Detection of Chlamydia trachomatis Ocular Infection in Trachoma-Endemic Communities by rRNA Amplification

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PURPOSE. Trachoma remains the leading infectious cause of blindness worldwide. The World Health Organization (WHO) recommends mass antibiotic distributions in its strategy to eliminate blinding trachoma. To determine the most effective antibiotic treatment strategy, it is essential to have a diagnostic test that can correctly measure the true status of ocular Chlamydia trachomatis infection in individuals, particularly after treatment. A newer ribosomal ribonucleic acid (rRNA)-based amplification test was compared with the current DNA-based polymerase chain reaction (PCR) for the detection of C. trachomatis.

METHODS. An rRNA-based assay and PCR were performed on swab specimens taken from the right upper tarsal conjunctiva of 240 children aged 1 to 5 years living among 16 endemic villages in the Gurage Zone, Ethiopia.

RESULTS. The rRNA-based test detected ocular C. trachomatis infection in 142 (59%) subjects compared with 67 (28%) detected by PCR (McNemar’s test, P < 0.0001). The rRNA-based test gave positive results for all subjects who were positive by PCR and detected infection in 75 (31%) additional subjects.

CONCLUSIONS. The rRNA-based test appears to have significantly greater sensitivity than PCR for the detection of ocular C. trachomatis infection in children in trachoma-endemic villages. The increased sensitivity of the rRNA-based test may be due to its ability to detect low levels of C. trachomatis infection in individuals, which can occur especially after antibiotic treatment. Data from past studies in which PCR was used to assess the prevalence of infectious trachoma after community-wide antibiotic treatments could have underestimated the true prevalence of infection. (Invest Ophthalmol Vis Sci. 2009;50: 90–94) DOI:10.1167/iovs.08-2247

Trachoma remains the leading infectious cause of blindness worldwide, and continues to be the target of a global effort by the WHO to eliminate the disease as a public health concern by the year 2020.1 Several countries in which trachoma is endemic have implemented national trachoma-control programs; a major component of these programs is community-wide antibiotic treatment aimed at reducing the prevalence of active disease.2 Recent studies have shown that mass antibiotic distribution can dramatically decrease the prevalence of ocular chlamydial infection in trachoma-endemic villages.3–7 Indeed, some suggest that repeated mass administration of antibiotics could eliminate infection from a community. However, to accurately assess the feasibility of eliminating trachoma, it is essential to have a diagnostic test that can correctly measure the true status of ocular C. trachomatis infection in individuals, especially after antibiotic treatment. Currently, DNA amplification by PCR is commonly used for detection of ocular chlamydial infection. In recent years, newer nucleic acid amplification tests (NAATs) based on amplification of RNA have been developed. Amplification of RNA targets provides a potential advantage, since bacterial rRNA is present at up to 10,000 times the copy number of genomic DNA and 1,000 times that of plasmid DNA.8 This difference may become especially important in cases of low chlamydial load, such as in asymptomatic9,10 or recently treated patients.11 Prior studies comparing different NAATs for detecting genital chlamydial infection found that RNA amplification tests possessed significantly greater sensitivity and comparable specificity to PCR.12–14 However, there are few studies evaluating RNA-based NAATs for diagnosing ocular C. trachomatis infection. Burton et al.15 reported that a homebrew rRNA amplification test was much less sensitive than DNA amplification for detection of ocular chlamydial infection in the Gambia. The authors suggested that their findings may have been due to the presence of dead or subviable C. trachomatis that the rRNA test could not detect, presumably because of a lack of RNA stability in nonviable organisms. Alternatively, a recent pilot study in Ethiopia showed that a commercially available rRNA amplification test detected significantly more infection than PCR, even in a small sample size.16 This discrepancy with the results in Burton et al.15 was believed to be due to the different rRNA assays used (commercially available ACT assay versus homebrew assay).

The primary goal of this study was to evaluate an rRNA amplification test for the diagnosis of C. trachomatis in ocular swabs taken from subjects living in a trachoma-endemic area. A superior diagnostic test would allow for more accurate monitoring of the effectiveness of treatment programs. We com-

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pared the 16s rRNA-based APTIMA C. trachomatis (ACT) assay (Gen-Probe, Inc., San Diego, CA) to the DNA-based Amplicor PCR assay (Roche Molecular Systems, Branchburg, NJ). Both tests are commonly used for diagnosis of genital infection with C. trachomatis.

METHODS

The study was performed across 16 villages from March to April 2006 in the Gurage Zone, Ethiopia. Research and ethical clearances for all procedures were obtained from the Ethiopian Science and Technology Commission and the Committee for Human Research of the University of California, San Francisco. The study was performed in accordance with the tenets of the Declaration of Helsinki.

Participants

The sample population consisted of 240 children living among 16 hyper-endemic villages in the Gurage Zone, Ethiopia. Eight villages had no history of prior antibiotic distributions; the other villages had received mass azithromycin treatments (1 g in adults or 20 mg/kg in children) biannually for 2 years, with the last dose administered 18 months before the study commenced. For each village, 15 children were randomly selected for inclusion in the study from a census of all children aged 1 to 5 years living in the village. Children were selected because this age group is the most likely to harbor infection.

Clinical Examination

Experienced ophthalmologists or trained ophthalmic assistants performed a clinical examination. For each patient, the right upper eyelid was everted and the tarsal conjunctiva was examined with a 2.5 × binocular loupe. The WHO’s simplified trachoma grading system was used to identify signs of trachoma: normal, no signs of trachoma; TF, follicular trachomatous inflammation; TI, intense trachomatous inflammation. This grading scheme was selected to provide a dichotomous definition of clinical activity for our analysis.

For each patient, specimens were collected from the right upper tarsal conjunctiva for testing by the rRNA-based ACT test and PCR-based Amplicor. A swab was passed firmly across the conjunctiva along its full length three times, rotating approximately 120° between each pass. Specimens for the ACT assay were collected with swabs and transport tubes provided in the APTIMA Unisex Swab Specimen Collection Kit (Gen-Probe, Inc.). Specimens for Amplicor PCR were collected with sterile Dacron-tipped plastic swabs (Fisher, Middletown, VA) that were broken off into sterile 1.5-mL microcentrifuge tubes and stored at 80°C before processing. Specimens for ACT testing were stored at room temperature (~21°C) until processed, as specified by the manufacturer. Amplification and detection using ACT and Amplicor were performed according to the manufacturers’ instructions. Positive laboratory controls were included according to protocol for all assays.

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swab that yielded the negative result was subjected to confirmatory testing by repeat ACT. Negative field controls (a swab passed 2.5 cm above but not touching the conjunctiva) were also performed to evaluate for the possibility of field contamination. Specimens for Amplicor testing were stored at 4°C in the field and transferred to −20°C within 8 hours of collection. Samples were then transferred to −80°C, transported to the University of California, San Francisco at 4°C, and stored at −80°C before processing. Specimens for ACT testing were stored at room temperature (~21°C) until processed, as specified by the manufacturer. Amplification and detection using ACT and Amplicor were performed according to the manufacturers’ instructions. Positive laboratory controls were included according to protocol for all assays.

Dilution Experiments

For selected swab specimens, serial 10-fold dilutions from 10⁰ to 10⁻⁵ were prepared from each swab. The diluted samples were subjected to testing by ACT to evaluate the detection threshold of the test. Results for ACT were measured in relative light units (RLU). Test results were classified according to the following thresholds, as specified by the manufacturer: >5000 RLU, positive; 100 to 5000 RLU, low-positive; 50 to <100 RLU, equivocal; and <50, negative.

Statistical Analysis

To determine whether one test was more likely to be positive than another, we performed a McNemar’s test. Pearson’s correlation coefficients were used to quantify the association between two tests, with P < 0.05 considered statistically significant. A logistic regression model was used to evaluate whether the order in which swabs were taken affected the likelihood that swab would be positive. All statistical analyses were performed using commercial software (STATA 8.0; StataCorp LP, College Station, TX).

RESULTS

The sample population consisted of 121 (50.4%) girls and 119 (49.6%) boys, with a mean age of 3.3 years (Table 1). There was no statistically significant difference in the distribution of sex or age between the control and treated villages. Of the 120 subjects living in control villages, 73 (60.8%) had clinically active trachoma (TF and/or TI). Twenty of 120 (16.7%) were found to have clinically active disease. Of these, 21 (17.5%) were found to have TF and 36 (30.0%) TI (Table 1).

ACT and Amplicor results correlated significantly (r = 0.52; P < 0.0001). However, the number of discrepant results was also significant (McNemar’s test, P < 0.0001). Of the 240 samples positive by ACT, 75 (31.3%) were Amplicor negative. ACT detected chlamydial infection in 142 (59.2%) subjects and Amplicor in 67 (27.9%). All 67 Amplicor-positive samples were also positive by ACT (Table 2).

Table 2. Comparison of ACT and Amplicor for Detection of C. trachomatis

<table>
<thead>
<tr>
<th>rRNA Amplification Test Result</th>
<th>DNA Amplification Test Result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT+</td>
<td>Amplicor+</td>
<td>67</td>
</tr>
<tr>
<td>ACT-</td>
<td>Amplicor+</td>
<td>75</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>142</td>
</tr>
<tr>
<td>ACT-</td>
<td>Amplicor−</td>
<td>0</td>
</tr>
<tr>
<td>ACT−</td>
<td>Amplicor−</td>
<td>98</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>98</td>
</tr>
<tr>
<td>Correlation coefficient, r = 0.52 (P &lt; 0.0001).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Both ACT and Amplicor displayed high detection rates in subjects with clinically active disease and relatively lower detection rates in subjects with no active disease. The correlation between ACT and Amplicor decreased with decreasing severity of clinical disease (no active disease < TF < TI). Table 3 displays the results of ACT and Amplicor stratified by clinical activity. Of the 89 subjects with TI, Amplicor was positive in 50 (56.2%) and ACT in 64 (71.9%). The correlation between ACT and Amplicor was significantly lower in subjects with TF than in subjects with TI (r = 0.43 vs. r = 0.71, respectively; P = 0.02). Amplicor detected infection in 10 (24.4%) of 41 subjects with TF; ACT detected infection in 26 (63.4%). In subjects without clinically active disease, ACT and Amplicor were significantly less correlated as compared with subjects with active disease (P = 0.0004). In the 110 subjects without clinically active disease, Amplicor detected infection in only 7 (6.4%), whereas ACT detected infection in 52 (47.3%).

The correlation between ACT and Amplicor results was also significantly lower in subjects who had been previously treated, compared with those with no prior history of antibiotic treatment (P = 0.03). Results are shown in Table 4. In control villages, ACT detected infection in 80 (66.7%) subjects, as compared with 49 (40.8%) by Amplicor (r = 0.41; P < 0.0005). In previously treated villages, ACT detected infection in 62 (51.7%) subjects and Amplicor in 18 (15.0%; r = 0.43, P = 0.005). Of those, 7 (46.7%) samples were ACT positive up to a 10-fold dilution. The remaining five samples were ACT negative upon repeat testing of the undiluted concentration but negative at further dilutions. Three (20%) samples were ACT positive at the undiluted concentration but negative at further dilutions. The remaining five samples were ACT negative upon repeat testing of the undiluted concentration.

All negative field control swabs were negative. Sixteen (20%) of the duplicate control swabs were not concordant with the ACT results of the initial swab. Test results were not affected by the order in which swabs were taken. The effect on positivity of a swab being taken first was not statistically significant (OR = 0.96; 95% CI, 0.66 -1.40; P = 0.83).

**DISCUSSION**

In this comparison, ACT detected ocular C. trachomatis infection significantly more often than Amplicor. ACT was positive for all Amplicor-positive samples, and it detected infection in an additional 75 (31.3%) subjects. The results of this study suggest that ACT may be more sensitive than Amplicor for detecting ocular chlamydial infection. An alternative explanation is that the ACT assay is not more sensitive but instead less specific, and the discrepant results are in fact false positives of the test. False positives could result from detection of other bacterial rRNA. However, prior and much larger studies of Gen-Probe’s rRNA-based tests for detecting genital C. trachomatis showed increased sensitivity and comparable specificity when compared to Amplicor. A recent study comparing ACT with Amplicor as a screening assay for detection of genital C. trachomatis found significantly higher sensitivity and specificity with ACT.

Comparison of an rRNA-based test to Amplicor for detection of oropharyngeal and rectal C. trachomatis infection showed higher sensitivity and specificity with the rRNA-based test, even in asymptomatic patients. Our results differ from those of Burton et al. who found that their rRNA-based test detected infection in fewer subjects than did PCR. They suggested that an rRNA-based test could discriminate metabolically active organisms from nonviable bacteria, possibly due to a lack of rRNA stability in nonviable organisms. Although this is a possibility, it appears more likely that the discrepancy between our findings and those of Burton et al. is due to differences in the rRNA assay used (commercially available ACT assay versus homebrew assay). Given that bacterial rRNA is present in quantities several orders of magnitude higher than that of DNA, the ACT assay would be expected to be capable of detecting lower levels of Chlamydia that are beyond the detection ability of Amplicor PCR. Larger studies comparing ACT and other rRNA-based assays to PCR for detection of genital, rectal, and oropharyngeal C. trachydia are consistent with this notion.

In an individual with a low level of C. trachomatis, the amount of chlamydial DNA present may not be sufficient to be detected by Amplicor. However, in this same individual, bacterial rRNA would typically be present at exponentially higher levels so that the amount of rRNA may be well above the

**Table 4. Comparison of ACT and Amplicor for Detection of C. trachomatis Stratified by Treatment Group**

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>ACT</th>
<th>Amplicor</th>
<th>Correlation between ACT and Amplicor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Previously treated villages</td>
<td>62</td>
<td>18</td>
<td>r = 0.41, P &lt; 0.00001</td>
</tr>
<tr>
<td>Control villages</td>
<td>80</td>
<td>49</td>
<td>r = 0.59, P &lt; 0.00001</td>
</tr>
</tbody>
</table>

**Table 5. C. trachomatis Detection by ACT for Serially Diluted Samples**

<table>
<thead>
<tr>
<th>Original Test Result</th>
<th>n</th>
<th>10^0</th>
<th>10^-1</th>
<th>10^-2</th>
<th>10^-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT+, Amplicor+ (n = 7)</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ACT+, Amplicor- (n = 15)</td>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ACT-, Amplicor- (n = 7)</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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detection threshold of ACT. Amplicor and ACT assays would therefore be more likely to yield discrepant results in such subjects with low chlamydial load, resulting in lower correlation between the two tests for these individuals. Alternatively, in subjects with high chlamydial load, high levels of both chlamydial DNA and rRNA would be expected to yield positive results for both Amplicor and ACT, respectively. Since both tests would be more likely to give positive results in individuals with high chlamydial load, these tests would be expected to show a higher level of correlation in such individuals.

An ideal analysis of the sensitivities of ACT and Amplicor would compare the two tests in different individuals with various chlamydial loads as determined by quantitative assays. However, current quantitative assays are less sensitive than PCR, making it impossible to evaluate ACT and Amplicor in this manner, as these two tests appear to be the most sensitive tests for *C. trachomatis* currently available. Instead, we compare ACT and Amplicor based on indicators of chlamydial load that have been established in past studies. Previous studies in trachoma-endemic communities have shown that clinical signs of active trachoma are generally positively correlated with chlamydial load. The proportion of subjects with high chlamydial load significantly increases with severity of clinical sign (TI > TF > no clinical disease). Assuming the same characteristic in our study population, we would expect that those with TI would have the greatest proportion of subjects with high chlamydial loads, while those with no clinical disease would have the greatest proportion of subjects with low loads. For subjects without clinical disease, a more sensitive assay would be expected to detect infection disproportionately more often than a less sensitive test that could not pick up low levels of infection. As expected, in our study, Amplicor and ACT displayed the lowest correlation for subjects with no clinical active disease (r = 0.28). Amplicor was positive for only 6% of these subjects, whereas ACT was positive for almost half. The two assays displayed a higher correlation in subjects with TF (r = 0.43) and a significantly higher correlation in subjects with TI (r = 0.71) compared with subjects with TF (P = 0.02). These different detection rates between ACT and Amplicor for subjects with different levels of clinical activity appear to be the result of differences in underlying chlamydial loads. ACT may be detecting low levels of *C. trachomatis* that are below the detection threshold of Amplicor. This difference becomes more pronounced in populations with low chlamydial loads—in this case, those without clinically active disease.

Similarly, we compared the sensitivities of ACT and Amplicor by evaluating the ability of each assay to detect infection in subjects treated with azithromycin and never treated with antibiotics. In past studies trachoma-endemic populations treated with mass azithromycin distributions displayed a dramatic decrease in chlamydial load. Therefore, a more sensitive test would be expected to detect infection more frequently in treated populations, since there would be more individuals with low chlamydial loads that are undetectable by a less sensitive assay. In our study, ACT detected infection more frequently in both treated and untreated populations, but the difference in detection ability of ACT and Amplicor was significantly more pronounced for treated subjects. As expected, the correlation between ACT and Amplicor was significantly lower (P = 0.03) in the group that had been previously treated with azithromycin (r = 0.41), compared with those who had never been treated (r = 0.59).

We also performed dilution experiments on specific samples to further evaluate the detection threshold of ACT relative to that of Amplicor. Samples that were initially positive by both ACT and Amplicor were serially diluted to ascertain whether they would remain ACT-positive at lower concentrations. Since these samples were Amplicor-positive, they presumably had amounts of *C. trachomatis* well above the detection threshold of the ACT test. Even at 1:1000 of the original concentration all samples remained ACT-positive, suggesting that samples positive by Amplicor contained relatively high levels of *C. trachomatis* which were easily detected by ACT. Conversely, samples that were ACT-positive but Amplicor-negative presumably had sufficient organisms to be detectable by ACT but too few to be identified by Amplicor—representing a relatively low *C. trachomatis* load. That none of the samples were positive by ACT beyond a 10^-1 dilution suggests that these ACT-positive, Amplicor-negative samples represented low-level positives that were near the detection threshold of ACT, the more sensitive test in our comparison.

It appears that the RNA-based ACT assay offers significantly higher sensitivity for ocular *C. trachomatis* than current DNA-based PCR tests. The possibility remains that the higher detection rates displayed by ACT, particularly in populations with low chlamydial load, may represent false positives due to detection of other bacteria. Although prior comparisons of NAATs for detecting genital chlamydial infection have shown superior specificity with rRNA-based tests, more studies are needed to exclude the possibility that other bacterial rRNA are not detected. If RNA-based assays indeed detect ocular *C. trachomatis* infection with high specificity as in genital *Chlamydia*, then there could be significant implications. Previous estimates of trachoma prevalence using PCR could have underestimated infection prevalence, especially in communities that had been treated with antibiotics or that largely comprised individuals without clinically active disease.

A more sensitive test would allow for improved accuracy in studies evaluating the effectiveness of mass administration of antibiotics for trachoma elimination, but the epidemiologic significance of the newer rRNA-based tests remains unclear for ocular *Chlamydia*. Do low chlamydial loads detectable only by ACT represent viable and infectious chlamydial organisms, or can rRNA-based tests detect nonchlamydial or nonviable organisms? What proportion of low-level infection leads to high chlamydial loads or clinically significant disease?

Recent longitudinal studies have demonstrated a gradual return of infection after mass antibiotic distributions. It is possible that low-level infection, previously missed by DNA-based tests, is a cause of such infection re-emergence as that witnessed in communities mass-treated with antibiotics. A more sensitive and highly specific rRNA-based test would better detect such low-level infection and provide a more accurate assessment of the effectiveness of trachoma elimination efforts.

References


24. West SK, Munoz B, Mkocha H, Gaydos C, Quinn T. Trachoma and ocular Chlamydia trachomatis were not eliminated three years after two rounds of mass treatment in a trachoma hyperendemic village. *Invest Ophthalmol Vis Sci.* 2007;48:1492–1497.