JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2002, p. 4056–4059 Vol. 40, No. 11
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.11.4056–4059.2002
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Confirmation by 16S rRNA PCR of the COBAS AMPLICOR CT/NG Test for Diagnosis of *Neisseria gonorrhoeae* Infection in a Low-Prevalence Population

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Received 7 May 2002/Returned for modification 14 June 2002/Accepted 15 August 2002

The COBAS AMPLICOR CT/NG test is widely used for the diagnosis of *Neisseria gonorrhoeae* infection using genital swabs or urine samples. Although highly specific, cross-reactivity occurs with some nonpathogenic strains of *Neisseria* and *Lactobacillus* species. In low-prevalence populations, even highly specific assays may require confirmatory testing of positive results. We assessed the positive predictive value (PPV) of this test in a low-prevalence (0.5%) setting. Genital and urine specimens testing positive using the COBAS AMPLICOR NG test were retested using an investigational 16S rRNA PCR assay. Additionally, 737 specimens were tested in parallel by both culture and the above PCR protocol. Of 9,772 specimens tested in-house, 168 were positive by the AMPLICOR test; in addition, 62 AMPLICOR-positive specimens were referred to our laboratory for confirmatory testing, yielding 230 positive specimens. Of these, 72 were confirmed positive by 16S rRNA PCR, yielding a specificity of 98.7% and a PPV of 31.3%. Specificity was similar for all specimen types, whereas PPV varied with prevalence: specimens from males, females, urine specimens, and genital swabs had PPVs of 70.8, 13.3, 51.9, and 20.1%, respectively. The PPV was higher when the initial AMPLICOR optical density (OD) was ≥3.5 versus initial and repeat OD readings in an equivocal zone of ≥0.2 to <3.5 (65.1 versus 10.1%; *P* < 0.001). On repeat testing of specimens with ODs in the equivocal zone, 54 gave ODs of ≥0.2 and <2.0, 35 gave ODs of ≥2.0 and <3.5, and 12 gave ODs of ≥3.5, with 3.7, 20, and 33.3% confirmed positive, respectively (*P* = 0.004). Comparing PCR to culture as the "gold standard," specificity increased from 96.8 to 99.9% when 16S rRNA PCR was performed on specimens positive by the COBAS AMPLICOR NG test. Confirmatory testing with a more specific method such as 16S rRNA PCR should be considered in low-prevalence populations, especially for specimens with an OD in the equivocal zone.

Neisseria gonorrhoeae remains the second most prevalent sexually transmitted bacterial infection after *Chlamydia trachomatis*, with approximately 650,000 new infections occurring annually in the United States (2). A significant proportion of these infections, especially in women, are asymptomatic and, if they remain undiscovered, may result in such long-term consequences as pelvic inflammatory disease, chronic pelvic pain, ectopic pregnancy, and infertility (9). Furthermore, silent and untreated infection facilitates the spread of this organism to unsuspecting sexual partners. Therefore, accurate diagnosis of both symptomatic and asymptomatic infection is critical.

A number of techniques have been developed to detect genital infections caused by *N. gonorrhoeae*. The current "gold standard" for diagnosis of infection is by culturing endocervical or urethral swab specimens on selective media. However, even under optimal laboratory conditions, the sensitivity of gonococcal cultures ranges from 85 to 95% for acute infection (17) and falls to approximately 50% for females with chronic infections (1). Poor specimen collection, transport, and storage may contribute to false-negative results. Recently, nucleic acid amplification-based techniques, including the ligase chain reaction and PCR, have been shown to have both high sensitivity and specificity for the detection of *N. gonorrhoeae* (3, 11, 14, 15, 18). The COBAS AMPLICOR test for *C. trachomatis* and *N. gonorrhoeae* (COBAS AMPLICOR CT/NG; Roche Molecular Systems, Branchburg, N.J.) is a fully automated multiplex PCR-based test that allows simultaneous detection of both pathogens in clinical specimens.

The COBAS AMPLICOR CT/NG test has demonstrated high sensitivity for detecting *C. trachomatis* infection using both urethral and endocervical swabs and urine specimens (6, 16, 19, 23). Studies on the utility of this test in diagnosing infection with *N. gonorrhoeae* have demonstrated sensitivities ranging from 92.4 to 100% for endocervical or urethral swabs and from 42.3 to 94.1% for urine specimens, while specificity has been high (95.9 to 99.9%) for all specimens (4, 5, 10, 13, 14, 20–22). The majority of these studies were performed in populations with a high prevalence (4.4 to 20.1%) of infection, resulting in positive predictive values (PPV) greater than 80%.

In areas of low prevalence, however, even highly specific assays may need confirmatory testing of positive results. In this study, the sensitivity, specificity, and PPV of the NG portion of the test (COBAS AMPLICOR NG) for the detection of *N. gonorrhoeae* infection was assessed using both genital swabs and urine specimens collected from subjects from a low-prevalence (0.5%) population by comparing results to those of culture and an investigational 16S rRNA PCR assay developed by Roche Molecular Systems.

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MATERIALS AND METHODS

Patient population. Specimens were collected from consecutive patients undergoing investigation for *N. gonorrhoea* infection (both symptomatic and asymptomatic screening) at a diverse array of outpatient clinics affiliated with a large Montreal tertiary-care center serving both adults and children, as well as at two health centers in northern Québec serving a primarily Native Canadian population. Based on *N. gonorrhoea*e culture data from 1999, the overall prevalence of infection in this combined population was approximately 0.4%. In addition, a number of specimens were sent to our laboratory for confirmation of a positive COBAS AMPLICOR NG test performed elsewhere. Urethral or endocervical samples for *N. gonorrhoeae* culture were also collected from a subset of patients from the hospital-based clinics. Specimens were collected between June 1999 and November 2000.

Specimen collection and storage. (i) DNA amplification.

Urethral, cervical, and first-pass urine specimens were collected, stored, and treated as described in the COBAS AMPLICOR CT/NG product insert (Roche Diagnostic Systems Inc.). Samples collected from communities in northern Québec were frozen at −20°C for up to 1 week pending transportation to our laboratory.

(ii) *N. gonorrhoeae* culturing. Urethral and endocervical swabs were collected and immediately placed in charcoal transport medium. Specimens were transported to the laboratory within 8 h, and often within 1 h, where they were inoculated onto modified Thayer-Martin and chocolate agar plates.

PCR methods. The COBAS AMPLICOR *N. gonorrhoeae* PCR was performed on the automated COBAS instrument according to the manufacturer's instructions. For each processed specimen, the *N. gonorrhoeae*, *C. trachomatis*, and internal control (IC) target DNAs were amplified simultaneously in a single reaction mixture containing two primer pairs, one specific for *C. trachomatis* and the IC and one specific for *N. gonorrhoeae*. Amplification products were detected separately by hybridization to magnetic microparticles coated with *N. gonorrhoeae*-, *C. trachomatis*-, and IC-specific oligonucleotide probes. The 16S rRNA assay is an investigational method supplied by Roche Molecular Systems. The assay uses the *N. gonorrhoeae* 16S rRNA gene described by Rossau et al. that targets a region of the *N. gonorrhoeae* genome that is different from that targeted by COBAS AMPLICOR and is thought to be highly conserved within the species (16a). It was performed according to instructions from Roche Molecular Systems. Briefly, PCR was performed on a Perkin-Elmer GeneAmp 9600 thermocycler, with 38 cycles of 10 s at 91°C, 50 s at 62°C, and 35 s at 72°C. Amplicons were denatured and detected in an enzyme immunoassay system using microwell plates coated with specific DNA probes and an avidin-horseradish peroxidase conjugate.

Interpretation of results. For the COBAS AMPLICOR NG test, specimens yielding an optical density (OD) of ≥0.2 units were interpreted as presumptively positive (as recommended by the manufacturer), regardless of the IC result. Specimens yielding an OD of <0.2 were considered negative, provided that the IC signal was equal to or above 0.2. Specimens yielding ODs below 0.2 for both the IC- and *N. gonorrhoeae*-specific reactions were interpreted as inhibitory. These specimens were retested by freezing and thawing an aliquot of the original specimen. Specimens were excluded from the analysis if they were repeatedly inhibitory.

Specimens with an *N. gonorrhoeae* signal of ≥0.2 and <3.5 were considered equivocal and were retested. Initially equivocal specimens were considered presumptively positive if two or more tests yielded OD readings of ≥0.2. All specimens considered presumptively positive by the COBAS AMPLICOR NG test then underwent testing by the 16S rRNA PCR assay. Interpretation of the results obtained from 16S rRNA PCR was similar to that of the COBAS AMPLICOR NG test, except that specimens with an *N. gonorrhoeae* signal of ≥0.2 and <0.8 were considered equivocal and repeated in duplicate. Initially equivocal specimens were interpreted as positive if at least two of three tests (including the original one) yielded *N. gonorrhoeae* signals of ≥0.2. Initially equivocal specimens were interpreted as negative if fewer than two tests yielded an OD of ≥0.2.

Gonorrhea culture. Cultures were examined following 48 and 72 h incubation at 35°C in 5% CO_2. *N. gonorrhoeae* was presumptively identified based on the appearance of typical colonies of gram-negative diplococci and positive oxidase and catalase reactions. The identities of presumptive *N. gonorrhoeae* isolates were confirmed by carbohydrate utilization profiles.

Statistical analysis. The specificity and PPV of the COBAS AMPLICOR CT/NG PCR compared to the 16S rRNA PCR were calculated for each type of specimen and according to the gender of the patient. Since some of the clinics performed their own COBAS AMPLICOR PCR and sent only positive specimens for 16S rRNA PCR testing, these specimens were not included in the specificity calculations (but were included in the PPV calculations) as the total number of COBAS AMPLICOR tests performed was unavailable for these laboratories. The significance of differences in specificity and PPV between subsets of specimens was assessed by the chi-square or Fisher's exact test.

TABLE 1. Specificity of COBAS AMPLICOR NG test versus 16S rRNA PCR test for detection of *N. gonorrhoeae* in clinical specimens

Specimen	*n*	Specificity (%)	95% CI
Female endocervical	5,727	98.3	97.9–98.6
Male urethral	1,257	98.8	98.0–99.3
Female urine	2,397	99.2	98.8–99.5
Male urine	391	99.2	97.8–99.8
Total	9,772	98.7	98.4–98.9

RESULTS

From June 1999 until November 2000, 9,772 specimens from both symptomatic and asymptomatic patients were tested for *N. gonorrhoeae* using the COBAS AMPLICOR CT/NG test. Of these, 168 (1.7%) specimens from 167 separate patient visits were presumptively positive and underwent confirmatory testing using the 16S rRNA assay. In addition, 62 specimens were referred in from other centers for confirmation (using the 16S rRNA assay) of a positive COBAS AMPLICOR NG test, yielding a total of 230 specimens that were presumptively positive. Of these specimens, 158 were from females and 59 were from males. There were 149 urethral or cervical swab specimens and 81 urine specimens.

Seventy-two specimens identified as presumptively positive for *N. gonorrhoeae* by COBAS AMPLICOR were confirmed positive by the 16S rRNA assay, yielding a specificity of 98.7% (95% confidence interval [CI], 98.4 to 98.9%) and a PPV of 31.3% (95% CI, 25.4 to 37.7%) for the COBAS AMPLICOR NG test. Specificities were similar for all specimen types: 98.3% (95% CI, 97.9 to 98.6%) for female endocervical swabs, 98.8% (95% CI, 98.0 to 99.3%) for male urethral swabs, 99.2% (95% CI, 98.8 to 99.5%) for female urine specimens, and 99.2% (95% CI, 97.8 to 99.8%) for male urine specimens (Table 1). Specificities for the different specimen types were not significantly different except when female urine specimens and female endocervical swabs were compared ($P = 0.001$).

When analyzed according to gender and type of specimen, the PPV of the COBAS AMPLICOR result compared to the 16S rRNA assay varied with prevalence (Table 2). For specimens from men and women, the PPVs were 70.8% (95% CI, 58.9 to 81.0%) and 13.3% (95% CI, 8.4 to 19.6%), respectively, while for urine specimens and urethral or cervical specimens, the PPVs were 51.9% (95% CI, 40.5 to 63.1%) and 20.1% (95% CI, 14.0 to 27.5%), respectively. The PPV was highest when restricted to urine specimens from men (Table 2), at 82.9% (95% CI, 66.4 to 93.4%),and lowest for endocervical swabs obtained from women, at 7.1% (95% CI, 3.1 to 13.6%). The PPV for urine specimens from females was 28.3% (95% CI, 16.0 to 43.5%) and 59.5% (95% CI, 42.1 to 75.2%) for urethral swabs from men.

Eighty-six specimens had an initial COBAS AMPLICOR OD reading of 3.5 or more, whereas 144 had initial readings in the equivocal zone of ≥0.2 and <3.5. One hundred two specimens with readings in the equivocal zone were retested by

TABLE 2. PPV of COBAS AMPLICOR NG test compared to that of 16S rRNA PCR

Specimen	n[a]	PPV (%)	95% CI
Male	72	70.8	58.9–81.0
Urethral	37	59.5	42.1–75.2
Urine	35	82.9	66.4–93.4
Female	158	13.3	8.4–19.6
Endocervical	112	7.1	3.1–13.6
Urine	46	28.3	16.0–43.5
Urine	81	51.9	40.5–63.1
Cervix or urethra	149	20.1	14.0–27.5
Total	230	31.3	25.4–37.7

[a] The number of specimens positive by COBAS AMPLICOR NG PCR that underwent confirmatory testing by 16S rRNA PCR.

COBAS AMPLICOR before undergoing 16S rRNA PCR testing; the remainder underwent 16S rRNA testing before a second COBAS AMPLICOR test was performed (the PPV of the subset of samples that did not have a repeat COBAS AMPLICOR test was not significantly different from those that did [data not shown] [$P = 0.5$]). The PPV of the COBAS AMPLICOR NG test was significantly higher when the OD was ≥3.5 on initial testing (65.1% [95% CI, 54.8 to 75.4%]) than when both initial and repeat OD readings were within the equivocal zone (10.1% [95% CI, 3.7 to 16.5%]; $P < 0.001$) (Table 3). Upon repeat COBAS AMPLICOR NG testing of 102 specimens with an initial OD in the equivocal zone, 1 was inhibited. Fifty-four specimens gave ODs that were ≥0.2 but <2.0, 35 gave ODs that were ≥2.0 but <3.5, and 12 gave ODs that were ≥3.5. Within these subgroups, two (3.7%), seven (20.0%), and four (33.3%) specimens, respectively, were confirmed to be positive for *N. gonorrhoeae* DNA by 16S rRNA testing ($P = 0.004$).

Comparison between PCR and culture. From the 9,772 specimens that were tested using the COBAS AMPLICOR NG test, 737 endocervical and urethral specimens were also tested by conventional culture. These were collected from patients undergoing screening for sexually transmitted diseases from whom a specimen was collected for both COBAS AMPLICOR CT testing and *N. gonorrhoeae* culture, prior to the routine offering of the COBAS AMPLICOR NG test in our institution. Twenty-nine specimens were positive by the COBAS AMPLICOR NG test, of which seven were culture positive; no specimens were positive by culture but negative by COBAS AMPLICOR. This yielded a specificity of 96.8% (95% CI, 95.5 to 98.1%) and sensitivity of 100% for the COBAS AMPLICOR NG test (using a cutoff of 0.2). However, when 16S rRNA PCR confirmation was performed on specimens presumptively positive by COBAS AMPLICOR, the specificity of this testing algorithm increased to 99.9% (95% CI, 99.2 to 100%).

TABLE 3. PPV of COBAS AMPLICOR NG test based on OD

OD	n[a]	PPV (%)	95% CI
≥3.5	86	65.1	54.8–75.4
0.2–3.5	89[b]	10.1	3.7–16.5

[a] The number of specimens positive by COBAS AMPLICOR NG PCR that underwent confirmatory testing by 16S rRNA PCR.
[b] The number of specimens that were within the equivocal range both on initial and repeat COBAS AMPLICOR NG testing.

DISCUSSION

The results of this study confirm the high sensitivity (100%) and specificity (98.3 to 99.2%) of the COBAS AMPLICOR CT/NG test for the detection of *N. gonorrhoeae* in endocervical, urethral, and urine specimens. Previous studies have demonstrated high sensitivities (92.4 to 100%) for endocervical swab specimens from women and urine specimens from symptomatic men, whereas sensitivities for urine specimens from women and asymptomatic men were somewhat lower, ranging from 42.3 to 88.3% (4, 14, 21). We could not make a precise estimate of sensitivity due to the low number of true positives in the 737 specimens that were evaluated by both culture and PCR.

The specificity of the COBAS AMPLICOR NG test was high for all types of specimens tested, ranging from 98.3% for endocervical swab specimens from women to 99.2% for urine specimens. Previous studies have reported similar specificities, with corresponding PPVs of more than 80% (4, 10, 13, 14). Although these specificities are high, there is controversy in the literature with respect to the methods used to calculate them, particularly in regards to the use of discrepant analysis (7, 8). In fact, Hadgu asserts that using a second DNA amplification test to resolve the discrepancy between a positive DNA amplification test and negative culture result may overestimate the specificity of the first PCR-based assay.

Regardless of this controversy, all of the previous studies reporting high PPVs for the COBAS AMPLICOR NG test were performed in populations with relatively a high prevalence of *N. gonorrhoeae* infection (4.4 to 20.1%). In contrast, the prevalence of infection in our subject population was 0.5% over the duration of the study period. Not surprisingly, therefore, the PPV of the COBAS AMPLICOR NG test was only 31.2% in this study. The PPV was highest for urine specimens from men (82.9%) and lowest for cervical swab specimens obtained from women (6.7%). These differences were entirely secondary to differences in prevalence, as the specificities were not markedly different between the different specimen types.

The use of an equivocal zone for the COBAS AMPLICOR NG test has been advocated by Martin et al. and Van Der Pol et al. based on the results of a large multicenter trial (14, 20). In this study evaluating the performance of the COBAS AMPLICOR NG test in the clinical setting, specificities ranged from 98.8 to 99.9% depending on the source of the specimen. This high specificity was obtained using an algorithm that required retesting specimens with OD readings that fell within a broad equivocal zone of 0.2 to 3.5 OD units; only those samples for which at least two out of three tests (including the initial test) produced signals of ≥2.0 OD units were interpreted as positive. Without this retesting algorithm, and using a cutoff of 0.2 OD units for a positive result, specificity fell to a range of 96.2 to 98.9%, depending on the type of specimen.

In the present study, all specimens yielding OD values within the equivocal zone were retested by 16S rRNA PCR. The PPV for specimens with an initial OD value of ≥3.5 was 65.1%, whereas for specimens with an OD consistently below 3.5 it was significantly lower (10.1%; $P < 0.001$). Our study confirms

the finding that specificity increases continuously as the cutoff is increased from 0.2 to 3.5 OD units, to the detriment of sensitivity. This supports the adoption of Martin et al.'s algorithm using a higher cutoff of 2.0 OD units; however, this would have missed two (2.8%) true positives in our study. On the other hand, confirmatory testing of all specimens yielding COBAS AMPLICOR NG test OD readings within the equivocal zone between 0.2 and 3.5 would not compromise sensitivity.

The necessity of including a broad equivocal zone in the COBAS AMPLICOR NG testing algorithm may derive from cross-reactivity of the test's primers with certain nonpathogenic strains of *Neisseria* such as *N. subflava* and *N. cinerea* (5, 20). Although these organisms are considered commensal organisms of the upper respiratory tract, it has been postulated that transient carriage in the genital tract may occur (12). Specimens containing cross-reactive *Neisseria* strains may give signals above the negative cutoff of 0.2 units, but it has been suggested that due to minor DNA base pair differences in the probe region and low organism load, detection of the nonpathogenic *Neisseria* strains will give OD readings below 3.5 (20). Cross-reactivity has also been shown with *Lactobacillus* species, another commensal organism of the female genital tract (21). Interestingly, in the present study, specificity was slightly lower for female endocervical specimens, which would be the most likely to be contaminated with *Lactobacillus.*

In the study by Martin et al., only 2.9% of their specimens gave COBAS AMPLICOR NG test results within the equivocal zone versus 11% that had an OD of ≥3.5, leading them to assert that the additional testing burden of their retesting algorithm would be small. However, in our study, a much greater proportion (144 out of 230) of specimens with an OD of ≥0.2 had readings within the equivocal zone. This variability in the number of specimens yielding equivocal results likely relates to differences in NG prevalence—0.5% in our study versus 13% in the study of Martin et al.—between the two populations that were studied.

In summary, the COBAS AMPLICOR NG test is highly sensitive and specific for the diagnosis of infection with *N. gonorrhoeae*. However, in low-prevalence populations, the PPV of the test is low; this may be secondary to cross-reactivity with certain nonpathogenic strains of *Neisseria* or *Lactobacillus*, which becomes a more important concern as the prevalence of *N. gonorrhoeae* infection declines. There is no alternate OD cutoff or testing algorithm based solely on the use of COBAS AMPLICOR which would improve PPV while maintaining sensitivity; therefore, confirmatory testing with another, more specific assay such as the 16S rRNA PCR assay should be performed on all specimens with an OD of more than 0.2 in populations with a low prevalence of infection. Additionally, even in areas of higher prevalence, specimens yielding an OD reading between 0.2 and 3.5 should be considered for confirmatory testing, given the lower PPV of results within this equivocal zone.

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