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# Reproducibility Problems with the AMPLICOR PCR *Chlamydia trachomatis* Test

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**In an attempt to use an expanded "gold standard" in an evaluation of an antigen detection test for *Chlamydia trachomatis*, the AMPLICOR (Roche Diagnostics Systems, Inc., Branchburg, N.J.) PCR *Chlamydia trachomatis* test and culture were used with 591 sets of cervical specimens. Of the 591 specimens assayed, 35 were retested due to either an equivocal result by the PCR (19 samples) or a discrepancy between the results of culture, PCR, and the antigen detection method. During the repeat testing of the samples with equivocal and discrepant results, all but one interpretation change was due to the PCR result. In addition, upon repeat testing the PCR assay value measured in optical density units varied widely for 13 of these specimens. These 13 specimens were then tested in triplicate by the manufacturer with primers to the chlamydia plasmid and in duplicate with primers to the major outer membrane protein. Only 3 of the 13 specimens gave the same interpretation with these five replicates. In summary, reproducibility problems with the AMPLICOR test should be considered before it is incorporated as part of routine testing or used as an expanded gold standard for chlamydia testing.**

*Chlamydia trachomatis* is a leading cause of sexually transmitted diseases. This pathogen is primarily associated with cervicitis and urethritis. Chlamydia infections can involve the fallopian tube, where they can cause tubal scarring and eventual infertility (8, 12). In addition, infants born to infected mothers are at risk for conjunctival and respiratory infections caused by this organism (4). Many female patients infected with *C. trachomatis* are asymptomatic and are in the early stages of their reproductive lives (15). Therefore, due to the morbidity associated with these infections, screening of individuals at greatest risk has been proposed (7).

The "gold standard" for the diagnosis of chlamydial infections has been cell culture. However, culture is technically demanding, not standardized, and not widely available, and due to the fastidious nature of chlamydiae, if the transport of specimens is not tightly controlled, culture results could be compromised (1, 10, 11). As a result there is great laboratory-to-laboratory variation in the sensitivity of tissue culture methods (18). More recently, it has been suggested that for evaluations of new tests for the detection of *C. trachomatis*, a combined gold standard of culture and a nucleic acid amplification test be used (16). In a recent evaluation of an antigen detection method, our study design was to use an expanded standard by using the AMPLICOR (Roche Diagnostics Systems, Inc., Branchburg, N.J.) *Chlamydia trachomatis* PCR test and culture. However, due to reproducibility problems with PCR, it could not be used as part of an expanded gold standard. This report summarizes the problems encountered with the PCR results.

Cervical specimens were obtained from patients seen in the Obstetrics and Gynecology Clinic at the University of California, Irvine, Medical Center. Data for patients who had been receiving antibiotics 14 days before samples were obtained were excluded from the data analysis. After cleansing of the cervix, swab specimens were obtained for each of the different detection methods used in the study. The order of collection of the swab specimens was rotated every 50 patients. For culture, a cotton swab (American Scientific Products, McGaw Park, Ill.) was used to collect the sample and was placed in 2.0 ml of 0.2 M sucrose, 0.02 M phosphate, and 5 mM glutamate (pH 7.2) transport medium, which contained amphotericin B (25 μg/ml) and gentamicin (50 μg/ml). Swabs and transport media for PCR were provided with the AMPLICOR assay kit. Culture was performed within 24 h of sample collection as described previously (13). Briefly, 24- to 48-h-old monolayers of McCoy cells contained in glass vials (15 by 45 mm) were inoculated with 0.2 ml of specimen and centrifuged at 1,000 × *g* at 30°C for 1 h. Afterward, 1 ml of Eagle's minimum essential medium containing fetal bovine sera (10%), gentamicin (50 μg/ml), and cycloheximide (1.0 μg/ml) was added. After 48 h of incubation at 37°C, the cultures were fixed in ethanol and were stained with the reagent provided in the MicroTrak *Chlamydia trachomatis* Culture Confirmation Test kit (Syva Co., San Jose, Calif.). Cultures that appeared toxic or contaminated were treated with vancomycin (25 μg/ml) and subcultured. Specimens for the PCR assay were frozen at −40°C and were tested on a weekly basis.

PCR was performed according to the manufacturer's instructions and as described previously (3). Optical density (OD) values greater than 0.25 were considered positive; however, initial values falling between 0.20 and 0.50 were considered equivocal and the test was repeated in duplicate. Upon duplicate testing the interpretation for two of the three determinations was considered the result.

Any sample with a culture or PCR result that was discrepant with the results obtained by the other two methods was tested again. If the repeat culture or PCR result agreed with the results of the other methods, the discrepancy was considered resolved; if it did not, it remained a discrepant result. In addition, as stated above, with the PCR, all samples with initial equivocal results were tested again, in duplicate, in accordance with the instructions in the package insert. If the result of the test performed to resolve the initial equivocal result was discrepant with the results of the other two tests, the sample was tested again. With some samples the PCR result was so vari-

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TABLE 1. Overall results

| Group | Result | | No. of specimens | |
|---|---|---|---|---|
| | Culture | PCR | Initial | Resolved |
| A | − | − | 533 | 553 |
| B | + | + | 21 | 26 |
| C | + | − | 6 | 3 |
| D | − | + | 12 | 9 |
| E | − | E[a] | 18 | 0 |
| F | + | E | 1 | 0 |

[a] E, equivocal.

able that a positive or a negative result could not be assigned to the sample (see below). These samples were sent to the laboratories of Roche Diagnostics Systems, where PCR was performed in triplicate with the plasmid primers and in duplicate with major outer membrane protein (MOMP) primers. For these samples, for the purpose of data analysis, the final result was determined to be that which was the same for at least three of the five tests performed at Roche Diagnostics Systems.

Included in the study were 591 sets of cervical specimens, of which 29 were positive by culture, for a prevalence of 4.9%. The overall results for the initial and repeat tests are presented in Table 1. Initially, 21 specimens were positive by both culture and PCR. By repeat testing, and the resolution of discrepant results, 26 positive samples showed agreement between the two systems. Between the initial and repeat tests, the interpretation for only one culture changed (Table 2). This culture, which was originally read as negative, had a subconfluent monolayer, possibly due to contamination or toxicity.

In contrast, the PCR results on repeat testing showed greater variability and accounted for all but 1 of the 35 changes in the initial and resolved results (Table 2). In the initial test, 3.2% (19 of 591) of the samples tested gave equivocal results by PCR, of which 14 were determined to be negative. Initially, six samples were positive by PCR, but upon repeat testing, they proved to be negative. In addition, three samples initially negative by PCR were shown to be positive upon repeat testing. Thirteen samples with discrepant or equivocal PCR results showed wide variations in the OD values in both the initial and the repeat test(s) and thus were sent to Roche Diagnostics Systems for further testing. In addition, we sent in five other samples for further testing because while the repeat PCR results were the same as the initial results, they were discrepant compared to the culture results. All of these samples that were tested by Roche were subjected to five tests: three with the same plasmid primers supplied with the AMPLICOR assay and two with primers to the MOMP. As can be seen from the data in Table 3, for 10 of these 18 samples the results were also variable upon testing by Roche. For purposes of analysis of the data from the five tests performed by Roche, at least three of the repeat PCR assays had to be positive for the sample to be considered positive.

TABLE 2. Distribution of discrepant or equivocal results

| Group[a] | No. of specimens | | Test result | |
|---|---|---|---|---|
| | Initial | Resolved | Culture | PCR |
| C | 6 | | + | − |
| B | | 3 | + | + |
| D | 12 | | − | + |
| A | | 6 | − | − |
| B | | 1 | + | + |
| E | 18 | | − | E[b] |
| A | | 14 | − | − |
| D | | 4 | − | + |
| F | 1 | | + | E |
| B | | 1 | + | + |

[a] Groups correspond to those in Table 1.
[b] E, equivocal.

TABLE 3. Results obtained by Roche for samples with discrepant results[a]

| Sample no. | Result (O D) with primers for: | | | | | No. of positive assays |
|---|---|---|---|---|---|---|
| | Plasmid | | | MOMP | | |
| | Trial 1 | Trial 2 | Trial 3 | Trial 1 | Trial 2 | |
| 1 | 0.253 | 0.186 | 0.069 | >3 | 0.071 | 2 |
| 2 | 0.290 | 0.346 | 0.080 | 0.072 | 0.067 | 2 |
| 3 | 0.155 | 0.425 | 0.252 | 0.067 | 0.055 | 2 |
| 4 | 0.165 | 0.221 | 0.639 | >3 | 0.059 | 2 |
| 5 | 0.366 | 0.057 | 0.409 | 0.061 | 0.059 | 2 |
| 6 | 0.055 | 0.312 | 0.063 | 0.064 | >3 | 2 |
| 7 | 0.338 | 0.270 | 0.769 | 0.068 | 2.537 | 4 |
| 8 | 0.280 | 0.287 | 0.075 | 0.059 | 0.071 | 2 |
| 9 | 0.404 | >3 | 0.099 | >3 | >3 | 4 |
| 10 | 0.061 | 1.394 | 0.056 | 0.058 | 0.070 | 1 |

[a] The samples gave variable results at the University of California, Irvine, Medical Center and were therefore sent to Roche Diagnostics Systems and were tested there. An assay result was considered positive if the sample had an OD of ≥0.250 at least three of the five times tested.

It has been reported that DNA amplification techniques have increased sensitivity compared to that of culture (2, 3, 5, 17). However, there are also definite drawbacks to the AMPLICOR PCR assay that may cast doubt on whether some of the increased sensitivity by this assay is due to false-positive results. We, as others, have found that the results obtained by this PCR assay may not be reproducible (3, 5). This was exemplified by the samples that we sent to Roche Laboratories for further testing. Here, as can be seen with samples 1 and 4, values obtained with the same specimen ranged from the upper positive limit of the assay (>3) to a low OD value that was considered a negative result (Table 3). Primers to both the MOMP and the chlamydial plasmid were used. Since a *C. trachomatis* isolate has been reported to lack this plasmid, it is possible that MOMP primers would yield a positive reaction while plasmid primers would yield a negative reaction (14). However, this was not the case for the isolates with discrepant results in this study, because a positive and a negative value could be obtained by duplicate assays with either the plasmid primer or the MOMP primer. In our study we only repeat tested samples with discrepant or equivocal results and did not run each test in duplicate. It is possible that this lack of reproducibility could also have been shown for samples that were not tested again. This point was also raised by Hadgu (6) in a recent review of discrepant analysis work published on DNA amplification methods for the detection of *C. trachomatis*. Possible causes of these erratic results could be either false-positive hybridization during the detection assay, nonspecific priming in the amplification phase, amplicon contamination, inhibition of the PCRs, or a very low concentration of chlamydia (3, 5). Kellogg et al. (9), in trying to overcome the problem of specimen inhibition with the AMPLICOR PCR assay, compared specimens transported on dry swabs to those

placed immediately in transport medium after collection. They found an increase in positivity with the dry swab. Verkooyen et al. (18) tested patient specimens by the AMPLICOR test and then spiked the samples with *C. trachomatis*. The spiked samples were then pretreated by a variety of methods prior to being tested by the AMPLICOR PCR. They reported that certain treatments reduced the level of inhibition of the reactions, resulting in a positive result. However, those investigators performed only a single PCR assay with each pretreated sample and no studies on reproducibility. In light of our findings, it would be important to test these samples multiple times to determine if the reported improvement was reproducible. Further work on this problem is warranted if DNA amplification techniques are to be useful in a clinical laboratory setting where repeat testing is seldom performed. In addition, if DNA amplification techniques are to be used in an expanded gold standard, they must be reproducible.

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