Evaluation of Gen-Probe APTIMA-based Neisseria gonorrhoeae and Chlamydia trachomatis Confirmatory Testing in a Metropolitan Setting of High Disease Prevalence

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Running title: APTIMA confirmatory testing in high-prevalence population

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ABSTRACT

Prompted by reports challenging the validity of low-positive *Neisseria gonorrhoeae* (Ng) and *Chlamydia trachomatis* (Ct) results generated by the APTIMA Combo 2 Assay (Gen-Probe, Incorporated) and by a CDC recommendation to confirm Ng- or Ct-positive screens by using an alternative amplification target, we report a comparison of this means of confirmation with an in-house algorithm of repeat testing. Primary clinical specimens yielding Ng- or Ct-specific luminescent values between 100,000 and 1,000,000 were repeat tested in duplicate. A subset of specimens was forwarded for confirmatory assays (Gen-Probe) individualized for alternative Ng or Ct targets. An eighteen-month audit revealed that 230 of 29,977 Ct screens (0.8%) and 41 of 29,064 Ng assays (0.1%) yielded low-positive data. When a subset of 40 low-positive Ng screens was repeat tested, 20 (50.0%) remained positive; 22 (55.0%) of the screens remained positive following performance of the confirmatory assay. In contrast, repeat testing of 153 low-positive Ct screens yielded a positive result in fewer specimens (n = 97; 63.4%) than when commercial confirmatory testing was employed (n = 124; 81.0%). However, confirmation of additional Ct screens using an alternative target did not translate into significant differences in calculated overall Ct-positive screen rates (7.39% using repeat testing versus 7.52% via confirmatory assay; *P* = 0.53). Furthermore, utilization of the confirmatory assay raised positive predictive value only 1.8% over that of repeat testing. Molecular confirmatory testing did not significantly enhance the reliability of Ct- or Ng-specific nucleic acid amplification testing in this metropolitan setting when compared to repeat testing.
INTRODUCTION

Deficiencies in culture and antigen detection assay sensitivity [6, 13, 17, 21, 31], as well as result reproducibility [18, 24], have, in part, characterized past problems with laboratory testing for *Chlamydia trachomatis*. Sensitivity of culture assays for the fastidious pathogen *Neisseria gonorrhoeae* can be impacted by specimen collection device [14], viability of organisms in certain transport systems [27], and utilization of various selective media [33]. Such factors contributed to the development of molecular diagnostic assays for these sexually-transmitted agents. Along with rapid generation of results, *C. trachomatis*- [20, 25, 31] and *N. gonorrhoeae*-specific [20, 22, 25] nucleic acid hybridization assays provide equivalent analytical sensitivity to that of culture. Commercial availability of nucleic acid amplification tests (NAATs) has greatly enhanced the detection of both of these agents [1, 7, 11, 15, 29, 30] in genital swab specimens submitted to clinical laboratories.

The advent of these highly-sensitive assays has raised the potential for generation of false-positive results. Studies have documented the reactivity of a commercial *N. gonorrhoeae*-specific assay with non-gonococcal *Neisseria* species [9, 19]. Recent data have questioned the validity of low-positive *C. trachomatis* and *N. gonorrhoeae* screens generated by a second commercial system [8, 32]. Furthermore, problems with reproducibility have been described for both *C. trachomatis*- and *N. gonorrhoeae*-specific assays [12, 23]. The Centers for Disease Control and Prevention (CDC) addressed a number of these concerns by recommending performance of additional testing for confirmation of a positive result [3]. This may be accomplished by repeating the original assay on the original specimen (with or without blocking...
antibody or competitive probe) or by assaying original/new clinical material with a different test that uses a different antigen, phenotype, or target and a different format.

Schachter et al. [26] cited a number of valid limitations inherent to this paradigm and demonstrated in low- and moderate-prevalence disease populations that C. trachomatis confirmatory testing is unnecessary. In this report, we summarize our experiences with C. trachomatis and N. gonorrhoeae confirmatory testing, comparing utilization of secondary commercial C. trachomatis- and N. gonorrhoeae-specific NAATs to an algorithm of duplicate repeat testing in a large metropolitan population with documented high disease prevalence.

MATERIALS AND METHODS

Setting. A 2005 analysis of data from United States metropolitan statistical areas (MSAs) [4] reported that the Milwaukee-Waukesha-West Allis (Wisconsin) MSA had a chlamydia rate of 684.0 per 100,000 population. This figure ranked second-highest in the United States and was 94% higher than the national cumulative MSA rate of 352.2 per 100,000 population. In the same year, Milwaukee County reported a gonorrhea rate of 426.0 per 100,000 population (ranked eighth in the United States) [4]. When analysis was broadened to include a metropolitan population, the Milwaukee-Waukesha-West Allis MSA had the second-highest gonorrhea rate of United States MSAs (265.9 per 100,000 population; more than double that of the national MSA total rate of 128.2).

Wheaton Franciscan and Midwest Clinical Laboratories serve four Milwaukee metropolitan hospitals (with satellite urgent care facilities) and an approximate 700-clinic outpatient clientele in a nine-county region of southeastern Wisconsin. An eighteen-month audit of molecular testing in our laboratory revealed that the number of \textit{C. trachomatis} and \textit{N. gonorrhoeae} NAATs performed was 29,977 and 29,064, respectively; 24% of the testing was performed on specimens collected by emergency departments of urban hospitals.

Primary screening assays. The APTIMA Combo 2 Assay (AC2; Gen-Probe, Incorporated, San Diego, CA) was utilized for routine screening of primary clinical specimens. The assay, based on the principles of target capture, transcription-mediated amplification, and chemiluminescent hybridization protection, was executed per manufacturer specifications. Monthly environmental
samplings from fourteen sites endogenous to and in proximity to the assay system revealed no amplicon contamination during the course of the investigation.

**In-house algorithm of duplicate repeat testing.** Specimens yielding positive screen results with concomitant *N. gonorrhoeae* - or *C. trachomatis*-specific relative light unit (RLU) values between 100,000 and 1,000,000 (termed low-positive screens) were subject to an in-house algorithm of repeat testing using AC2. Duplicate results (positive/positive or negative/negative) were considered the final reportable result for the assay. Specimens yielding variable results were reported as equivocal.

**Assessment of confirmatory assays.** A randomly-selected subset of low-positive *C. trachomatis* and *N. gonorrhoeae* screens were cryptically coded and forwarded for the APTIMA CT Assay or APTIMA GC Assay (Gen-Probe, Incorporated), performed per manufacturer specifications. Results from the confirmatory assays were compared to both the primary AC2 result and the result generated via duplicate repeat testing.

**Statistical analysis.** The significance test of proportions [28] determined if changes in positive screen percentage were significant. The alpha level was set at 0.05 before investigations commenced and all *P* values are two-tailed.
RESULTS

AC2 for *C. trachomatis* with in-house algorithm of duplicate repeat testing. 2234 specimens were reported positive for *C. trachomatis* (7.45% rate). 2070 (92.7%) of these specimens yielded an RLU value greater than 1,000,000 while 230 specimens were subject to duplicate repeat testing. A positive result was retained for 164 (71.3%) of the low-positive *C. trachomatis* screens (Table 1), while 66 false-positive results would have been reported without duplicate repeat testing. Only 41.7% of screens yielding RLU values ≤ 300 remained positive following duplicate repeat testing (data not illustrated).

AC2 for *N. gonorrhoeae* with in-house algorithm of duplicate repeat testing. 788 specimens were reported positive for *N. gonorrhoeae* (2.71% rate). A greater proportion of these specimens (97.0%; *P* < 0.001) yielded an RLU value greater than 1,000,000 when compared to *C. trachomatis*. Only 41 low-positive screens were subject to duplicate repeat testing, with 24 (58.5%) remaining positive and 17 being false-positive results (Table 1). Less than one-third of specimens yielding RLU values ≤ 400 remained positive following duplicate repeat testing (data not illustrated).

Characterization of subset of primary specimens forwarded for APTIMA CT and GC Assays. Analysis of a subset of low-positive *C. trachomatis* screens forwarded for the APTIMA CT Assay revealed no significant difference in the percentage of specimens that remained positive following duplicate repeat testing (63.4%; Table 2) when compared to all *C. trachomatis* low-positive screens encountered in the audit (Table 1; *P* = 0.10). In similar fashion, no
significant difference was demonstrated (via duplicate repeat testing) in the percentage of low-positive *N. gonorrhoeae* screens that repeated positive in the subset of specimens forwarded for the APTIMA GC Assay (50.0%; Table 2) when compared to all *N. gonorrhoeae* low-positive screens (Table 1; \( P = 0.44 \)). Furthermore, no significant difference was observed in the frequency at which low-positive *C. trachomatis* and *N. gonorrhoeae* screens generated a negative result upon duplicate repeat testing (\( P > 0.26 \); Table 2).

**AC2 Assay with APTIMA confirmatory testing.** A greater percentage of the subset of low-positive *C. trachomatis* screens repeated positive with the APTIMA CT Assay (81.0%; Table 3) when compared to duplicate repeat testing (63.4%; \( P < 0.001 \)). No such difference was observed in the subset of low-positive *N. gonorrhoeae* screens. An increased percentage of both low-positive *C. trachomatis* and *N. gonorrhoeae* screens yielded a negative result (Table 3) when compared to duplicate repeat testing (Table 2), although this difference was not significant (\( P > 0.22 \)).

Specificity of the APTIMA CT and GC Assays was further demonstrated by generation of negative results from 18 and 46 randomly-selected specimens, respectively, which were negative when initially screened with AC2 (data not illustrated). A randomly-selected subset of specimens that screened positive (RLU > 1,000,000 by AC2) for *C. trachomatis* (n = 13) or *N. gonorrhoeae* (n = 40) all yielded positive results through respective APTIMA CT and GC assays.
Extrapolation of confirmatory testing results to local history of *C. trachomatis* and *N. gonorrhoeae* AC2 testing. When the percentages of specimens having confirmed positive screen results based upon duplicate repeat (63.4%) and confirmatory (81.0%) testing of specimens in the low-positive screen subset were extrapolated to the 230 low-positive *C. trachomatis* screens encountered over the 18-month audit, 186 were classified as true-positive screens via the confirmatory method (Table 4). When added to the 2070 *C. trachomatis*-positive screens with RLU values exceeding 1,000,000, the combination of AC2 with the APTIMA CT Assay yielded a positive predictive value of 98.1% for *C. trachomatis*. The extrapolated *C. trachomatis*-positive screen rate (7.52%) was not significantly increased over the rate determined by duplicate repeat testing (*P* = 0.53). No enhancement of the aforementioned parameters was observed with *N. gonorrhoeae* confirmatory testing (Table 4).
DISCUSSION

Challenges related to assay sensitivity and organism viability during specimen transport have, in part, promoted the development of molecular diagnostic modalities for the sexually-transmitted agents *C. trachomatis* and *N. gonorrhoeae*. NAATs, such as AC2, have become the laboratory standard for detection of these agents. Gaydos *et al.* [11] reported that AC2 was 94.2% and 99.2% sensitive for the detection of *C. trachomatis* and *N. gonorrhoeae*, respectively, from endocervical specimens. Additional studies [2, 5] noted equivalent-to-enhanced sensitivity of AC2 on both male and female genital swab specimens when compared to other NAATs. A number of studies [2, 5, 10, 11, 16] demonstrated the utility of AC2 for detection of *C. trachomatis* and/or *N. gonorrhoeae* from female and/or male urine specimens.

Chernesky *et al.* [5] prepared mock swab specimens containing propagated *C. trachomatis* elementary bodies and showed that the analytical sensitivity of AC2 was 1000-fold greater than that of ProbeTec ET (Becton Dickinson Diagnostic Systems) and 10-fold greater than that of AMPLICOR (Roche Molecular Systems). The AC2 had 100-fold greater sensitivity than the two comparators in analogous mock urine specimens. Despite data revealing excellent specificity of the AC2 for *C. trachomatis* and/or *N. gonorrhoeae* in urine and/or genital specimens [10, 11], increased analytical sensitivity of AC2 may potentiate false-positive results.

Wang *et al.* [32] noted that 37.5% of low-positive *C. trachomatis* AC2 screens (defined as RLU values less than 500,000) failed to repeat positive. Dunham *et al.* [8] used a Washington, D.C. population (clinical sample size of 25,200) to report that only 42.4% of low-positive *C.
trachomatis AC2 screens (defined as RLU values between 100,000 and 1,000,000; n = 434) repeated positive using a duplicate repeat testing algorithm and that 31.6% of low-positive N. gonorrhoeae screens (n = 19) repeated positive. Our data corroborate these findings in that: 1) low-positive C. trachomatis and N. gonorrhoeae results occur infrequently in routine AC2 screening (less than 1% in our study); 2) low-positive AC2 screens occur far more frequently with C. trachomatis than with N. gonorrhoeae; and importantly, 3) a significant percentage of these low-positive screens fails to remain positive via duplicate repeat testing. With the benefit of performing testing in an area with a higher prevalence of gonorrhea, enough data were generated in our audit to show that no significant difference existed between the percentages of low-positive C. trachomatis and N. gonorrhoeae screens that remained positive in duplicate repeat testing ($P = 0.10$).

A second mechanism to address the potential for false-positive NAAT results is utilization of an analogous method to amplify an alternative target [3]. Data from a low-prevalence population (cumulative five-state chlamydia and gonorrhea rates of 312.7 and 52.8 per 100,000 population, respectively) reveal that repeat testing and Gen-Probe analyte-specific reagent confirmatory testing of AC2 C. trachomatis- and N. gonorrhoeae-positive screens demonstrated 95% and 90% concordance, respectively [34]. Schachter et al. [26] used low- to moderate-prevalence California populations (as a point of reference, chlamydia rate of 336.7 per 100,000 population in the San Francisco-Oakland-Fremont MSA; [4]) to show that 97.7% of positive C. trachomatis AC2 screens confirmed by repeat testing, while 99.5% of specimens in this subset were confirmed by the APTIMA CT Assay.
Given the high-prevalence of *C. trachomatis* and *N. gonorrhoeae* genital infections in the Milwaukee metropolitan area and the previous discussion of performing follow-up testing on low-positive AC2 screens, we hypothesized that APTIMA CT and GC Assays have the potential to confirm more screens than duplicate repeat testing. The subset of 193 specimens forwarded for *C. trachomatis* and *N. gonorrhoeae* confirmatory testing was representative of previous laboratory experience with duplicate repeat testing (*P* > 0.10 for positive *C. trachomatis* and *N. gonorrhoeae* result retention). While confirmatory testing did not enhance detection of low-positive *N. gonorrhoeae* AC2 screens over that of duplicate repeat testing, between 10% and 18% more low-positive *C. trachomatis* screens were confirmed by the APTIMA CT Assay (Table 3) than by repeat testing (Tables 1 and 2). However, when these data are analyzed in the context of positive AC2 screens for *C. trachomatis* with RLU values greater than 1,000,000 (i.e., specimens not subjected to follow-up testing) and extrapolated to past AC2 experience of this laboratory, overall detection rates derived from AC2 in conjunction with the APTIMA CT Assay exhibited only a modest increase over analogous rates generated by duplicate repeat testing (*P* = 0.53; Table 4) and positive predictive value increased only 1.8%.

A paucity of equivocal results encountered during confirmatory testing translated into an increase in both the percentage of negative and positive results generated by APTIMA CT and GC Assays (Table 3). This change was unlikely a byproduct of specimen source as only 5% of specimens subjected to confirmatory testing in this study were urine specimens. One possible explanation for the increased percentage of specimens yielding positive results by confirmatory testing could have been related to amplification efficiency of the alternative *C. trachomatis* and *N. gonorrhoeae* targets. Conversely, a low-positive result phenomenon, analogous to that
observed with AC2, could also be present in the APTIMA CT and GC Assay. Yet if this were to occur at a rate (~1%) similar to the AC2 rate reported in this study and others [8, 32], this phenomenon probably would not have been realized in the subset of 193 specimens forwarded for confirmatory testing.

Our data confirm and extend the findings of Schachter, Zanto, and colleagues [25, 34] to a population of high gonorrhea and chlamydia prevalence. Limitations to adopting the CDC follow-up testing recommendations include clinicians not routinely providing two specimens for evaluation, the lack of feasibility in calling a patient back for specimen recollection, and the prohibitive expense for some laboratories to either modify an existing molecular assay to target a different nucleic acid sequence or validate a second NAAT [26]. Even when NAATs are utilized as a means of confirmation, Schachter et al. [26] reported differences in performance characteristics of these assays, deficiencies in result reproducibility for a given specimen using the same testing modality, and potential differences in sensitivity related to heterologous specimen collection media/transport devices. As a result, additional generated data may be difficult to interpret, especially when considering the extremely high rates of sensitivity and specificity already inherent to these screening assays [2, 5, 10, 11, 16]. A low-positive result phenomenon with APTIMA CT and GC Assays, should it be demonstrated, could hypothetically contribute to the interpretation conundrum.

In conclusion, while no significant differences were demonstrated between the APTIMA GC Assay and duplicate repeat testing for confirmation of low-positive *N. gonorrhoeae* screens, the APTIMA CT Assay confirmed significantly more low-positive *C. trachomatis* screens than
duplicate repeat testing. Despite the enhanced confirmation rate of the APTIMA CT Assay, only minimal differences in positive predictive value and the rate of true-positive screening in this community of high sexually-transmitted disease prevalence were noted upon extrapolation of confirmatory assay data to recent laboratory experience. Potential clinical and social benefits of *C. trachomatis* - and *N. gonorrhoeae*-specific confirmatory assays, even in high-prevalence locales, may need to be weighed against logistical and financial considerations before such testing algorithms are implemented into the screening approaches for these agents.
ACKNOWLEDGMENT

We express sincere appreciation to Gerald A. Hanson, M.D. for critical review of the manuscript.
REFERENCES


<p>| Table 1: Results of duplicate repeat testing used to resolve all low-positive <em>Chlamydia trachomatis</em> or <em>Neisseria gonorrhoeae</em> screens encountered in an eighteen-month audit of APTIMA Combo 2 Assay performance. |</p>
<table>
<thead>
<tr>
<th>Organism</th>
<th>n</th>
<th>Positive [number (%)]</th>
<th>Equivocal [number (%)]</th>
<th>Negative [number (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>230</td>
<td>164 (71.3)</td>
<td>43 (18.7)</td>
<td>23 (10.0)</td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>41</td>
<td>24 (58.5)</td>
<td>4 (9.8)</td>
<td>13 (31.7)</td>
</tr>
</tbody>
</table>
TABLE 2: Results of duplicate repeat testing used to resolve a subset of low-positive *Chlamydia trachomatis* or *Neisseria gonorrhoeae* APTIMA Combo 2 Assay screens.
<table>
<thead>
<tr>
<th>Organism</th>
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<th>Result Following In-house Algorithm of Duplicate Repeat Testing</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Positive [number (%)] Equivocal [number (%)] Negative [number (%)]</td>
</tr>
<tr>
<td>Chlamydia trachomatis</td>
<td>153</td>
<td>97 (63.4)          35 (22.9)          21 (13.7)</td>
</tr>
<tr>
<td>Neisseria gonorrhoeae</td>
<td>40</td>
<td>20 (50.0)          4 (8.0)            16 (32.0)</td>
</tr>
</tbody>
</table>
TABLE 3: Results of APTIMA CT and GC (confirmatory) Assays used to resolve a subset of low-positive *Chlamydia trachomatis* or *Neisseria gonorrhoeae* APTIMA Combo 2 Assay screens.
<table>
<thead>
<tr>
<th>Organism</th>
<th>n</th>
<th>Positive [number (%)]</th>
<th>Equivocal [number (%)]</th>
<th>Negative [number (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>153</td>
<td>124 (81.0)</td>
<td>0 (0.0)</td>
<td>29 (19.0)</td>
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<tr>
<td><em>Neisseria gonorrhoeae</em></td>
<td>40</td>
<td>22 (55.0)</td>
<td>0 (0.0)</td>
<td>18 (45.0)</td>
</tr>
</tbody>
</table>
TABLE 4: Extrapolation of data generated from evaluation of a subset of low-positive *Chlamydia trachomatis* and *Neisseria gonorrhoeae* screens using duplicate repeat testing or APTIMA confirmatory testing to data generated from an eighteen-month audit of APTIMA Combo 2 performance.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Chlamydia trachomatis</th>
<th>Neisseria gonorrhoeae</th>
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</thead>
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<tr>
<td></td>
<td>Duplicate Repeat Testing</td>
<td>Confirmatory Method</td>
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<tr>
<td>True-positive results†</td>
<td>146</td>
<td>186</td>
</tr>
<tr>
<td>False-positive results†</td>
<td>84</td>
<td>44</td>
</tr>
<tr>
<td>Positive predictive value‡</td>
<td>96.3%</td>
<td>98.1%</td>
</tr>
<tr>
<td>Rate of true-positive screen‡</td>
<td>7.39%</td>
<td>7.52%</td>
</tr>
</tbody>
</table>

† Derived from follow-up testing of low-positive *C. trachomatis* and *N. gonorrhoeae* APTIMA Combo 2 screens
‡ Derived from analysis of combined APTIMA Combo 2 Assay and follow-up testing data