Ligase Chain Reaction To Detect *Chlamydia trachomatis* Infection of the Cervix

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We performed a multicenter evaluation of ligase chain reaction (LCR) in the diagnosis of *Chlamydia trachomatis* infection of the cervix. This LCR provides an amplification of target sequences within the chlamydial cryptic plasmid. The LCR results were compared with those of isolation in cell culture. Discrepant (tissue culture-negative and LCR-positive) test results were resolved by the application of a direct immunofluorescent-antibody test to detect chlamydial elementary bodies and by the use of alternate DNA primers that targeted the chlamydial major outer membrane protein gene. A total of 234 of 2,132 specimens (10.9%) could be confirmed as containing *C. trachomatis*. Of these, 152 were detected by isolation in cell culture and 221 were detected by LCR. The corresponding sensitivities were 94% for LCR and 65% for cell culture. There was greater variability among study site results for cell culture sensitivity (52 to 92%) than for LCR sensitivity (87 to 98%). The specificity of each test was greater than 99.9%. Thus, LCR offers a highly sensitive nonculture method for detecting chlamydial infection of the cervix.

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen (4). It is a major cause of pelvic inflammatory disease and can cause serious disease in newborn infants exposed during passage through an infected birth canal. In the United States, with an estimated four million infections occurring each year and costs running into several billion dollars, it is imperative that effective *Chlamydia* control programs be developed (11). Symptomatic men and women are likely to receive appropriate therapy because Centers for Disease Control and Prevention guidelines now call for the inclusion of antichlamydial therapy into treatment regimens for those conditions which are associated with chlamydiae. A truly effective *Chlamydia* control program must be aimed at reducing the reservoir of infected asymptomatic individuals who make up the bulk of prevalent infections and are responsible for maintaining transmission of the infection within a community.

Treatment is relatively simple and straightforward, with a week's treatment with doxycycline being highly effective (13). The recent introduction of single-dose therapy with azithromycin heralds even more readily acceptable treatment regimens (8). Thus, the challenge continues to be the accurate diagnosis of chlamydial infection.

For many years, isolation in tissue culture (TC), usually with cycloheximide-treated McCoy cells, has been the diagnostic test of choice (9). This has not been a readily available procedure because it requires considerable technical expertise and the maintenance of a cell culture facility. The introduction of nonculture tests, such as direct fluorescent-antibody assay (DFA) and enzyme immunoassays, to detect chlamydial antigens in clinical specimens has greatly increased access to

chlamydial diagnostic tests (9, 12). However, these tests have been recognized as relatively insensitive compared with culture and, as is true for most nonculture methods, can yield false-positive results that could have important social consequences. In addition, during evaluations of nonculture diagnostic tests, it became increasingly apparent that culture itself was far from perfect as a diagnostic test. While its specificity, by definition, is essentially 100%, the sensitivity of culture, even in excellent laboratories, seldom exceeds 90%, is typically between 75 and 85% (10), and sometimes falls below 50%.

Thus, there continues to be a need for better nonculture diagnostic tests, with improved sensitivity and specificity compared with those of antigen methods. Some nucleic acid probes have been made available, but they have not been shown to be markedly more sensitive than the antigen detection methods (5). The use of amplified DNA techniques, such as ligase chain reaction (LCR) and PCR, offer the possibility of increasing sensitivity through amplification (1, 2, 3, 6, 7). Both techniques increase the number of specific target DNA sequences to enhance sensitivity. Amplified DNA products can be detected by enzyme immunoassay by using standard photometric detection of a color product.

We report here the results of a multicenter evaluation of LCR for the detection of *C. trachomatis* in women. The target DNA sequence used for this assay lies within the cryptic plasmid. There are 7 to 10 copies per chlamydial particle, thus theoretically providing a further increment in sensitivity over chromosomal DNA detection.

MATERIALS AND METHODS

Patient population. Patients were enrolled at the following institutions: Johns Hopkins University, Baltimore, Md.; University of Alabama at Birmingham; University of Washington at Seattle; University of California at San Francisco. The patients in Seattle and Baltimore were seen in sexually transmitted disease clinics, the patients in Birmingham were from

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obstetrics and gynecology clinics, and the patients in San Francisco were examined in an emergency room or family planning clinic. Abbott Laboratories, Chicago, Ill., processed some LCR samples, with the matching TC specimens being processed at two of the study sites (Birmingham and San Francisco). The other results presented are for specimens for which both LCR and TC were done locally.

Specimen collection. Specimens were collected in a randomized order for isolation or LCR. Special transport medium was supplied for each test. For cell culture, the medium used was 2SP or TC medium containing antibiotics (50 μg of streptomycin per ml or 10 μg of gentamicin per ml, 100 μg of vancomycin per ml, and 10 U of nystatin per ml) to kill contaminating bacteria or fungi (9). Specimens were collected after the endocervical canal had been cleaned by the removal of discharge with large cotton swabs or sponges. Then, either a cytobrush or a dacron swab was rubbed against the endocervical canal. Swabs were left in the proprietary LCR transport medium.

Specimens were transported to the laboratory on the day of collection and were stored at 4°C prior to the inoculation of cells. Isolation attempts were performed within 24 h of collection. The remaining isolation specimen was then frozen at −70°C in case it was needed for further tests. LCR specimens were transported to the laboratory within 24 h of collection. If specimens could be processed within 4 days, they were stored at 4°C; otherwise, they were stored at −70°C (up to 2 weeks) and processed in batches. Specimens tested in Chicago were shipped frozen. After the initial LCR test, the remaining LCR specimen was frozen at −70°C.

TC isolation. Each of the participating centers used its own standard cell culture isolation procedure. In Seattle, the method involved the use of McCoy cells planted in 96-well plates and no blind passage. In Baltimore, 96-well plates were used and blind passage was performed. In San Francisco and Birmingham, 1-dram (1 dram = 3.697 ml) shell vials and blind passage were used. All study sites used a fluorescent-antibody stain (either Syva culture confirmation reagent [San Jose, Calif.], Kallestad culture confirmation reagent [Chaucer, Minn.], or an in-house-produced species-specific monoclonal antibody) to detect chlamydial inclusions.

LCR assay. The LCR assay has been previously described (6). If they had been frozen, specimens were thawed and tested in batches. For sample preparation, specimens were placed in a heat block at 100°C for 15 min. For DNA amplification, 100 μl of each sample and each control was added to a microcentrifuge tube containing a predispensed LCR mix of four oligonucleotide probes, nucleotides, and a thermostable enzyme (ligase). Tubes were inserted into a Perkin-Elmer model 480 thermocycler programmed for 40 cycles. Each cycle contained a denaturation step of 97°C for 1 sec and lower-temperature steps at 55°C for 1 s and 62°C for 1 min to allow the annealing, gap filling, and ligation of the oligonucleotides. An automated microparticle enzyme immunoassay was used to detect amplicons. Each detection probe was labeled at one end. The 3′ ends of probes 1 and 2 had the same hapten, hapten A. The 5′ ends of probes 3 and 4 had a different hapten, hapten B. Upon ligation, the resulting full-length amplicon was labeled at both ends with haptens. Antibody-coated (anti-hapten A) microparticles, held by a filter, captured ligated and unligated probes 1 and 2. Unligated probes 3 and 4 were washed into the absorbent layer below. The amplicons were then detected by an alkaline phosphatase-labeled conjugate directed against hapten B on probes 3 and 4. The latter catalyzes the hydrolysis of 4-methylumbelliferyl phosphate (a fluorescent substrate) to 4-methylumbelliferone. Samples were

read at 448 nm. Total assay time was 4 to 5 h per 48 samples. LCR was done by technicians without knowledge of culture results. LCR-positive controls were included in each run. They consisted of *Chlamydia* (serovar L2)-infected McCoy cells. Negative controls consisted of irrelevant DNA. LCR calibrators consisted of DNA extracted from chlamydial elementary bodies (EBs).

Resolution of discrepant results. Whenever the cell culture and LCR results differed, the specimens were subjected to further analysis. The procedures used were as follows. (i) If the specimen was positive by TC but negative by LCR, a repeat LCR was performed with the LCR medium and a 1:10 dilution of the LCR medium, with the same transport medium as diluent. If the repeat LCR was not positive, then an LCR was performed with the unused remnants of the cell culture isolation medium. The tests were done to assess the presence of inhibitors and used the same plasmid primers as the original test. The original negative LCR result was considered a false-negative outcome, and the result of this analysis did not change the evaluation. (ii) If the specimen was negative by TC but positive by LCR, a cytospin-DFA analysis was performed with the remnants of the cell culture isolation medium. The specimen was centrifuged at 18,000 × g for 20 min. The sediment was resuspended, placed on slides, air dried, fixed with methanol, and stained with the species-specific Syva anti-major outer membrane monoclonal antibody (14). The detection of two or more EBs was considered a DFA-positive result and a confirmation of the original LCR-positive result. If this DFA test was negative, another LCR test using primers for the major outer membrane protein (MOMP) gene was done with the original specimen and dilutions of this specimen. If the MOMP gene was detected, the original LCR-positive result was considered to be a true positive. If neither the MOMP gene nor EBs were detected, the original positive (plasmid-based) LCR result was considered to be a false-positive result.

The appropriateness of the MOMP gene LCR for discrepant analysis had already been shown by a side-by-side comparison with the plasmid-based LCR in a low-prevalence (3.1%) population. Both test configurations had a specificity of 100%. The MOMP gene LCR had a sensitivity of 83% (10 of 12), compared with 92% (11 of 12) for the plasmid-based LCR and 67% (8 of 12) for culture. The plasmid-based LCR test was chosen for the final assay because it gives a stronger signal in direct comparisons and has the theoretical advantage of multiple plasmid copies per chlamydial particle.

RESULTS

A total of 2,132 cervical samples were tested by both TC and LCR. In the different laboratories, the sensitivity of LCR compared with that of cell culture isolation of chlamydia ranged from 81 to 98%. The results at individual laboratories are presented in Table 1. When discrepant samples (TC negative and LCR positive) were further analyzed, the overall performance of LCR increased. Of the 84 original LCR false positives, 82 contained either EBs by DFA staining (n = 48) or the MOMP gene by LCR (n = 34). Thus, there were two false-positive LCR results. See Tables 1 and 2 for revised sensitivities and specificities. The sensitivity of TC at different laboratories ranged from 52 to 92%.

Of 2,132 specimens, 39% (834 of 2,132) were collected from symptomatic patients (women being evaluated for abdominal pain, discharge, or dysuria) and 61% (1,298 of 2,132) were from asymptomatic patients. The LCR test performed margin-

TABLE 1. Performance profile of Abbott Laboratories LCR for detection of *C. trachomatis* in cervical specimens[a]

Study site	Prevalence (%)	LCR sensitivity (%)	LCR specificity (%)	TC sensitivity (%)
Baltimore (*n* = 196)	11.7 (12.8)	91.3 (92.0)	98.8 (100)	92.0
Seattle (*n* = 296)	4.1 (4.7)	91.7 (92.9)	99.3 (100)	85.7
Birmingham (*n* = 446)	9.6 (18.6)	93.0 (96.4)	89.6 (99.5)	51.8
San Francisco (*n* = 589)	5.4 (7.5)	81.2 (86.7)	97.7 (100)	71.1
Abbott Laboratories (*n* = 605)	6.9 (11.1)	97.6 (98.5)	95.6 (100)	62.7
All sites (*n* = 2,132)	7.1 (10.9)	91.4 (94.4)	95.8 (99.9)	65.0

[a] Results based only on TC positives appear without parentheses; results after revision for discrepant specimens which were TC negative, LCR positive, and either DFA or LCR MOMP positive appear within parentheses.

ally better with specimens from symptomatic patients. Compared with true positives, the sensitivity and specificity of LCR were 95.8 and 99.8%, respectively, for symptomatic patients and 91.3 and 100%, respectively, for asymptomatic patients.

Of 234 specimens positive for chlamydia, LCR failed to detect 13 that were culture positive. The presence of inhibitors of LCR in some specimens could be demonstrated. Seven samples did test positive 3 to 7 days after the initial negative result, and one sample tested positive only after dilution of the original specimen. Subsequently, all 13 tested LCR positive with the frozen remnants of the original TC specimens.

DISCUSSION

Although a traditional comparison of LCR amplification technology with culture results demonstrated good sensitivity and specificity, an expansion of the gold standard showed its true potential as a diagnostic test. The majority (48 of 84) of the discrepant results were readily resolved by DFA. Thus, 57% of the LCR-positive and TC-negative specimens were found to have EBs by DFA and likely represented the testing of specimens that contained dead EBs, perhaps because of the loss of viability by chlamydiae during storage or transit. A smaller subset (34 of 84) of LCR-positive and TC-negative specimens were DFA negative but could be shown to contain chlamydial genes because subsequent LCRs for the MOMP gene were positive. These specimens probably represent the real increment in sensitivity for LCR DNA amplification over those of enzyme immunoassays, DFA, and direct DNA probe technology. These specimens were both TC and DFA negative but tested positive for two different chlamydial genes (cryptic plasmid and MOMP). The specimens that could be verified by DFA actually represent those specimens for which LCR is more efficient than culture in that it detects dead particles and controls for variations in laboratory culture technique and the conditions of transport. The detection of dead chlamydiae detracts from the use of LCR as the test of cure if one collected

TABLE 2. Comparison of Abbott Laboratories LCR and TC for the detection of *C. trachomatis* in cervical specimens

LCR result	TC	
	+	−
+	139 (221)[a]	84 (2)[b]
−	13	1,896

[a] Of the 84 apparent false positives, 48 were confirmed positive by DFA of TC sediment, 28 were confirmed positive by LCR for the MOMP gene, 6 were confirmed positive by LCR for the MOMP gene after dilution.

[b] Two specimens were unconfirmed and remained false positives.

specimens before the body could clear the dead particles or if plasmid DNA persisted in situ. The use of PCR for the test of cure found it took up to 3 weeks to clear chlamydial genes (15).

Some of the participating laboratories had previously evaluated DFA procedures and always found them to be less sensitive than TC. The DFA procedure in this study was somewhat different in that the specimen was a high-speed sediment from a transport medium. We used DFA results to resolve discrepant results, creating a de facto (albeit interim) gold standard of TC positive or of TC negative but DFA and LCR positive. This is consistent with the Centers for Disease Control and Prevention guidelines for confirmatory testing that call for verification based on the detection of a totally different chlamydial structure (4). The same principle was followed in our final verification step, detecting the presence of MOMP or its gene to confirm the detection of a plasmid gene sequence. This extends the limits of currently available diagnostic technology. The use of two different genes (i.e., MOMP gene detection to confirm the detection of plasmid nucleotide sequences) represents a technological increment in sensitivity. A qualitatively similar increment has been shown with PCR, suggesting that DNA amplification procedures may generically provide this enhanced sensitivity. A direct head-to-head comparison of different amplification methods will be needed to determine which test has the superior performance profile.

Of the specimens that were TC positive and LCR negative, approximately half (7 of 13) were reported as LCR positive when they were tested 3 to 7 days after the original test. There was one sample which became LCR positive only after the sample was diluted. The other five specimens did not become LCR positive but were found to be positive with TC sediment rather than the original LCR material. Thus, it is likely that the failure of these specimens to be LCR positive reflected either the presence of inhibitors in the sample or possibly sampling variation. This was a demonstrated problem with approximately 3% (8 of 234) of all the positive specimens. Cervical specimens have been found by some workers to contain inhibitors of PCR tests for chlamydiae (2).

The overall LCR sensitivity was 94%, and its specificity was 99.9%. The corresponding sensitivity and specificity of culture were 65% and, by definition, 100%, respectively. The LCR test obviously offers a highly sensitive and specific way of detecting the *C. trachomatis* cryptic plasmid in clinical specimens. The excellent results seen with specimens shipped to Chicago (Table 1) for LCR testing indicates the potential usefulness of this technology for surveys performed at distant locations.

The basic advantage of LCR appears to be its ability to detect nearly all the specimens that are chlamydia positive by culture as well as those that had sufficient EBs to be DFA positive. Beyond this, there is a modest increase in the sensitivity of LCR over that of existing technology (culture and

DFA additively) in that 15% of specimens were confirmed only by other gene amplification procedures with a different chlamydia target sequence. Nevertheless, the basic advantage of the LCR is its efficient detection of highly positive specimens. Its specificity was very high (99.9%), meaning the predictive values of positive results would be over 80%, even in low-prevalence (2%) populations.

Although the LCR test for cervical chlamydial infection appears to be the most sensitive (94%) of the commercially developed nonculture tests evaluated by these investigators, it could still be improved upon. A small subset (5.5%) of positive specimens obtained by culture were not positive by LCR. The subsequent positive tests for chlamydial genes in these specimens suggest that labile inhibitors are involved. Whether LCR or PCR is more sensitive or more susceptible to inhibitors must await suitably designed parallel evaluations.

In addition, there is a potential for DNA contamination with the use of LCR. Two of the five facilities did experience this problem. It was detected when negative controls tested positive. However, by employing strict guidelines for specimen flow, isolation, and routine cleanup, laboratories engaged in DNA amplification procedures can avoid DNA contamination. Incoming specimen (target) areas and product areas should be kept separate, and airflow should be limited. The amplified product should be destroyed at the end of routine processing. It is likely that the routine cleaning of equipment and work areas with bleach would be useful. Certainly, the inclusion of appropriate controls is mandatory, and environmental sampling may be warranted.

The exceptional performance profile of the LCR test suggests that it may well become a test of choice for the diagnosis of chlamydial infection of the genital tract. Similarly good results were obtained with first-catch urine and urethral swab specimens from men with and without urethritis and, perhaps of even greater interest, with urine specimens from women. Those results will be reported elsewhere. However, even when only the results with cervical specimens presented here are considered, it is quite obvious that the LCR test is a serious candidate to be one of the tests of choice for the diagnosis of chlamydial infection.

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