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# 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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**Prompted by reports challenging the validity of the low-positive *Neisseria gonorrhoeae* and *Chlamydia trachomatis* results generated by the APTIMA Combo 2 assay (Gen-Probe, Incorporated) and by a Centers for Disease Control and Prevention recommendation to confirm *N. gonorrhoeae*- or *C. trachomatis*-positive screens by using an alternative amplification target, we report on a comparison of this means of confirmation with an in-house algorithm of repeat testing. Primary clinical specimens yielding *N. gonorrhoeae*- or *C. trachomatis*-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 *N. gonorrhoeae* or *C. trachomatis* targets. An 18-month audit revealed that 230 of 29,977 *C. trachomatis* screens (0.8%) and 41 of 29,064 *N. gonorrhoeae* assays (0.1%) yielded low-positive data. When a subset of 40 low-positive *N. gonorrhoeae* 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 *C. trachomatis* screens yielded a positive result for fewer specimens ($n$ = 97; 63.4%) than when commercial confirmatory testing was used ($n$ = 124; 81.0%). However, confirmation of the results for additional *C. trachomatis* screens by use of an alternative target did not translate into significant differences in the calculated overall *C. trachomatis*-positive screen rates (7.39% by repeat testing versus 7.52% by the confirmatory assay; $P$ = 0.53). Furthermore, use of the confirmatory assay raised the positive predictive value only 1.8% over that of repeat testing. Molecular confirmatory testing did not significantly enhance the reliability of *C. trachomatis*- or *N. gonorrhoeae*-specific nucleic acid amplification testing in this metropolitan setting compared to the reliability of repeat testing.**

Deficiencies in culture and antigen detection assay sensitivities (6, 13, 17, 21, 32), as well as result reproducibility (18, 24), have, in part, characterized past problems with laboratory testing for *Chlamydia trachomatis*. The sensitivities of culture assays for the fastidious pathogen *Neisseria gonorrhoeae* can be affected by the specimen collection device used (14), the viability of organisms in certain transport systems (27), and the various selective media used (33). Such factors contributed to the development of molecular diagnostic assays for these sexually transmitted agents. Along with the rapid generation of results, *C. trachomatis*-specific (20, 25, 32) and *N. gonorrhoeae*-specific (20, 22, 25) nucleic acid hybridization assays provide analytical sensitivities equivalent to that of culture. The 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 the generation of false-positive results. Studies have documented the reactivity of a commercial *N. gonorrhoeae*-specific assay with nongonococcal *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, 31). 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 the performance of additional testing for the confirmation of a positive result (3). This may be accomplished by repeating the original assay with the original specimen (with or without blocking antibody or competitive probe) or by assaying original/new clinical material by 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 with 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 the use of secondary commercial *C. trachomatis*- and *N. gonorrhoeae*-specific NAATs to an algorithm of duplicate repeat testing in a large metropolitan population with a documented high disease prevalence.

(The results of this work were previously presented, in part, at the 106th General Meeting of the American Society for Microbiology, Orlando, FL, 21 to 25 May 2006 [20a].)

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TABLE 1. Results of duplicate repeat testing used to resolve all low-positive *Chlamydia trachomatis* or *Neisseria gonorrhoeae* screens encountered in an 18-month audit of AC2 performance

| Organism | Total no. of samples tested | No. (%) of samples with the following result by use of in-house algorithm of duplicate repeat testing: | | |
|---|---|---|---|---|
| | | Positive | Equivocal | Negative |
| *Chlamydia trachomatis* | 230 | 164 (71.3) | 43 (18.7) | 23 (10.0) |
| *Neisseria gonorrhoeae* | 41 | 24 (58.5) | 4 (9.8) | 13 (31.7) |

TABLE 2. Results of duplicate repeat testing used to resolve a subset of low-positive *Chlamydia trachomatis* or *Neisseria gonorrhoeae* AC2 screens

| Organism | Total no. of samples tested | No. (%) of samples with the following result by use of in-house algorithm of duplicate repeat testing: | | |
|---|---|---|---|---|
| | | Positive | Equivocal | Negative |
| *Chlamydia trachomatis* | 153 | 97 (63.4) | 35 (22.9) | 21 (13.7) |
| *Neisseria gonorrhoeae* | 40 | 20 (50.0) | 4 (8.0) | 16 (32.0) |

## 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* infection rate of 684.0 per 100,000 population. This figure ranked the 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 the analysis was broadened to include a metropolitan population, the Milwaukee-Waukesha-West Allis MSA had the second-highest gonorrhea rate among United States MSAs (265.9 per 100,000 population; more than double the national MSA total rate of 128.2 per 100,000 population).

Wheaton Franciscan and Midwest Clinical Laboratories serve four Milwaukee metropolitan hospitals (with satellite urgent-care facilities) and an approximately 700-clinic outpatient clientele in a nine-county region of southeastern Wisconsin. An 18-month audit of molecular testing in our laboratory revealed that the numbers of NAATs performed for *C. trachomatis* and *N. gonorrhoeae* were 29,977 and 29,064, respectively; 24% of the tests were performed with specimens collected by the emergency departments of urban hospitals.

**Primary screening assays.** The APTIMA Combo 2 assay (AC2; Gen-Probe, Incorporated, San Diego, CA) was used 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 according to the manufacturer's specifications. Monthly environmental samplings from 14 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 by the use of 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 for *C. trachomatis* and the APTIMA GC assay for *N. gonorrhoeae* (Gen-Probe, Incorporated), respectively, which were performed according to the manufacturer's specifications. The results of the confirmatory assays were compared to both the primary AC2 result and the result generated by duplicate repeat testing.

**Statistical analysis.** The significance test of proportions (28) was used to determine if the changes in the positive screen percentages were significant. The alpha level was set at 0.05 before the investigations commenced, and all *P* values are two tailed.

## RESULTS

**AC2 for *C. trachomatis* with in-house algorithm of duplicate repeat testing.** A total of 2,234 specimens were reported to be positive for *C. trachomatis* (rate, 7.45%). A total of 2,070 (92.7%) of these specimens yielded RLU values 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 the 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.** A total of 788 specimens were reported to be positive for *N. gonorrhoeae* (2.71% rate). A greater proportion of these specimens (97.0%; $P < 0.001$) than the proportion of specimens positive for *C. trachomatis* yielded an RLU value greater than 1,000,000. Only 41 low-positive screens were subject to duplicate repeat testing, with 24 (58.5%) remaining positive and 17 having false-positive results (Table 1). Less than one-third of the 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) compared to the percentage of all *C. trachomatis* low-positive screens encountered in the audit (Table 1; $P = 0.10$). In a similar fashion, no significant difference was demonstrated (by duplicate repeat testing) in the percentage of low-positive *N. gonorrhoeae* screens that retested positive among the subset of specimens forwarded for the APTIMA GC assay (50.0%; Table 2) compared to the percentage of all *N. gonorrhoeae* low-positive screens (Table 1; $P = 0.44$). Furthermore, no significant difference in the frequency at which low-positive *C. trachomatis* and *N. gonorrhoeae* screens generated a negative result upon duplicate repeat testing was observed ($P > 0.26$; Table 2).

**AC2 with APTIMA confirmatory testing.** A greater percentage of the subset of low-positive *C. trachomatis* screens retested positive by the APTIMA CT assay (81.0%; Table 3) compared to the percentage that were positive upon duplicate

TABLE 3. Results of APTIMA *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (confirmatory) assays used to resolve a subset of low-positive *Chlamydia trachomatis* or *Neisseria gonorrhoeae* AC2 screens

| Organism | Total no. of samples tested | No. (%) of samples with the following result after confirmatory testing: | | |
|---|---|---|---|---|
| | | Positive | Equivocal | Negative |
| *Chlamydia trachomatis* | 153 | 124 (81.0) | 0 (0.0) | 29 (19.0) |
| *Neisseria gonorrhoeae* | 40 | 22 (55.0) | 0 (0.0) | 18 (45.0) |

TABLE 4. Extrapolation of data generated from evaluation of a subset of low-positive *Chlamydia trachomatis* and *Neisseria gonorrhoeae* screens by using duplicate repeat testing or APTIMA confirmatory testing to data generated from an 18-month audit of AC2 performance

| Organism and test | No. of samples with: | | Positive predictive value (%)[b] | Rate (%) of true-positive screen[b] |
|---|---|---|---|---|
| | True-positive results[a] | False-positive results[a] | | |
| *Chlamydia trachomatis* | | | | |
| Duplicate repeat testing | 146 | 84 | 96.3 | 7.39 |
| Confirmatory test method | 186 | 44 | 98.1 | 7.52 |
| *Neisseria gonorrhoeae* | | | | |
| Duplicate repeat testing | 20 | 21 | 97.4 | 2.70 |
| Confirmatory test method | 23 | 18 | 97.8 | 2.71 |

[a] Derived from follow-up testing of low-positive *C. trachomatis* and *N. gonorrhoeae* AC2 screens.
[b] Derived from analysis of combined AC2 and follow-up testing data.

repeat testing (63.4%; $P < 0.001$). No such difference was observed for 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) compared to the percentage that were negative upon duplicate repeat testing (Table 2), although this difference was not significant ($P > 0.22$).

The specificities of the APTIMA CT and GC assays were further demonstrated by the generation of negative results from 18 and 46 randomly selected specimens, respectively, which were negative when they were initially screened by 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* ($n = 40$) yielded positive results by the APTIMA CT and GC assays, respectively.

**Extrapolation of confirmatory testing results to local history of *C. trachomatis* and *N. gonorrhoeae* AC2 testing.** When the percentages of specimens with confirmed positive screen results based upon duplicate repeat testing (63.4%) and confirmatory testing (81.0%) 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 by the confirmatory method (Table 4). When these results were added to the 2,070 *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 the 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 swab specimens. Additional studies (2, 5) noted the equivalent to enhanced sensitivity of AC2 with both male and female genital swab specimens compared to the sensitivities of other NAATs. A number of studies (2, 5, 10, 11, 16) demonstrated the utility of AC2 for the 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 1,000-fold greater than that of the ProbeTec ET assay (Becton Dickinson Diagnostic Systems) and 10-fold greater than that of the AMPLICOR assay (Roche Molecular Systems). AC2 had a 100-fold greater sensitivity than the two comparators with analogous mock urine specimens. Despite the availability of data revealing the excellent specificity of AC2 for the detection of *C. trachomatis* and/or *N. gonorrhoeae* in urine and/or genital specimens (10, 11), the increased analytical sensitivity of AC2 may potentiate false-positive results.

Wang et al. (31) noted that 37.5% of low-positive *C. trachomatis* AC2 screens (defined as RLU values less than 500,000) failed to retest positive. Dunham et al. (8) used a Washington, DC, population (clinical sample size, 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; $n = 434$) retested positive by the use of a duplicate repeat testing algorithm and that 31.6% of low-positive *N. gonorrhoeae* screens ($n = 19$) retested positive. Our data corroborate those findings, in that (i) low-positive *C. trachomatis* and *N. gonorrhoeae* results occur infrequently in routine AC2 screening (less than 1% in our study); (ii) low-positive AC2 screens occur far more frequently with *C. trachomatis* than with *N. gonorrhoeae*; and (iii) importantly, a significant percentage of these low-positive screens fail to remain positive by duplicate repeat testing. With the benefit of performing testing in an area with a higher prevalence of gonorrhea, our audit generated enough data to show that no significant difference existed between the percentages of low-positive *C. trachomatis* and *N. gonorrhoeae* screens that remained positive by duplicate repeat testing ($P = 0.10$).

A second mechanism that can be used to address the potential for false-positive NAAT results is the use of an analogous method to amplify an alternative target (3). Data from a low-prevalence population (cumulative five-state *Chlamydia* infection 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 of results, respectively (34). Schachter et al. (26) used low- to moderate-prevalence California populations (as a point of reference, the *Chlamydia* infection rate was 336.7 per 100,000 population in the San Francisco-Oakland-Fremont MSA [4]) to show that 97.7% of positive results for *C. trachomatis* AC2 screens could be confirmed by repeat testing, while the results for 99.5% of the 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 on the performance of follow-up testing with samples with low-positive AC2 screens, we hypothesized that the APTIMA CT and GC assays have the potential to confirm more screen results than duplicate repeat testing. The results obtained with the subset of 193 specimens forwarded for *C. trachomatis* and *N. gonorrhoeae* confirmatory testing were 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 the detection of low-positive *N. gonorrhoeae* AC2 screens over that of duplicate repeat testing, the results for 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 the past experience with AC2 of our laboratory, the overall detection rates derived from AC2 in conjunction with the APTIMA CT assay exhibited only a modest increase over the analogous rates generated by duplicate repeat testing ($P = 0.53$; Table 4), and the positive predictive value increased only 1.8%.

A paucity of equivocal results encountered during confirmatory testing translated into an increase in the percentages of both negative and positive results generated by the APTIMA CT and GC assays (Table 3). This change was unlikely a by-product of the specimen source, as only 5% of the 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 the amplification efficiencies 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 assays. Yet, if this were to occur at a rate (~1%) similar to the rate for AC2 reported in this and other studies (8, 31), 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 et al. (25) and Zanto et al. (34) to a population with a high prevalence of gonorrhea and *Chlamydia* infection. Limitations to the adoption of the CDC follow-up testing recommendations include the lack of the routine provision of two specimens for evaluation by clinicians, 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 used as a means of confirmation, Schachter et al. (26) reported differences in the performance characteristics of these assays, deficiencies in result reproducibility for a given specimen by the same testing modality, and potential differences in sensitivity related to the use of heterologous specimen collection media and transport devices. As a result, the additional data that are generated may be difficult to interpret, especially when the extremely high rates of sensitivity and specificity already inherent to these screening assays are considered (2, 5, 10, 11, 16). A low-positive-result phenomenon with the APTIMA CT and GC assays, should it be demonstrated, could hypothetically contribute to the interpretation conundrum.

In conclusion, while no significant differences between the APTIMA GC assay and duplicate repeat testing for confirmation of the results for low-positive *N. gonorrhoeae* screens were demonstrated, the APTIMA CT assay confirmed the results for 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 the positive predictive value and the rate of true-positive screening in this community with a high prevalence of sexually transmitted diseases were noted upon extrapolation of the confirmatory assay data to recent laboratory experience. The 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 approaches for screening for these agents.

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