalis* was detected in three patients, and *Neisseria gonorrhoeae* was detected in two patients.

Finally, screening of the male population can easily be performed by the PCR. The invasive sampling technique can be replaced by first-catch urine analysis, which is less inconvenient. The chlamydial antigen is detected in first-catch urine samples (12), and the PCR can be performed on urine sediments, as shown previously for human papillomavirus (11).

For the detection of *C. trachomatis*, the culture technique can be replaced by the PCR. The PCR can become a major technique in the laboratory diagnosis of other microorganisms as well.

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