Clinical Evaluation of a New Polymerase Chain Reaction Assay for Detection of *Chlamydia trachomatis* in Endocervical Specimens

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A clinical evaluation of the Amplicor polymerase chain reaction (PCR) assay for the detection of *Chlamydia trachomatis* in endocervical swabs (Roche Molecular Systems, Branchburg, N.J.) is described. This new clinical system used one-step sample preparation, amplification with biotinylated cryptic plasmid primer pairs (CP24-CP27), uracil-N-glycosylase (AmpErase), and a microtiter format for amplicon capture and detection. Culture with McCoy cells in duplicate 1-dram (3.697-ml) vials with fluorescent immunostaining was the reference system. Endocervical swab samples from 945 women provided 74 culture-positive specimens, of which PCR detected 71. The initial PCR result was positive for 12 additional specimens. Arbitration of the PCR-positive, culture-negative samples by PCR with major outer membrane protein primers, duplicate culture, elementary body direct fluorescent-antibody staining, and DNA extraction PCR showed that all 12 samples were positive for chlamydia, raising the number of truly positive samples from 74 to 86. After arbitration the true sensitivities of PCR and culture were 96.5 and 86%, respectively (*P* = 0.02). Specificities for both were 100%. For PCR, the positive and negative predictive values were 100 and 99.7%, respectively. Total test efficiency was 99.7%. A high-test-volume (121 samples) timing study with all items included in the College of American Pathologists work load method indicated that this PCR format took approximately 3 min per sample. Because of the high sensitivity, specificity, and improved ease of handling, we found PCR to be a good alternative to culture for detection of *C. trachomatis*.

*Chlamydia trachomatis* is a small obligate intracellular parasite frequently implicated in sexually transmitted diseases such as cervicitis, nongonococcal urethritis, and pelvic inflammatory disease (1, 4, 23). Infertility and ectopic pregnancy in women and conjunctivitis in newborns are associated sequelae (1, 4). The clinical and epidemiologic implications associated with undetected infections in women have increased demand for a rapid and accurate laboratory screening test (4, 18, 19, 23). Culture on McCoy cell monolayers, especially in 1-dram (3.697-ml) vials, is considered to be the reference method for chlamydial detection, but the method is laborious and slow. A number of rapid test kits are commercially available, including a DNA probe, an enzyme-linked immunosorbent assay, and direct fluorescent-antibody staining (1, 4, 19, 21, 26). These tests are good, but improvements in test sensitivity are an ongoing quest, especially for women with minimal or no symptoms.

Polymerase chain reaction (PCR) is an in vitro technique of amplifying specific DNA fragments over 109-fold through cycles of primer annealing and enzymatic extension of target sequences by DNA polymerase at specific temperatures (7, 20). Investigators have described several PCR-based assays that use primer pairs directed to the cryptic plasmid (CP) (5, 6, 8, 11, 12, 14–17), the major outer membrane protein (MOMP) gene (2, 3), or rRNA (5, 6, 25); these assays have higher sensitivities than cell culture or enzyme immunoassay (EIA) (8, 11, 12, 14, 15, 25). The purpose of the present study and report was to perform an independent clinical evaluation of the new Roche Amplicor chlamydia cryptic plasmid PCR assay and compare it with the 1-dram (3.697-ml) vial McCoy cell culture as the reference method.

**MATERIALS AND METHODS**

**Specimens.** A total of 1,135 endocervical specimens along with clinical histories were collected from women seen on initial consultation in the obstetrics-gynecology clinic of Thomas Jefferson Hospital, Philadelphia, Pa. These women were considered to be at increased risk for sexually transmitted disease because they had three or more risk factors such as age group, no barrier method of contraception, and multiple partners. Our clinic had a 7.8% culture-positive rate for chlamydiae during the present study. Two samples obtained with dacron-tipped swabs were collected from each patient in random order after cleaning the cervix. Samples were immediately stored at 4°C unless testing was to be delayed over 24 h, in which case they were stored in a freezer at −75°C. The specimens were divided into three study groups, depending on the technique used for PCR specimen collection and handling, as follows: study group I consisted of 300 swabs stored frozen at −75°C in 200 μl of 0.9% saline. The swabs were transferred to Amplicor Specimen Transport Medium (STM) containing sodium dodecyl sulfate just prior to processing. Study group II consisted of 190 swabs stored in Amplicor STM until they were processed. Study group III consisted of 645 swabs that were immediately mixed in STM for more than 10 s at the time of specimen collection, and then the swab was discarded.

**McCoy cell culture assay for chlamydiae.** Swabs for culture were collected in chlamydial transport medium (Bartels, Deerfield, Ill.). Before processing, the specimens were mixed for 1 min by using a vortex mixer (Baxter Scientific
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Products, McGaw Park, Ill.) and were then inoculated into duplicate 1-dram (3.697-ml) vials containing McCoy cell monolayers (BioWhittaker, Walkersville, Md.) and spun in a centrifuge at 3,900 \( \times g \) for 1 h. After centrifugation, the inoculum was removed and the vials received growth medium containing cycloheximide (Bartels). They were incubated for 48 h at 37°C in 5% CO\(_2\). After incubation, the cell monolayers were fixed in cold methanol for 10 min. One vial per patient specimen was stained with a direct fluorescent monoclonal antibody stain (Ortho Diagnostic Systems, Raritan, N.J.) following the manufacturer’s instructions. The number of inclusions per coverslip was counted by using a fluorescence microscope (\( \times 400 \)). The presence of one or more typical inclusions was considered a positive result. The second coverslip was frozen at -75°C until needed. If the initial culture stain was negative but the PCR result was positive, the second coverslip was stained and was reported as the duplicate culture result.

PCR assay. All reagents and equipment for performance of the PCR assay were supplied by Roche Molecular Systems, Branchburg, N.J. This test was performed according to draft package insert instructions for the Amplicor chlamydia kit. Sample preparation consisted of the addition of 1 ml of specimen diluent containing Tween 20 and magnesium ion to each specimen tube containing 1 ml of STM. This was mixed with a vortex mixer and was allowed to stand at room temperature for 10 min. All swabs were removed prior to the addition of diluted reagents. Reagents were allowed to equilibrate to room temperature before use. Master mixture reagent was prepared by the addition of 100 \( \mu \)l of uracil-N-glycosylase enzyme (AmpErase) to the master mixture reagent containing the deoxynucleotide triphosphates dATP, dGTP, dCTP, and dUTP, biotinylated primer pairs CP24 and CP27, and thermostable Taq polymerase just prior to the amplification process. A 50-\( \mu \)l aliquot of master reagent mixture was dispensed into each reaction tube of a tray (MicroAmp; Perkin-Elmer, Norwalk, Conn.); this was followed by the addition of 50 \( \mu \)l of the appropriate specimen or control sample. Single-well amplification was performed on patient samples. Positive and negative controls provided with the kit were amplified in duplicate. The reaction tubes were sealed with plastic caps and were immersed into a Perkin-Elmer 9600 thermocycler programmed for one cycle of 5 min at 95°C and then 1 min at 60°C. Subsequently, there were 29 cycles of 30 s at 95°C plus 1 min at 60°C. There was a final hold temperature of 72°C. After performance of the CP PCR amplification, the amplified products were immediately de- 

There was a final hold temperature of 72°C. After performance of the CP PCR amplification, the amplified products were immediately de-natured for 10 min with 100 \( \mu \)l of denaturant solution containing sodium hydroxide. A 100-\( \mu \)l amount of hybridization solution was added to each well of a 96-well microtiter plate containing a specific plate-immobilized oligonucleotide probe (CP35); this was followed by the addition of 25 \( \mu \)l of the denatured reaction mixture. The plate was incubated at 37°C for 1 h, and then each well in the 96-well plate was washed five times with wash buffer and an automated plate washer. A 100-\( \mu \)l aliquot of avidin-labeled horseradish peroxidase conjugate was then added to each well, and the plate was incubated for 15 min at 37°C. After incubation with conjugate, each well in the 96-well plate was washed five times with wash buffer, and a 100-\( \mu \)l amount of prepared substrate containing four parts of reagent A (hydrogen peroxide) and one part of reagent B (tetramethylbenzidine) was added to each microtiter well. After incubation for 10 min, each well received 100 \( \mu \)l of stop reagent, and the \( A_{400} \) of each well in the 96-well plate was measured. Samples were classified as PCR positive or negative according to an optical density (OD) cutoff value of 0.250. Samples with initial readings of between 0.200 and 0.500 OD units were considered borderline during this evaluation phase and were classified as positive or negative on the basis of retesting in duplicate.

Avoidance of PCR amplicon contamination. Pre-PCR reagent preparation, specimen processing, and amplification were performed in one room. Post-PCR amplicon detection procedures were performed in a separate room. The pre-PCR room did not have controlled air pressure relative to the post-PCR room. A dedicated biological safety cabinet was used for pre-PCR specimen processing. All work areas were wiped with 10% Clorox before and after use. The biological safety cabinet was also subjected to UV light after use. All pipettes, equipment, and supplies were dedicated to each work area. Positive-displacement and filter-tipped negative-displacement pipettes were used when appropriate. Frequent glove changing was maintained, but the PCR technologist did not routinely change laboratory coats when moving from room to room. These laboratory rooms were busy workplaces for a variety of other clinical microbiology laboratory procedures, so it was impossible to restrict the movement of other personnel between these rooms.

Resolution of discrepant samples. If the CP PCR result was positive and the culture result was negative, the duplicate culture coverslip was stained. If the duplicate culture coverslip result was positive, then the specimen was considered truly positive. If the duplicate culture coverslip result was negative, further analysis of discrepant results was performed by Roche Molecular Systems by using alternate primer (MOMP) PCR and DNA phenol-chloroform extraction PCR, either of which was considered to provide a truly positive result. For MOMP PCR-negative specimens, DNA extraction was performed and the purified sample was retested by PCR. DNA extraction and MOMP PCR were performed as described by Loeffelholz et al. (11). MOMP PCR was performed essentially as described above for the CP PCR, except for increased cycling times and the use of MOMP-derived primer and probe oligonucleotide sequences. The use of alternate DNA target sequences was to rule out falsely positive PCR results because of amplicon contamination. Discrepant specimens which were positive by culture but which had a negative CP PCR result were phenol-chloroform extracted, ethanol precipitated, and then retested by PCR. This was to detect the presence of possible PCR inhibitors in the specimen.

A final method of arbitration used in study groups II and III involved direct staining of the residual culture specimen transport medium by using specific fluorescent antibody for elementary bodies. An aliquot (0.2 ml to 0.5 ml) of the culture transport fluid was spun in a high-speed microcentrifuge for 15 min. The supernatant was removed, and the pellet was resuspended in 100 \( \mu \)l of phosphate-buffered saline by mixing with a vortex mixer. The procedure was repeated, and then a drop of the resuspended pellet was delivered to a microscope slide and air dried. The dried pelleted material was fixed with methanol and then stained according to the manufacturer’s instructions (Sanofi Diagnostics/Kallestad, Chaska, Minn.). Known positive and negative specimens were run as controls.

Analysis of data. Significance testing was done by using the modified chi-square test described by McNemar (15).
TABLE 1. Comparison of PCR with cell culture for detection of _C. trachomatis_ by three methods of specimen collection*  

<table>
<thead>
<tr>
<th>Study group and PCR result</th>
<th>No. of specimens with the following initial culture result:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>PCR positive</td>
<td>31</td>
</tr>
<tr>
<td>PCR negative</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td></td>
</tr>
<tr>
<td>PCR positive</td>
<td>9</td>
</tr>
<tr>
<td>PCR negative</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td></td>
</tr>
<tr>
<td>PCR positive</td>
<td>40</td>
</tr>
<tr>
<td>PCR negative</td>
<td>2</td>
</tr>
</tbody>
</table>

* Amplicor PCR with CP primers and McCoy 1-dram (3.697-ml) vial cell culture.

** Results **

The results for study groups I, II, and III are presented in Table 1. Even without arbitration of discrepant specimens, there was good correlation between culture and PCR. For study group I, 31 specimens tested positive by PCR and cell culture and 262 tested negative by both methods. Of the six specimens that initially tested PCR positive and culture negative, all were found to be truly positive (Table 2), bringing the total number of _Chlamydia_-positive samples from 32 to 38 for this group. After arbitration as described in Materials and Methods, the sensitivities of PCR and culture for this group were 97 (37 of 38 specimens) and 84% (32 of 38 specimens), respectively. The specificities of PCR and culture were both 100% (262 of 262 specimens).

For study group II, an attempt was made to make sample processing more convenient by placing specimen swabs directly into Amplicor STM rather than saline. Specimens were held for several hours at 4°C or were frozen for several days prior to testing. This group initially contained 12 culture-positive specimens, of which CP PCR detected 9. Three specimens were falsely negative by PCR, indicating an increase in the proportion of falsely negative PCR specimens compared with the proportion in study group I specimens. Roche Molecular Systems stopped specimen collection by this method after they found that the kit’s specimen collection swabs could exert an inhibitory effect on the subsequent PCR if left in STM for prolonged periods of time. The three specimens which were positive by CP PCR but culture negative were all found to be truly positive, bringing the number of truly positive samples from 12 to 15 for study group II specimens.

Study group III initially contained 42 culture-positive specimens, of which 40 tested PCR positive. There were an additional six PCR-positive specimens that were initially culture negative. All were confirmed as truly positive, bring-
TABLE 3. Comparison of Amplicor PCR and culture with total resolved specimens from study groups I and IIIa

<table>
<thead>
<tr>
<th>Test and result</th>
<th>No. of resolved specimens</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>PCR Positive</td>
<td>83</td>
<td>0</td>
<td>96.5</td>
</tr>
<tr>
<td>PCR Negative</td>
<td>3</td>
<td>859</td>
<td>100</td>
</tr>
<tr>
<td>Culture Positive</td>
<td>74</td>
<td>0</td>
<td>86.0</td>
</tr>
<tr>
<td>Culture Negative</td>
<td>12</td>
<td>859</td>
<td>100</td>
</tr>
</tbody>
</table>

a Results for all specimens were classified as truly positive or negative on the basis of complete agreement of both test methods or arbitrated results when differences occurred.

DISCUSSION

We performed a premarketing evaluation of a new clinical PCR kit, Amplicor, for the detection of C. trachomatis in endocervical samples. The study population was an obstetrics and gynecology clinic, with a culture prevalence rate of 7.8% in patients with qualifying risk factors. Our study protocol differed in several ways from that of a previously published study by Loeffelholz et al. (11). There was a difference in the order of reagent addition for specimen, diluent, and the master mixture. Our procedure followed the instructions in the kit package insert. We used reagents identical to those that will be found in the marketed production kits. In addition, we evaluated alternative methods of specimen collection. This evaluation also reflected the performance of the kit in a routine clinical microbiology laboratory setting. Our sensitivity and specificity results were consistent with those in the report by Loeffelholz et al. (11). From our study of 945 specimens which were not affected by the inhibitory effects of prolonged storage of the swab in STM (Table 3), the Amplicor assay gave greater sensitivity (96.5%) than culture (86%). The difference was statistically significant (P = 0.02). The specificity was 100%, the positive predictive value was 100%, the negative predictive value was 99.7%, and the total test efficiency was 99.7%.

Current non-PCR diagnostic tests have been shown to perform well in symptomatic or high-prevalence groups and tend to be less effective in low-prevalence or asymptomatic populations (10, 22, 23). One strength of a PCR-based assay is the potential to detect infection in lower-prevalence groups (prevalence <10%), in which infection may be asymptomatic or the number of infecting organisms may be low. Most PCR assays described in the literature for detection of C. trachomatis in clinical specimens have had good (94 to 100%) sensitivity and specificity (8, 11, 12, 16, 25). Mahony et al. (12) reported PCR to be 100% sensitive and 99.5% specific compared with the blocked Chlamydiazyme EIA on male urine specimens. Ostergaard et al. (16) tested 228 clinical samples and found PCR to be 100% sensitive in comparison with cell culture and to have a corrected specificity of 99% when compared with culture and EIA. Two premarketing evaluations of the Amplicor assay have been described. Jaszek et al. (8) reported that the Amplicor test had a revised sensitivity and specificity of 95 and 99.8%, respectively, in comparison with culture or MOMP PCR when they tested 530 male urinary-urethral sample pairs. Loeffelholz et al. (11) reported a revised PCR sensitivity of 97% and specificity of 99.7% compared with culture or MOMP PCR when they tested 503 cervical specimens. They also reported a revised PCR sensitivity and specificity of 100% when compared with the Chlamydiazyme EIA by using 375 specimens. In conclusion, most studies have reported higher sensitivities for PCR than for cell culture (8, 11, 14, 15), EIA (11, 12, 15, 25), or elementary body direct fluorescent-antibody assay (3) when testing various urogenital and eye specimens.

In our study, 4% (3 of 74) of the culture-positive specimens were initially negative by PCR because of the presence of PCR inhibitors (this value excludes data from study group II). Failure of specimens to initially amplify because of interference from PCR inhibitors in the specimen have been reported by others (3, 11, 25). Some inhibitors could be removed by sample dilution (3, 25) or heating to greater than 95°C (11). Our data from study group II suggested a possible inhibition caused by prolonged exposure of the dacron-tipped plastic specimen collection swab to the detergent in the STM. In this group, assay sensitivity appeared to decrease to 79% from 97%. This trend did not reach statistical significance before the termination of specimen collection for this group. Experiments performed at Roche Molecular Systems indicated that PCR inhibition could occur after prolonged exposure of the swab to STM, but the exact nature of this inhibition has not been elucidated. Inhibition was minimized when the collection protocol described for study group III was followed. Swabs could be placed directly into STM for a brief mixing time and could be removed promptly afterward. This is the method currently recommended by the manufacturer. We also found that swabs could be stored frozen at −75°C in 0.2 ml of saline for...
prolonged periods and still give a good PCR test correlation with culture results.

The avoidance of false-positive Chlamydia test results is important because of social implications and the costs associated with unnecessary treatment (10, 19). Since PCR has the potential to amplify even one molecule of DNA to detectable levels, desirable PCR work practices must be maintained to prevent amplicon carryover (9). The high overall specificity that we achieved indicated that the AmpErase system, plus physical separation of pre- and post-PCR areas, were adequate to prevent intrunrun amplicon contamination. The possibility of intrarun specimen contamination or well-to-well cross-contamination cannot be prevented by incorporation of AmpErase into the assay. Therefore, adherence to meticulous setup and transfer techniques along with frequent glove change should be maintained. In addition, the use of separate workstations with dedicated pipettes and supplies is important.

Nonisotopic detection systems for the detection of PCR products in microtiter wells and tubes have been described; those systems use chemiluminescent and fluorometric labels (2, 3, 24) and chromogenic substrates (8, 11). Bobo et al. (2) described successful amplicon detection by hybridization in combination with an enzyme label in a microtiter plate assay. Traditional detection systems use gel electrophoresis and ethidium bromide staining (12, 25) and gel electrophoresis and then Southern blot hybridization (5, 6, 12, 14–16) and are excellent detection methods, but they are less adaptable than microtiter formats to high-volume clinical testing.

The Amplicor test can be read by using standard EIA equipment which may minimize additional capital equipment purchases. We found that the Amplicor assay resulted in OD values that were widely separated for the positive and negative groups of specimens, making interpretation easy. Most positive specimens had OD values of ≥1.0, and negative specimens usually had OD values of ≤0.1. For the present evaluation, an equivocal zone between 0.2 and 0.5 OD units was used. All specimens with values that fell within this zone were repeated tested. A cutoff value of 0.250 OD units was determined by the manufacturer to be optimal. A total of 4 of 945 (0.4%) specimens fell within the equivocal zone. One of the four specimens had an initial OD reading of 0.233 (below cutoff), but duplicate repeat testing resulted in positive values greater than 0.250 OD units. The remaining three specimens confirmed the initial test (positive if the initial result was greater than 0.250 OD units or negative if the initial result fell to less than 0.250 OD units). All four specimens had repeat-test values in agreement with culture results. This wide separation between positive and negative readings is an advantage of the Amplicor assay and is a characteristic not always found in EIAs for chlamydiae (19). The PCR assay described by Bobo et al. (2) also showed a clear distinction between positive and negative values by using a fluorescent endpoint.

We evaluated the ease of performance of the Amplicor PCR assay and found that it was convenient for testing large numbers of samples, and testing could be completed in 4.5 h. Automated PCR amplification in the Perkin-Elmer 9600 programmable thermocycler was able to accommodate a 96-well tray of reaction tubes. For specimens collected and transported to the laboratory in the STM tube, sample preparation consisted of one step, the addition of diluent to the STM tube. In contrast, sample preparation described in the literature included DNA extraction methods (5, 6, 25), detergent and protease treatment and then heating (2, 12, 15, 16), and centrifugation and then boiling (14). One future advantage of the Amplicor assay format is its potential for increased automation.

We measured the total hands-on time for performance of a typical 121-sample run for chlamydia testing by using the criteria of the American Society of Clinical Pathologists work load recording method. Included in this timing were initial laboratory specimen accessioning and handling, actual test processing time, recording and reporting of results, plus estimates of daily and periodic maintenance, repair, and technical supervision. The total technologist time to perform this large run averaged 3 min per specimen. Additional timing studies need to be performed at other sites, especially those that use runs with fewer numbers of samples.

This first PCR test designed to be commercially available in kit form for use in clinical microbiology laboratories should be readily accepted. With this simple format, we feel that PCR assays for infectious diseases should be done in the microbiology laboratory rather than in another section, such as a chemistry or a special molecular biology laboratory serving various disciplines. Patient care will benefit when those performing the test are the most informed individuals regarding the interpretations and implications of various microbiology test results and infectious disease issues. Because of the technical ease of the assay format, especially with carryover prevention with AmpErase, it is suitable for use in routine microbiology laboratories with relatively minor adjustments in work flow. Most technologists currently working in microbiology laboratories will need some PCR-specific education and training. When that is accomplished, a PCR assay such as the one described here could readily be transferred from the research laboratory to the high-volume clinical laboratory. We expect PCR to become routinely used if it can demonstrate that it gives more true-positive results than the existing standard test or if it gives the same degree of sensitivity and specificity but is easier or faster than the current reference detection method. Because of the high sensitivity and specificity and the improved ease of handling, we found the Amplicor PCR assay described here to be a good alternative to culture or immunoassay for detection of C. trachomatis.

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REFERENCES


