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Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by Enzyme Immunoassay, Culture, and Three Nucleic Acid Amplification Tests

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The purpose of this study was to evaluate and compare three commercially available nucleic acid amplification tests (NAATs) for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Roche PCR and Becton Dickinson strand displacement amplification (SDA) were performed on 733 endocervical swab specimens from commercial sex workers. Abbott ligase chain reaction (LCR) was performed on a subset of 396 samples. Endocervical specimens from all women were also tested by culture for *N. gonorrhoeae* and by Syva MicroTrak enzyme immunoassay (EIA) for *C. trachomatis*. A positive *N. gonorrhoeae* result was defined as a positive result by culture or by two NAATs, and a positive *C. trachomatis* result was defined as a positive result by two tests. According to these definitions, the sensitivities and specificities for the subsample of 396 specimens of *N. gonorrhoeae* culture, PCR, SDA, and LCR were 69.8, 95.2, 88.9, and 88.9% and 100, 99.4, 100, and 99.1%, respectively; the sensitivities and specificities of *C. trachomatis* EIA, PCR, SDA, and LCR were 42.0, 98.0, 94.0, and 90.0% and 100, 98.0, 100, and 98.6%, respectively. The performance characteristics of *N. gonorrhoeae* culture, PCR, and SDA and *C. trachomatis* EIA, PCR, and SDA for all 733 specimens were defined without inclusion of LCR results and by discrepant analysis after resolution of discordant *N. gonorrhoeae* PCR results and of discordant *C. trachomatis* EIA and PCR results by LCR testing. The sensitivities of *N. gonorrhoeae* culture, PCR, and SDA before and after LCR resolution were 67.8, 95.7, and 93.9% and 65, 95.8, and 90.0%, respectively. The sensitivities of *C. trachomatis* EIA, PCR, and SDA decreased from 39.4, 100, and 100% to 38.7, 98.7, and 94.7%, respectively. All three NAATs proved to be superior to *N. gonorrhoeae* culture and to *C. trachomatis* EIA. The accuracies of the different NAATs were quite similar. SDA was the only amplification assay with 100% specificity for detection of both *N. gonorrhoeae* and *C. trachomatis* in endocervical specimens.

Chlamydia trachomatis and *Neisseria gonorrhoeae* are two of the most prevalent sexually transmitted pathogens, with high rates of infection among female commercial sex workers in developing countries, a substantial proportion of whom have asymptomatic infections. Traditional laboratory diagnosis of these infections is done by culture for *N. gonorrhoeae* and cell culture or antigen detection for *C. trachomatis*. Recently, nucleic acid amplification tests (NAATs) have become widely used; these tests have shown a greater sensitivity and have improved the ability to detect *C. trachomatis* and *N. gonorrhoeae* infections. Several studies have shown that NAATs are more accurate than the former standard tests for *C. trachomatis* and *N. gonorrhoeae* (2–6, 11–13, 16, 22, 25–28, 30, 33). However, it is known that certain substances in clinical specimens may be associated with amplification inhibition and that NAATs may give false-positive results (7, 9, 16, 20, 21, 29, 31). Currently available commercial *C. trachomatis* and *N. gonorrhoeae* DNA amplification tests include PCR (Roche Molecular Systems, Branchburg, N.J.), the ligase chain reaction (LCR; Abbott Laboratories, Abbott Park, Ill.), and strand displacement amplification (SDA; Becton Dickinson, Sparks, Md.).

A major drawback for comparative studies of different commercialized NAATs for the detection of *N. gonorrhoeae* and *C. trachomatis* in genital swabs is the incompatibility of the various specimen collection and transport systems, each accompanied by particular handling instructions and DNA extraction procedures. To allow a scientifically valid comparison and a correct head-to-head evaluation of different diagnostic assays, all tests should be performed on the same single specimen and the study should include a substantial number of true-positive specimens.

The purpose of this study was to evaluate the performance of SDA, PCR, and LCR in a reference laboratory setting for the detection of *N. gonorrhoeae* and *C. trachomatis* in single endocervical swabs stored and transported in dry tubes. Locally performed *N. gonorrhoeae* culture and *C. trachomatis* enzyme immunoassay (EIA), used for management of patients, were also evaluated.

MATERIALS AND METHODS

Study population and clinical specimens. Between September 1996 and April 2000, a multicenter study on the effectiveness of a vaginal microbicide to prevent human immunodeficiency virus (HIV) infection among female commercial sex workers was conducted in Cotonou, Benin; Durban, South Africa; and Hat Yai, Thailand. After giving written informed consent, women were screened for HIV infection and other sexually transmitted diseases, and HIV-negative women were included in the study and followed up on a monthly basis. Dacron swabs were used to collect endocervical specimens each month for the detection of *N. gonorrhoeae* and *C. trachomatis*. A first swab was used for *N. gonorrhoeae* culture on modified Thayer Martin medium. A second swab was used for *C. trachomatis* EIA antigen detection (MicroTrak; Syva, San Jose, Calif.). A third swab was kept

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TABLE 1. Pattern of *N. gonorrhoeae* test results for 396 endocervical specimens

Result of:				No. of specimens
Culture	PCR	SDA	LCR	
+	+	+	+	38
−	+	+	+	14
+	−	−	−	2
+	+	+	−	2
+	+	−	−	1
+	−	−	+	1
−	+	+	−	2
−	+	−	+	3
−	+	−	−	2[a]
−	−	−	+	3[b]
−	−	−	−	328

[a] False positive by PCR.
[b] False positive by LCR.

dry in a sterile cryovial at 4°C after collection, stored at −20°C within 5 h, and shipped on dry ice to the Institute of Tropical Medicine, Antwerp, Belgium, for *C. trachomatis-N. gonorrhoeae* coamplification PCR testing (Amplicor; Roche Diagnostic Systems, Branchburg, N.J.). After arrival, swabs were stored at −20°C until testing.

Sample preparation and processing. Dry swabs (n = 733) collected consecutively between May 1999 and December 1999 (Cotonou, n = 317; Durban, n = 235; Hat Yai, n = 181) were used for comparison of different NAATs to detect *N. gonorrhoeae* and *C. trachomatis*. The first 396 specimens were tested in parallel by PCR (Amplicor; Roche), SDA (BDProbeTec ET; Becton Dickinson), and LCR (LCx; Abbott). The next 337 specimens were tested by PCR and SDA; *N. gonorrhoeae* culture-negative, PCR-SDA-discordant samples and samples positive by (*C. trachomatis*) EIA, PCR, or SDA only were tested by LCR for *N. gonorrhoeae* or *C. trachomatis*, respectively.

For processing, specimens were thawed and kept at room temperature for 2 h, and 1.2 ml of diluted phosphate-buffered saline (9 parts saline and 1 part phosphate-buffered saline) was added to each vial. Samples were vortexed vigorously at maximum speed for 2 min. After removal of the swabs, four aliquots of 250 μl of sample suspension were pipetted in small conic tubes and used for immediate testing or kept at −20°C for later testing.

For each amplification assay, a 250-μl sample was centrifuged at 12,000 × *g* for 10 min and the pellet was used for DNA extraction, as follows. (i) For PCR, 250 μl of Amplicor lysis buffer was added to the pellet. After vortexing for 30 s and incubation for 15 min at room temperature, the sample was mixed with 250 μl of specimen diluent. After another vortexing, 50 μl of the treated sample was used for the PCR assay following the instructions of the manufacturer. (ii) For SDA, 1 ml of test-specific diluent was added to the pellet. After vortexing for 30 s, the manufacturer's procedure was strictly followed and 100 μl of the treated specimen was used for amplification. (iii) For LCR, 1 ml of test-specific urine resuspension buffer was added to the pellet. After vortexing for 30 s and heating, 100 μl of the treated specimen was used for amplification according to the instructions of the manufacturer. The NAATs were performed blindly by three different technicians. Amplicons were detected according to the different test procedures, and specimens with assay values equal to or greater than the cutoff were considered positive.

Analysis of results. Specimens tested in parallel by PCR, SDA, and LCR were considered true positive for *N. gonorrhoeae* if they were positive by culture or by two NAATs and true positive for *C. trachomatis* if they were positive by any two tests (EIA, PCR, SDA, or LCR).

Specimens tested in parallel by PCR and SDA were considered *N. gonorrhoeae* true positive if they were positive by culture or by both amplification assays and *C. trachomatis* true positive if they were positive by any two tests (EIA, PCR, or SDA). For *N. gonorrhoeae* culture-negative, PCR-SDA-discordant samples and for *C. trachomatis* EIA-, PCR-, or SDA-only-positive samples, *N. gonorrhoeae* LCR or *C. trachomatis* LCR tests were performed, and LCR-positive samples were considered true positive in discrepant analysis.

The sensitivity and specificity of culture, PCR, SDA, and LCR for *N. gonorrhoeae* and of EIA, PCR, SDA, and LCR for *C. trachomatis* were calculated for specimens tested in parallel (n = 396) by all three NAATs. The sensitivity and specificity of *N. gonorrhoeae* culture, PCR, and SDA and of *C. trachomatis* EIA, PCR, and SDA were also calculated for all specimens (n = 733) before and after inclusion of supplemental LCR data obtained for samples with discordant results after parallel testing. Ninety-five percent confidence intervals (CIs) were calculated based on the binomial distribution of the observed test results.

RESULTS

***N. gonorrhoeae*.** All 733 specimens were tested by culture. The first 396 specimens were tested by PCR, SDA, and LCR in parallel, the next 337 specimens were tested by PCR and SDA, and LCR was performed on PCR-positive, SDA-negative, culture-negative samples only. None of the samples was found to be inhibitory when tested for the internal control in PCR or in SDA.

For the first 396 specimens, the pattern of test results is shown in Table 1: 328 specimens were negative by all tests, 63 (15.9%) specimens were *N. gonorrhoeae* positive, and 5 specimens were positive in one test only. Table 2 shows the sensitivities of culture, PCR, SDA, and LCR. The 95% CIs revealed no differences between the three NAATs. Culture was significantly less sensitive than PCR.

For the 337 samples tested by culture, PCR, and SDA, the results of all three tests were identical for 310 (92.0%), while 55 (16.3%) specimens were *N. gonorrhoeae* culture positive or positive by both PCR and SDA. Three culture-negative specimens were PCR positive but SDA negative. Table 2 shows the sensitivities of culture, PCR, and SDA. The 95% CIs revealed no differences between the two NAATs. Culture was significantly less sensitive.

Table 3 shows the combined results of culture, PCR, and SDA for all specimens (n = 733). According to the definitions, 115 samples were *N. gonorrhoeae* positive: 78 (68.2%) cases were detected by culture, and 37 (32.2%) were detected by the combination of PCR and SDA. In addition, 8 samples were PCR positive but negative by culture and by SDA: 5 of these were LCR positive (3 were detected among the first 396 sam-

TABLE 2. Sensitivity and specificity of *N. gonorrhoeae* diagnostic tests

Specimen group (n)	Test	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Tested by 4 techniques (396)	Culture	44/63 (69.8)	57.0–80.8	333/333 (100)	98.9–100
	PCR	60/63 (95.2)	86.7–99.0	331/333 (99.4)	97.8–99.9
	SDA	56/63 (88.9)	78.4–95.4	333/333 (100)	98.9–100
	LCR	56/63 (88.9)	78.4–95.4	330/333 (99.1)	97.4–99.8
Tested by 3 techniques (337)	Culture	34/55 (61.8)	47.7–74.6	282/282 (100)	98.7–100
	PCR	53/55 (96.4)	87.5–99.6	279/282 (98.9)	96.9–99.8
	SDA	52/55 (94.5)	84.9–98.9	282/282 (100)	98.7–100

TABLE 3. Combined results of culture, PCR, and SDA for all 733 endocervical swabs tested for gonococcal infection

Result of:			No. of specimens
Culture	PCR	SDA	
+	+	+	71
+	−	−	5
+	+	−	2
−	+	+	37
−	+	−	8[a]
−	−	−	610

[a] Five were LCR positive, and three were LCR negative

TABLE 5. Pattern of *C. trachomatis* test results for 396 endocervical specimens

Result of:				No. of specimens
ELISA	PCR	SDA	LCR	
+	+	+	+	19
−	+	+	+	23
+	+	+	−	1
+	−	−	+	1
−	+	+	−	4
−	+	−	+	2
−	+	−	−	7[a]
−	−	−	+	5[b]
−	−	−	−	334

[a] False positive by PCR.
[b] False positive by LCR.

ples, and 2 were detected among the 337 specimens, for which LCR was performed on 3 discordant PCR-positive samples only.

By combining all 733 specimens, the performance characteristics of *N. gonorrhoeae* culture, PCR, and SDA can be estimated with exclusion of all LCR data or with inclusion of the LCR results for the 8 PCR-positive discordant samples. Sensitivities and specificities of culture, PCR, and SDA before and after discrepant analysis by PCR are shown in Table 4. Before discrepant analysis the number of *N. gonorrhoeae*-positive samples was 115; sensitivities of culture, PCR, and SDA were 67.8, 95.7, and 93.9%, respectively. After additional testing by LCR of PCR-positive discordant samples, the number of *N. gonorrhoeae*-positive samples increased to 120 and the performance of culture, PCR, and SDA did not change significantly. Culture was significantly less sensitive than either NAAT.

***C. trachomatis*.** All specimens were tested by enzyme-linked immunosorbent assay (ELISA). The first 396 specimens were tested by PCR, SDA, and LCR, and the next 337 specimens were tested by PCR and SDA, with LCR being performed on specimens showing one positive result by either EIA, PCR, or SDA.

For the first 396 specimens, the pattern of test results is shown in Table 5: 50 (12.6%) samples were *C. trachomatis* positive, and there were 7 PCR-only-positive and 5 LCR-only-positive results. Table 6 shows the sensitivities and specificities of ELISA, PCR, SDA, and LCR. ELISA was significantly less sensitive than the NAATs.

For the 337 specimens tested by two NAATs (PCR and SDA), the results of ELISA, PCR, and SDA were identical for 314 (93.2%), while 24 (7.1%) specimens were *C. trachomatis* positive in at least two assays. Table 6 shows the sensitivities of ELISA, PCR, and SDA. The 95% Cls revealed no differences between the two NAATs. ELISA was significantly less sensitive.

The samples positive by one test only were additionally tested by LCR: one ELISA-only-positive sample was positive by LCR, and one out of seven PCR-only-positive samples was positive by LCR.

Table 7 shows the combined results of ELISA, PCR, and SDA for the total of 733 specimens. According to the definitions, 71 samples were *C. trachomatis* positive; all 71 were identified by both PCR and SDA, while ELISA identified 28 positive samples. One sample was positive by ELISA and negative by PCR and SDA but was positive by LCR; 16 samples were PCR positive and negative by ELISA and SDA, and 3 of these were LCR positive.

Because ELISA, PCR, and SDA were performed on all specimens, sensitivities and specificities of the three tests can be estimated with exclusion of all LCR results or with inclusion of the LCR results for ELISA-only-positive and PCR-only-positive samples. The performance characteristics of ELISA, PCR, and SDA before and after discrepant analysis by LCR are shown in Table 8. Before discrepant analysis, the number of *C. trachomatis*-positive samples was 71. After additional testing by LCR of the samples positive by one test only, the total number of *C. trachomatis*-positive samples increased to 75 and the sensitivities of ELISA, PCR, and SDA decreased slightly. The specificity of SDA remained 100%; for ELISA it changed from 99.8 to 100%, and for PCR it changed from 97.6 to 98.0%.

DISCUSSION

The performance and evaluation of NAATs for the detection of *N. gonorrhoeae* and *C. trachomatis* in genital specimens has been the subject of much study and controversy. We com-

TABLE 4. Performance characteristics of *N. gonorrhoeae* culture, PCR, and SDA before and after resolution of discordant results

Test	Discrepant analysis[a]	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Culture	A	78/115 (67.8)	58.5–76.4	618/618 (100)	99.4–100
	B	78/120 (65)	55.8–73.5	613/613 (100)	99.4–100
PCR	A	110/115 (95.7)	90.1–98.6	610/618 (98.7)	97.5–99.4
	B	115/120 (95.8)	90.5–98.6	610/613 (99.5)	98.6–99.9
SDA	A	108/115 (93.9)	87.9–97.5	618/618 (100)	99.4–100
	B	108/120 (90.0)	83.2–94.7	613/613 (100)	99.4–100

[a] A, before additional testing of discordant results; B, after resolution of discordant positive PCR results by LCR testing.

TABLE 6. Sensitivity and specificity of *C. trachomatis* diagnostic tests

Specimen group (*n*)	Test	Sensitivity (%)	95% CI	Specificity (%)	95% CI
Tested by 4 techniques (396)	ELISA	21/50 (42.0)	28.2–56.8	346/346 (100)	98.9–100
	PCR	49/50 (98.0)	89.4–99.9	339/346 (98.0)	95.9–99.2
	SDA	47/50 (94.0)	83.5–98.6	346/346 (100)	98.9–100
	LCR	45/50 (90.0)	78.2–96.7	341/346 (98.6)	96.7–99.5
Tested by 3 techniques (337)	ELISA	8/24 (33.3)	15.6–55.3	312/313 (99.7)	98.2–100
	PCR	24/24 (100)	85.8–100	306/313 (97.8)	95.4–99.1
	SDA	24/24 (100)	85.8–100	313/313 (100)	98.8–100

pared the results of PCR, SDA, and LCR for *N. gonorrhoeae* and *C. trachomatis* as well as those obtained by culture for *N. gonorrhoeae* and by antigen ELISA for *C. trachomatis*.

The specificity of DNA amplification assays can be ensured by retesting initially positive specimens by a different amplification method, as was done in the present study. It is more difficult to evaluate the sensitivity of NAATs (or any other assays) when they appear to be more sensitive than the conventional reference test. Many investigators repeat the test being evaluated on discordant specimens or subject them to additional tests, a strategy known as discrepant analysis. Discrepant analysis aims to identify, by an additional confirmatory assay, true-positive samples, negative by the reference method but positive by the test under evaluation. Since this procedure can improve the apparent sensitivity and specificity of the new test, selective supplemental testing favors the test being evaluated and introduces a data bias (8, 10). The size of the bias will depend on the sensitivity of the reference test and on the prevalence of disease. The lower the sensitivity of the reference test is, the higher the increase in specificity of the new test will be; the lower the prevalence of disease, the higher the increase in sensitivity (8, 15, 18). An alternative approach is to use a combination of tests to establish an expanded "gold standard" for the evaluation of a new diagnostic test (1). The introduction of an additional test to an expanded gold standard implies that it should be performed on all the specimens. This approach, however, is not common practice because it increases substantially the workload and the costs of a study (17, 19, 24). In this study the results were analyzed by applying an expanded gold standard and by discrepant analysis.

Commercial NAATs have their own specimen collection kits and transport media; unfortunately, these are incompatible, creating a difficulty for comparative studies. To overcome this inconvenience and to avoid problems of specimen collection order and bias due to interswab variation, we used single dry endocervical swabs. It has been shown that specimens transported on dry swabs have a higher positivity rate than swabs swirled for 15 s in transport medium and then discarded (14).

TABLE 7. Combined results of ELISA, PCR, and SDA for all 733 endocervical swabs tested for chlamydial infection

Result of:			No. of specimens
ELISA	PCR	SDA	
+	+	+	28
−	+	+	43
+	−	−	1[a]
−	+	−	16[b]
−	−	−	645

[a] Positive by LCR.
[b] Three were LCR positive, and 13 were LCR negative.

Various levels of DNA amplification inhibition with clinical specimens have been observed (20, 29, 31). Routine inclusion of an internal control as provided by PCR and SDA (not by LCR) allows the detection of amplification-inhibiting factors, validating the negative results. No inhibition was observed in our samples.

In vitro culture is still the reference method for the diagnosis of gonorrhea. The low sensitivity of *N. gonorrhoeae* culture (65%) found in our study is consistent with other publications reporting sensitivities ranging between 50 and 84% (2, 5, 6, 13). These data indicate that gonococcal infection in females as defined by culture is significantly underdiagnosed. Reasons for false-negative *N. gonorrhoeae* culture could include prior antimicrobial therapy, loss of viability of the organisms during transport, low concentrations of the organisms, or sampling error.

It has been shown in several recent studies that chlamydia culture, previously considered to be the gold standard, has a sensitivity ranging from 50 to 85% in expert laboratories (4, 12, 23). Because of logistic problems and limited resources, we did not perform *C. trachomatis* culture in this study but used classic *C. trachomatis* antigen detection by ELISA for rapid diagnosis and patient management. After confirmation of initially positive samples by a blocking assay, the specificity of this test was 100%, but the sensitivity was extremely low (38.7%). Most studies performed on endocervical specimens found sensitivities ranging between 45 and 70% (16, 25, 26, 32). In a study performed by Toye et al., 19 cases of *C. trachomatis* were detected by PCR, 8 were detected by culture, and none were detected by ELISA (29). In our study there was a wide variation of the ELISA sensitivity observed in the three participating centers, ranging from 12.8 to 63.6 to 71.4%. This variation most likely reflects a combination of differences in skills between the clinicians collecting the specimens, differences in transport and storage conditions, and variability in laboratory expertise.

In this study the performance of PCR, SDA, and LCR for the detection of *N. gonorrhoeae* and *C. trachomatis* infection was evaluated in 396 endocervical specimens, applying an expanded gold standard. No significant difference was observed between the sensitivities of PCR, SDA, and LCR, which were 95.2, 88.9, and 88.9%, respectively, for *N. gonorrhoeae* and 98, 94, and 90%, respectively, for *C. trachomatis*. For the detection of *N. gonorrhoeae* the specificities of the NAATs were more than 99% and statistically similar. For *C. trachomatis*, the spec-

TABLE 8. Performance characteristics of *C. trachomatis* ELISA, PCR, and SDA with and without supplemental testing of discordant results

Test	Discrepant analysis[a]	Sensitivity (%)	95% CI	Specificity (%)	95% CI
ELISA	A	28/71 (39.4)	28.0–51.7	661/662 (99.8)	99.2–100
	B	29/75 (38.7)	27.6–50.6	658/658 (100)	99.4–100
PCR	A	71/71 (100)	94.9–100	646/662 (97.6)	96.1–98.6
	B	74/75 (98.7)	92.8–100	645/658 (98.0)	96.6–98.9
SDA	A	71/71 (100)	94.9–100	662/662 (100)	99.4–100
	B	71/75 (94.7)	86.9–98.5	658/658 (100)	99.4–100

[a] A, before supplemental testing of discordant results; B, after resolution of discordant ELISA-positive and discordant PCR-positive results by LCR.

ificities of PCR and LCR were 98 and 98.6%, respectively, versus 100% for SDA; this difference was not significant.

For a second series of 337 samples PCR and SDA were performed in parallel, but LCR was done only on samples with discordant results for *N. gonorrhoeae* and *C. trachomatis*. By combining both series of samples, the performance characteristics of PCR and SDA on 733 samples were compared before and after resolution of discrepant results by LCR. For *N. gonorrhoeae* the initial sensitivities of PCR and SDA did not change significantly after resolution of discrepant results by LCR.

For *C. trachomatis* both PCR and SDA were 100% sensitive. After resolution of the PCR-SDA-discrepant results and one ELISA-positive, PCR-negative, SDA-negative result by LCR, the sensitivity of PCR decreased slightly to 98.7% and the sensitivity of SDA decreased significantly to 94.7%. After discrepant analysis the specificity of PCR increased slightly and that of SDA remained unchanged.

The disparities between the sensitivities of the three NAATs used in the present study may partly be explained by slight inoculum differences resulting from splitting of samples containing very low numbers of organisms; the use of multiple swab specimens, however, would probably have resulted in more disparities.

Although our results clearly show that sensitivity and specificity estimates for NAATs may vary slightly or significantly depending on the definition of the gold standard, it seems that all three assays are well suited to screening for genital gonorrhea and chlamydial infection in female endocervical specimens. SDA was the most accurate test in this study, being 100% specific for both *N. gonorrhoeae* and *C. trachomatis*.

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