The Use of Polymerase Chain Reaction Assay Versus Conventional Methods in Detecting Neonatal Chlamydial Conjunctivitis

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ABSTRACT

Purpose: To compare the performance of polymerase chain reaction versus conventional methods (cell culture and direct immunofluorescent assay) in diagnosing neonatal chlamydial conjunctivitis and their correlations to the severity of conjunctivitis.

Methods: Consecutive cases of neonatal conjunctivitis were recruited over a year. Both eyes were clinically graded according to the severity of conjunctivitis and investigated using the three aforementioned chlamydial tests. Neonatal chlamydial conjunctivitis was assumed if one of these three tests was positive and there was clinical improvement after treatment. Sensitivity and specificity of each of the tests were analyzed.

Results: Three hundred sixty-eight sets of chlamydial tests were done for 184 neonates. The percentage of positive results was 93.8% and 71.9% for polymerase chain reaction and conventional methods, respectively. Using positive results in either cell culture or direct immunofluorescent assay as a standard to diagnose neonatal chlamydial conjunctivitis, the sensitivity and specificity of polymerase chain reaction were 92.0% and 97.7%, respectively. If we used polymerase chain reaction as a standard, the sensitivity and specificity of cell culture were 73.3% and 99.7%, respectively. A discrepancy was noted in the number of positive results between polymerase chain reaction and conventional methods in milder disease.

Conclusions: Polymerase chain reaction might have a higher sensitivity and similar specificity in diagnosing neonatal chlamydial conjunctivitis compared to conventional methods, and it has an additional advantage as a diagnostic tool in mild disease.


INTRODUCTION

Diagnosis of Chlamydia trachomatis infection can be made by both culture and nonculture techniques, such as the antigen detection test and nucleic acid amplification. The newly developed automated polymerase chain reaction assay, COBAS AMPLICOR CT/NG test (Roche Diagnostics GmbH, Mannheim,
Germany), was approved by the U.S. Food and Drug Administration for cervical swabs from females, urethral swabs from males, and urine samples from males and females for the detection of *C. trachomatis* infection. This test was first used in eye disease in 1990.\(^1\) Several studies have shown that polymerase chain reaction was at least as sensitive as conventional techniques in the detection of *C. trachomatis* using conjunctival swabs.\(^2-5\) In adults, it has been shown that polymerase chain reaction was a good alternative to *C. trachomatis* cell culture in conjunctival scraping specimens and was sensitive and specific.\(^3\)

Previous studies on the use of the COBAS AMPLICOR test on neonatal ocular and nasopharyngeal specimens showed comparable results to those of cell culture in detection of *C. trachomatis*.\(^6\) However, correlation of this test to clinical presentation of neonatal chlamydial conjunctivitis is lacking. To have a better evaluation among cell culture, direct immunofluorescent assay, and polymerase chain reaction in diagnosing neonatal chlamydial conjunctivitis, direct comparison on the performance of all three tests and their clinical correlation to the severity of disease have been made in this article.

**Patients and Methods**

Consecutive cases of neonatal conjunctivitis, defined as a neonate with a gestational age of greater than 32 weeks with acute conjunctivitis acquired within the first 28 days of life who was born in our hospital, were recruited from May 1, 2004, to April 30, 2005. All cases were assessed by two assigned ophthalmologists. Each eye was scored according to the severity of purulent discharge, conjunctival congestion, the degree to which the eyelids were stuck together, edema, and eyelid erythema. Each sign was marked as 0 = absent, 1 = mild, 2 = moderate, and 3 = severe. The sum of these scores was calculated for each eye and finally graded as mild disease (sum = 1 to 3 inclusively), moderate disease (sum = 4 to 8 inclusively), and severe disease (presence of pseudo-membrane, corneal invasion, or dacyrocystitis) (sum = 9 or above). Those neonates who had prior topical antibiotics would have their medication stopped for 24 hours before specimen collection.

A set of chlamydial tests was done for each eye and each eye of the neonate was investigated separately. The set included *C. trachomatis* polymerase chain reaction, direct immunofluorescent assay, and cell culture. In our study, chlamydial infection was assumed if one of these three tests was positive. Oral erythromycin (50 mg/kg/day in four divided dose for 2 weeks) would then be given to *C. trachomatis* positive neonates. The response and compliance to oral erythromycin were also recorded.

A specimen from each eye was collected according to Centers for Disease Control and Prevention guidelines\(^7\) and was taken from the eye with less disease first. Any purulent exudate present was first removed from the eye using a cotton swab. A polyester-tipped swab was used to prepare a slide for direct immunofluorescent assay by smearing the materials from the palpebral conjunctival epithelium onto the slide. The slide was air dried and then fixed immediately in methanol. A second conjunctival swab was collected with a new polyester-tipped swab for cell culture and polymerase chain reaction. The swab was cut into *Chlamydia* transport medium (sucrose-phosphate buffer [pH 7.2] supplemented with inactivated fetal calf serum, 100 µg/mL of vancomycin, 10 µg/mL of gentamicin, and 25 Units/mL of nystatin), and transported to the laboratory on cold pack within the day of collection.

In the laboratory, a 200-µL aliquot of samples in transport medium was kept at -70°C until further processing for polymerase chain reaction. The remaining samples and the methanol-fixed direct immunofluorescent assay slides received were sent for cell culture and direct immunofluorescent assay.

Direct immunofluorescent assay was performed using MicroTrak *Chlamydia trachomatis* Direct Specimen Test (Syva MicroTrak, Palo Alto, CA) as recommended by the manufacturer. The slide was stained with fluorescein-conjugated monoclonal antibody specific to *C. trachomatis* major outer membrane protein and examined for the presence of elementary bodies under fluorescent microscope.

Cell culture was performed on cycloheximide-treated monolayer McCoy cells in a shell vial. One hundred fifty microliters of specimen in transport medium was inoculated and centrifuged onto the cell monolayer at 3,000 X g at 37°C for 1 hour. The inoculum was discarded and replaced with 1 mL of pre-warmed growth medium. The shell vials were then incubated at 37°C in a humidified incubator in the presence of 2.5% carbon dioxide. After 48 hours of incubation, the coverslip was examined for the presence of inclusion bodies by immunofluores-
ence using the MicroTrak Chlamydia trachomatis Culture Confirmation Test.

Polymerase chain reaction was performed using the COBAS AMPLICOR CT/NG test and the results were interpreted according to the manufacturer’s instructions. A 100-µL aliquot of specimen was mixed with 100 µL of CT/NG Lysis Buffer and incubated at room temperature for 10 minutes. After centrifugation, 200 µL of CT/NG Specimen Diluent was added to the mixture. The resultant mixture was further incubated at room temperature for another 10 minutes. Fifty microliters of processed sample was added to 50 µL of master mix. Amplification and detection were done on the COBAS AMPLICOR system.

The performance of the three chlamydial tests was analyzed in terms of positive rate of C. trachomatis (Table 1) and test sensitivity and specificity using conventional and molecular diagnostic criteria of C. trachomatis (Table 2). Positive results were correlated clinically with the severity of neonatal conjunctivitis (Table 3). Possible false-negative error in polymerase chain reaction assay caused by presence of inhibitor was stated to avoid overestimation of test performance (Table 2).

This study was conducted according International Conference on Harmonization Good Clinical Practice Guidelines with approval from the local ethics committee. Informed consent was obtained from the parent of the study neonate.

**RESULTS**

Three hundred sixty-eight sets of chlamydial tests were done for 184 neonates in the study period. Thirty-two sets from 24 neonates were diag-

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**TABLE 1**

**RESULTS OF CHLAMYDIAL TESTS IN CONJUNCTIVAL SPECIMENS**

<table>
<thead>
<tr>
<th>Chlamydia Test Results</th>
<th>PCR</th>
<th>DIF</th>
<th>Cell Culture</th>
<th>No. of Eyes (N = 368)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydia positive (n = 32)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>-ve</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-ve</td>
<td>-ve</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>+</td>
<td>-ve</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>lnh</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td>Chlamydia negative (n = 336)</td>
<td>lnh</td>
<td>-ve</td>
<td>-ve</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>299</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction assay; DIF = direct immunofluorescent assay; -ve = negative test result; lnh = presence of inhibitor in polymerase chain reaction assay.

**TABLE 2**

**PERFORMANCE OF DIFFERENT CHLAMYDIAL TESTS USING CONVENTIONAL AND MOLECULAR METHODS AS A STANDARD FOR DIAGNOSING NEONATAL CHLAMYDIAL CONJUNCTIVITIS**

<table>
<thead>
<tr>
<th>Standard of Diagnosis</th>
<th>Test Performance</th>
<th>Assuming Presence of Inhibitor as False-Negative Result (n = 368)</th>
<th>Assuming Presence of Inhibitor as Invalid Test and Excluded (n = 330)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional methoda</td>
<td>PCR</td>
<td>Sensitivity</td>
<td>92.0</td>
</tr>
<tr>
<td>Molecular methodb</td>
<td>Cell culture</td>
<td>Sensitivity</td>
<td>73.3</td>
</tr>
<tr>
<td></td>
<td>DIF</td>
<td>Sensitivity</td>
<td>70.0</td>
</tr>
</tbody>
</table>

PCR = polymerase chain reaction assay; DIF = direct immunofluorescent assay.

aUsing either positive cell culture or direct immunofluorescent assay as a standard for diagnosing neonatal chlamydial conjunctivitis. Numbers in bold indicate a higher sensitivity for polymerase chain reaction than for positive cell culture and direct immunofluorescent assay.

bUsing positive polymerase chain reaction as a standard for diagnosing neonatal chlamydial conjunctivitis.
nosed as having chlamydial infection based on the study criteria (Table 1). The percentage of positive results in conjunctival specimens was 93.8% (30 of 32), 71.9% (23 of 32), and 71.9% (23 of 32) for polymerase chain reaction, direct immunofluorescent assay, and cell culture, respectively.

Traditionally, chlamydial infection was diagnosed based on positive cell culture or direct immunofluorescent assay. If we used positive results in either of these two tests as the standard for diagnosing neonatal chlamydial conjunctivitis, the sensitivity and specificity of polymerase chain reaction was 92.0% and 97.7%, respectively. If we used polymerase chain reaction as the standard for diagnosing chlamydial infection, the sensitivity and specificity were 73.3% and 99.7%, respectively, for cell culture and 70.0% and 99.4%, respectively, for direct immunofluorescent assay (Table 2). Among the results of polymerase chain reaction tests, inhibitors were detected in 38 of 368 sets. Disregarding the specimens showing an inhibition, the performance of the COBAS AMPLICOR CT/NG test was as shown in Table 2.

Subgroup analysis was done according to the grade of neonatal conjunctivitis (Table 3). For moderate and severe disease, the performance of polymerase chain reaction, direct immunofluorescent assay, and cell culture were similar. However, a discrepancy was observed in the number of positive results between polymerase chain reaction and conventional diagnostic tests (direct immunofluorescent assay and cell culture) in milder disease. There was only 1 positive direct immunofluorescent assay and 1 positive cell culture among 8 sets of specimens in the asymptomatic and mild disease groups.

All cases of neonatal chlamydial conjunctivitis that were diagnosed according to the study criteria responded to oral erythromycin. They all had no signs or symptoms of conjunctivitis after a complete course of treatment.

**DISCUSSION**

Our study is one of the largest studies to directly compare the performance of polymerase chain reaction, direct immunofluorescent assay, and cell culture in a neonatal population and with clinical correlation on the severity of conjunctivitis. We employed the same specimen for cell culture and polymerase chain reaction to minimize possible sampling error due to the sequence of specimen collection. A detailed analysis of polymerase chain reaction performance with regard to the presence of inhibitors has also been made here. We hope this study can prove the practical value of a newer molecular technique (polymerase chain reaction) in diagnosing neonatal chlamydial conjunctivitis.

Regarding the performance of chlamydial tests, we employed either positive cell culture or direct immunofluorescent assay as a reference standard for the evaluation of sensitivity and specificity of polymerase chain reaction. This was a stringent standard to evaluate the performance of polymerase chain reaction, and yet polymerase chain reaction still has a much higher sensitivity (92.0 vs 73.3; 70.0%) and similar specificity (98.0 vs 99.7; 94.4%) when compared to cell culture and direct immunofluorescent assay, respectively, in diagnosing neonatal chlamydial conjunctivitis. If cell culture was regarded

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**Table 3**

Results of Chlamydial Tests From 24 Chlamydial Positive Neonates in Relation to Clinical Severity of Neonatal Chlamydial Conjunctivitis

<table>
<thead>
<tr>
<th>Disease Grade</th>
<th>No. of Chlamydia Negative Eyes (n = 12)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of Chlamydia Positive Eyes (n = 31)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>No. of Positive Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PCR</td>
</tr>
<tr>
<td>Asymptomatic</td>
<td>8</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Mild</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Moderate</td>
<td>0</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Severe</td>
<td>0</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup>PCR = polymerase chain reaction assay; DIF = direct immunofluorescent assay.

<sup>b</sup>There was a discrepancy in the number of positive results between polymerase chain reaction assay and conventional diagnostic tests (cell culture and direct immunofluorescent assay) in milder disease groups as shown in bold.

<sup>c</sup>5 of 48 eyes from 24 chlamydial conjunctivitis positive neonates were excluded due to presence of inhibitor in polymerase chain reaction assay.
as the gold standard, an even higher sensitivity for polymerase chain reaction was obtained (sensitivity: 95.6% and specificity: 97.7%). Furthermore, 7 of 32 eyes could only be identified as having chlamydial infection by polymerase chain reaction (Table 1), which implied that approximately 20% (7 of 32) of the eyes would probably be labeled as negative if polymerase chain reaction was not used as part of the diagnostic tests. This highlighted the effectiveness of polymerase chain reaction when compared to conventional methods.

So far, there has been little evidence of the correlation between clinical symptoms and microbiological diagnosis of neonatal chlamydial conjunctivitis. Studies of chlamydial infection in adults have shown that the clinical–microbiological correlation was low, regardless of the method used. Using the grading system in our study (Table 3), almost all eyes with moderate and severe disease had a positive result with all three chlamydial tests. The performance of polymerase chain reaction and cell culture in these two groups was thus similar. However, for eyes with asymptomatic and mild disease, the performance was much lower in direct immunofluorescent assay and cell culture. The positive rate was 100% for polymerase chain reaction in both the asymptomatic and mild disease groups. For both direct immunofluorescent assay and cell culture, it was 0% and 25% in the asymptomatic and mild disease groups, respectively. We demonstrated that cell culture and direct immunofluorescent assay had similar performance as polymerase chain reaction in more severe grades of disease but not for milder disease. On the basis of our study results, we think that an evaluation of the traditional standard for diagnosing neonatal chlamydial conjunctivitis is warranted.

Cell culture, the traditional gold standard in diagnosing chlamydial infection, is not without limitations. It requires a long processing time (approximately 5 days), tedious testing procedures, and special instrumentation. Problems associated with specimen handling and delay in inoculation may contribute to false-negative results due to the fastidious nature of the organisms. These problems may be overcome by the use of polymerase chain reaction, which does not require viable organisms to obtain a positive result. The additional value of polymerase chain reaction is that it can amplify the nucleic acid of an infectious organism at the early phase of the disease and in partially treated patients because the viable organism load is too low to be detected by cell culture in these conditions. The main disadvantage of polymerase chain reaction is the possibility of having a false-positive result. This may be caused by contamination at the time of either specimen collection or specimen processing in the laboratory. If proper procedures and precautions are followed, the chance of contamination could be greatly minimized.

In our study, the inhibition rate of polymerase chain reaction was 10.3% (38 of 368) and was higher than that detected in urogenital specimens, which ranges from 0.3% to 6.7%.

Because the ocular specimens were taken from inflamed conjunctiva, which was fragile and usually bloody, it was believed that the presence of blood might account for the higher inhibition rate. This figure also represented the percentage of indeterminate polymerase chain reaction results and tests may need to be repeated to make the diagnosis of chlamydial infection by polymerase chain reaction. Repeating the test with diluted processed samples that showed inhibition might partially solve the problem, but this would inevitably reduce the sensitivity of the test.

Our positive rate of polymerase chain reaction was comparable to those studies using the transport medium provided by the manufacturer. We believe the sensitivity of polymerase chain reaction results might have been even higher if the collected specimen had been inoculated directly into the kit’s transport medium provided by the manufacturer.

Neonates are a group of patients in whom detailed clinical examination and specimen collection are usually difficult. A test that has high sensitivity may be particularly important for this group so that the diagnosis is not missed. This study demonstrated that polymerase chain reaction might have a higher sensitivity and similar specificity in diagnosing neonatal chlamydial conjunctivitis than conventional methods, even though an identical culture medium was used for transport and storage. Polymerase chain reaction has an additional advantage as a diagnostic tool in mild disease. An evaluation of the traditional gold standard in confirming chlamydial infection is therefore warranted. Our work also suggested that polymerase chain reaction and cell culture could be transported in the same medium to facilitate specimen collection.

It is important to note that a higher inhibition rate in the polymerase chain reaction test was ob-
served in neonatal conjunctivitis specimens and caution should be used when interpreting the results. When comparing the sensitivity and specificity of polymerase chain reaction with that of other methods, each institution should consider its own needs and decide which tests should be used according to its own laboratory standard.

REFERENCES