

Performance of Transcription-Mediated Amplification and Ligase Chain Reaction Assays for Detection of Chlamydial Infection in Urogenital Samples Obtained by Invasive and Noninvasive Methods

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Based on the amplification of chlamydia-specific rRNA sequences and the ligase chain reaction (LCR), the performance characteristics of the Gen-Probe *Chlamydia trachomatis* transcription-mediated amplification (TMA) assay were evaluated with endocervical, urine, and vulval specimens from women and urethral and urine specimens from men and were compared with those for cultures on endocervical, vulval, and urethral swabs. Of the 308 women and 240 men tested, 25 (8.1%) and 44 (18.3%), respectively, were shown to be infected. By using the infected individual as the expanded "gold standard" for calculations, the TMA assay and LCR gave similar performances for the sensitivity of male urethral (93.2%) and urine (88.6 and 86.4%) samples, while culture detected only half of the 44 infected men. In women, the sensitivities of the TMA assay for endocervical and vulval samples were 88 and 92%, respectively, compared to values of 92% for the LCR on both sample types and of 52 and 8%, respectively, for culture. By using first-void urine for chlamydial diagnosis in women, LCR detected 24 (96%) and TMA assay detected 19 (76%) infected individuals, showing a significantly lower sensitivity for urine in women ($P = 0.0253$). The results indicate a high overall agreement for both amplifying techniques for all examined specimen types, except for female urine. Furthermore, they confirm the previous observation that vulval swabs are an effective alternative noninvasive sample type for the detection of *C. trachomatis* infection in women by nucleic acid-based amplification technologies.

Chlamydia trachomatis is one of the most common causes of sexually transmitted diseases (STDs) in the industrialized countries and has serious sequelae if the infection is left undiagnosed and untreated. The annual treatment costs for chlamydial infection and the adverse postdisease complications in patients and their children far outweigh the cost of an effective diagnostic and early intervention program. An effective chlamydial control program must be aimed at reducing the reservoir of infected asymptomatic individuals who are responsible for maintaining transmission of the infection within a community and constitute the target group of screening programs. The challenge for control of chlamydial infection, therefore, continues to be accurate diagnosis especially of asymptomatic individuals, since symptomatic men and women are likely to receive appropriate treatment with the recommended antibiotics.

Detection methodologies which make use of amplification of specific nucleic acid sequences have provided the clinical laboratory with powerful new tools with particular impact in the detection of *C. trachomatis*. Compared with traditional methods of cell culture or enzyme immunoassays, nucleic acid amplification technologies such as PCR or ligase chain reaction (LCR) have been highly sensitive and specific for the detection of *C. trachomatis* in genital specimens of symptomatic and asymptomatic men and women, detecting as many as 30% more infected individuals (4, 12, 15). When first-void urine

(FVU) was tested by both methods, they were also found to be highly effective in identifying chlamydial infections in individuals with or without symptoms of a genital chlamydial infection (1, 2, 10, 14, 16). In contrast to LCR or PCR, which target specific DNA sequences of the chlamydial organism, the Gen-Probe Transcription Mediated Amplification (TMA) system is an alternative nucleic acid-based technology in which specific rRNA sequences are amplified via DNA intermediates in an isothermal reaction. For amplification, the TMA assay uses two primers and two enzymes, i.e., the RNA polymerase and the reverse transcriptase. Reverse transcriptase creates first an RNA-DNA duplex and, second, a double-stranded DNA copy. The RNA polymerase recognizes the promoter sequence in the DNA template, and transcription is started for the synthesis of RNA amplicons. Detection of the amplified rRNA sequences is achieved by chemiluminescence detection of amplicons with an acridium ester-labeled DNA probe in the hybridization detection assay (HPA).

In the present study, the performance of the TMA assay was compared with that of LCR for detection of *C. trachomatis* infection in the genital tract with various invasive and noninvasive specimen types from men and women. Swabs from the endocervical canal and vulval region for women and from the urethra for men were tested by TMA, LCR, and culture. FVU specimens from both men and women were tested by both amplifying methods. In addition to comparing the performances of two molecular biological technologies that amplify different targets of the nucleic acid of *C. trachomatis*, the aim of this study was to evaluate whether vulval swabs and urine are suitable noninvasive samples when tested by an alternative amplifying technology.

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MATERIALS AND METHODS

Study population. Specimens were collected from a selected high-risk group of 308 women and 240 men attending an outpatients' center for STDs in the period from January to July 1996 because of symptoms in the genital tract, promiscuous behavior, suspicion of chlamydial infection of the partner, contact tracing, or treatment control. Persons enrolled in the study underwent a standard examination, including diagnosis for genital chlamydial and gonococcal infections, as well as for other sexually transmitted pathogens. Most of the men were examined because of urethritis (77.5%) or contact tracing (10.4%); 46.4, 24.4, and 9.4% of the women attended the center because of vaginal discharge, pelvic inflammatory disease (PID), and urethral symptoms of the partner, respectively.

Specimen collection and processing. Urethral specimens from men were first collected for culture by a Dacron-tipped swab which was placed into a transport vial containing sucrose-phosphate buffer, 10% fetal bovine serum, and antibiotics (2-SP medium). Two further specimens were collected in random order in respective collection buffers for testing by LCR and TMA. For each woman, the first endocervical swab was collected for culture after removal of the mucus from the cervix, and two further specimens were collected in random order for LCR and TMA. In addition, three separate samples were collected by the physician from the vulval region by swabbing the introital area and were placed into transport tubes with 2-SP medium for cell culture or in plastic tubes containing sample extraction buffer provided by the manufacturers for LCR and TMA. Specimen collection was restricted to two physicians for the purpose of consistency.

The patients were not to have urinated 2 h prior to specimen collection and were instructed to collect 20 to 30 ml of FVU in a sterile screw-cap plastic jar at the clinic after urogenital specimens had been taken. Urine specimens were aliquoted into four portions and immediately frozen at -20°C to store and process two of them under the same conditions for both amplifying methods and to store the third and fourth one for further analysis in case of discrepant results.

Cell culture. The cervical, urethral, and vulval specimens were either processed for cell culture within 24 h or stored at -70°C (20% of specimens) for as much as 1 week. Cell culture was performed on McCoy cell monolayers pretreated with cycloheximide in shell vials after a 1-h centrifugation at $3,000 \times g$. After fixation with 95% ethanol, the cells were stained with fluorescein-labeled monoclonal antibodies to *C. trachomatis* major outer membrane protein (MOMP; Mikrotrak Chlamydia Culture Reagent; Syva, San Jose, Calif.). Specimens were considered positive when one or more inclusions were present. No blind passage was performed.

LCR assay. The performance of the LCR assay (Abbott Park, Ill.) was processed according to the manufacturer's recommendation and has been described previously (10, 15). Genital samples for the LCR assay were transported to the laboratory within 3 h of collection and stored at 4°C before being tested within 1 to 4 days. One portion of FVU was thawed and vortexed, and a 1-ml portion was centrifuged at $11,000 \times g$ in a microcentrifuge for 15 min. The pellet was resuspended in 1 ml of urine resuspension buffer, lysed by heating at 95°C for 15 min, and tested according to the manufacturer's package insert.

TMA assay. The Gen-Probe TMA assay (Gen-Probe, Inc., San Diego, Calif.) procedures outlined in the Amplified CT Assay package insert were followed for each test run. Preparation of urethral, endocervical, and vulval specimens was performed with the Gen-Probe Amplified Swab Specimen Preparation kit. After centrifugation of specimens ($400 \times g$) for 5 min, $40 \mu\text{l}$ of reconstituted specimen preparation reagent was added and the mixture was incubated at 60°C for 10 minutes in a water bath. Afterwards, $20 \mu\text{l}$ of each prepared specimen was added to a polypropylene tube with specimen dilution buffer so as to be ready for the following amplification step.

Urine specimens were prepared with the Gen-Probe Amplified *C. trachomatis* Urine Specimen Preparation kit. Of each urine specimen, 1.5 ml was pipetted into a microfuge tube, warmed up in a 37°C incubator for 10 min, vortexed, and microfuged at $10,000 \times g$ for 5 min. Supernatant was decanted carefully, and specimen dilution buffer was added for further use in amplification.

For this step, $50 \mu\text{l}$ of the prepared specimen was briefly vortexed and added to the reconstituted enzyme reagent and placed in the 95°C dry heat bath for 10 min and then cooled down to a 42°C heat bath for 1 h until the amplification was terminated by the termination reagent. Detection of the amplicons occurred in the HPA with acridinium-ester-labeled DNA probes in the same way as for the Gen-Probe PACE 2 assay. Hybridization results were read with a luminometer (Leader 50; Gen-Probe, Inc.), and specimens producing signals greater than or equal to the cutoff value (50,000 relative light units [RLU]) were considered positive. Signals less than the cutoff value were considered negative. The amplification-negative and -positive controls produced values of $\leq 12,500$ RLU and $\geq 750,000$ RLU, respectively.

Expanded "gold standard" and resolution of discrepant results. Diagnosis of chlamydial infection was based on an expanded gold standard for infected patients, which was defined by a positive result in at least one sampling site by culture or by both amplifying methods, or by either the LCR or the TMA assay as confirmed by direct fluorescent-antibody assay (DFA) or by an alternative target sequence of chlamydial rRNA. For this confirmation testing, specimens were blinded and sent to the manufacturers for repeated testing by TMA with a target rRNA sequence located in a different gene than that used for screening.

TABLE 1. Comparison of results obtained by TMA, LCR, and culture on urogenital specimens of 240 men

Group of men and no. of specimens	TMA result		LCR result		Urethra culture result
	Urethra	Urine	Urethra	Urine	
Infected men ($n = 44$)					
19 ^a	+	+	+	+	+
14 ^b	+	+	+	+	-
3 ^b	+	+	+	-	-
1 ^b	+	+	-	+	-
1 ^b	+	-	+	+	-
3 ^b	+	-	+	-	-
2 ^b	-	+	-	+	-
1 ^b	-	-	+	+	-
Noninfected men ($n = 196$)					
190	-	-	-	-	-
1 ^c	-	-	+	-	-
1 ^c	-	-	-	+	-
2 ^c	+	-	-	-	-
2 ^c	-	+	-	-	-

^a DFA not done.

^b DFA from urethral and/or urine sediment positive.

^c Positive result could not be confirmed.

RESULTS

Identification of men infected with *C. trachomatis*. Of the 240 men tested, 44 (18.3%) were found to be infected based on the expanded gold standard. Most of the infected individuals had symptoms of urethritis (95.5%), such as discharge or dysuria. Table 1 shows the patterns of positive and negative results of the two amplifying methods for urethral and urine samples in men. Of the 44 infected men, the TMA assay and LCR detected 37 and 35 individuals, respectively, in both sample types. Both the TMA assay and LCR detected 41 individuals (93.2%) by testing urethral swabs only and 39 (88.6%) and 38 (86.4%) individuals, respectively, by testing only urine. Concordant positive results for both specimen types were observed for 33 men, positive results for urethral samples only were observed for 42 men, and positive results for urine samples only were observed for 36 men.

Of the 196 noninfected individuals, the TMA assay yielded four isolated highly positive RLU results (two in the urethral and two in the urine samples). These positive test results could not be confirmed by any other test, nor could positive results be repeated by a second run of the TMA assay. Therefore, they were considered falsely positive. LCR yielded one isolated positive result in the urethral sample and one in the urine sample, which could not be confirmed by further analysis. The performance characteristics of both methods are shown in Table 3. In a quantitative evaluation, the values of extinction rates (ER) of the LCR and RLUs of the TMA assay for true- and false-positive and negative results were compared. The ER for LCR results for infected and noninfected men ranged from 754.0 to 2,950.0 and from 8.2 to 29.3, respectively, with averages of 2,901 for positive results and of 12.5 for negative results. Four of the six false-negative LCR results for urine samples had ER which were clearly higher (45.6 to 441.1) than the average of ER of true-negative samples (10.5). The three false-negative urethral samples (14.0) did not differ from the ER of true-negative samples (13.2). The RLUs for TMA assay results of the FVU samples of infected men ranged from 57,228 to 2,529,508, with an average of 1,706,807. Only one of the false-negative TMA assay results with urine samples was in the borderline zone (36,000) and higher than usually observed for true-negative samples (6,920). However, retesting this sample

TABLE 2. Comparison of results obtained by TMA assay, LCR, and culture for urogenital specimens of 308 women

Group of women and no. of specimens	TMA result			LCR result			Culture result	
	Cervix	Vulva	Urine	Cervix	Vulva	Urine	Cervix	Vulva
Infected women (n = 25)								
2 ^a	+	+	+	+	+	+	+	+
9 ^a	+	+	+	+	+	+	+	-
6 ^b	+	+	+	+	+	+	-	-
2 ^b	+	+	-	+	+	+	-	-
2 ^a	+	+	-	+	+	+	+	-
1 ^c	+	+	-	+	+	-	-	-
1 ^b	-	+	+	+	+	+	-	-
1 ^b	-	+	+	-	-	+	-	-
1 ^b	-	-	-	-	-	+	-	-
Noninfected women (n = 283)								
278 ^a	-	-	-	-	-	-	-	-
1 ^d	+	-	-	-	-	-	-	-
1 ^d	-	+	-	-	-	-	-	-
2 ^d	-	-	+	-	-	-	-	-
1 ^d	-	-	-	+	-	-	-	-

^a DFA not done.^b DFA from cervical, vulval, and/or urine sediment positive.^c DFA negative.^d Positive result could not be confirmed.

gave an RLU result which was negative (9,755). The RLUs of all other false-negative TMA assay results with urine and urethra samples were clearly negative.

Identification of women infected with *C. trachomatis*. On the basis of the expanded gold standard, 25 (8.1%) of the 308 women were shown to be infected with *C. trachomatis*, most of whom had clinical symptoms of vaginal discharge (56%) and PID (8%) or who had a chlamydia-positive partner (36%). The patterns of concordant and discrepant results of different sampling sites for both amplifying methods and for culture are shown in Table 2. Of the 25 infected women, 17 (68%) and 22 (88%), respectively, were positive with all specimens by the TMA assay and LCR. By testing only endocervical swabs, 22 and 23 were positive by the TMA assay and by LCR, respectively. A concordance of 100% between the two techniques was observed with vulval swabs, with a detection rate of 92%. With urine samples, LCR detected 24 (96%) infected women, whereas the TMA assay gave positive results in only 19 (76%) of the infected individuals.

Of the 283 noninfected women, 279 and 282 tested negative for all specimens by the TMA assay and by LCR, respectively. The only isolated LCR-positive result of the endocervical specimen was obtained from a woman with a one-shot treatment with azithromycin 1 week before chlamydial examination; this could not be confirmed by any other method or any other

sampling site or by a reexamination 1 week later and, therefore, the result was considered a false-positive one. For the TMA assay, one sample each from the endocervix and vulva and two FVU specimens gave isolated positive results and could not be confirmed by any other method including the alternative TMA and a second run with the same specimen. The performance characteristics of all methods are shown in Table 3.

The quantitative evaluation of the LCR for the false-negative result of the urine sample of the infected woman showed an ER of 207.6, which was higher than that usually observed for negative results for female urine (10.9). In contrast, the RLU rates for the TMA assay of the six false-negative results of urine were clearly in the negative zone. This is in accordance with the observation obtained for false-negative results from men. For the four false-positive results obtained by the TMA assay, one RLU value was borderline (57,000), while the other three results gave isolated high RLU values (>950,000).

DISCUSSION

The application of the Amplified Mycobacterium Tuberculosis Direct Test (MTD) for the detection of *Mycobacterium tuberculosis* in respiratory as well as in nonrespiratory samples demonstrated a high sensitivity and specificity for *M. tuberculosis* compared with conventional culture (9, 19). Recently, the Gen-Probe TMA system was introduced for the diagnosis of chlamydial infections in the genital tracts of men and women. Data for the performance of the Gen-Probe *C. trachomatis* TMA assay with urine as the target sample type for the detection of *C. trachomatis* in infected individuals have already been reported (3, 11, 13). These results indicated that the TMA assay serves as a sensitive and reliable assay for the detection of *C. trachomatis* in urine specimens of men and women.

In contrast to FVU, only preliminary data on the performance of the TMA assay are available for cervical and urethral specimens (3). In the present study, we compared the performance of the TMA assay on different specimen types with that of the LCR assay as an alternative DNA amplification technique which has already shown a high sensitivity and specificity for various invasive and noninvasive specimens from men and women (1, 2, 4, 10, 15). The aim of the study was to evaluate the performance of the TMA assay for invasive and noninvasive sample types and to examine whether the amplification of the rRNA might improve the detection rate of *C. trachomatis*-infected individuals compared to chlamydial diagnosis by DNA amplification.

The present study shows that testing urine by TMA detects more infected individuals than does culture of endocervical or urethral swabs, which is in agreement with previous studies. Both amplification techniques gave similarly high sensitivities and specificities. There was very little variation in chlamydial detection rates for urethral, endocervical, and vulval specimens

TABLE 3. Performance characteristics of TMA assay, LCR, and culture for the detection of *C. trachomatis* in urogenital specimens^a

Specimen type	Sensitivity (%)			Specificity (%)			PPV (%)			NPV (%)		
	TMA	LCR	Culture	TMA	LCR	Culture	TMA	LCR	Culture	TMA	LCR	Culture
Endocervical	88.0	92.0	52.0	99.6	99.6	100.0	95.7	95.8	100.0	99.9	99.3	95.9
Vulvar	92.0	92.0	8.0	99.6	100.0	100.0	95.8	100.0	100.0	99.3	99.3	92.5
Urine (women)	76.0	96.0 ^b	ND	99.3	100.0	ND	90.5	100.0	ND	97.9	99.6	ND
Urethral (men)	93.2	93.2	50.0	99.0	99.5	100.0	95.3	97.6	100.0	98.5	98.5	89.9
Urine (men)	88.6	86.4	ND	99.0	99.5	ND	95.1	97.4	ND	97.5	97.0	ND

^a PPV, positive predictive value; NPV, negative predictive value; ND, not done.^b Significant ($P = 0.0253$). All other differences of sensitivity between TMA and LCR are not significant.

and male urine; the exception was female urine (Table 3), for which the sensitivity of the LCR assay was significantly higher ($P = 0.0253$; Wilcoxon matched-pairs signed ranks test) than that of the TMA assay. A discrepancy in the sensitivities of the TMA assay for male and female urine samples has already been observed in an earlier study (11). Although a high overall sensitivity (92.4%) of the TMA assay with urine samples from 1,000 individuals was reported, the authors observed a lower sensitivity for female than for male urine samples (84.3 versus 100%). Different sensitivity values were also shown in an evaluation of FVU samples tested by the LCR, TMA, and COBAS AMPLICOR methods (5). In contrast to the sensitivity with male urine (92.4%), that with female urine was 77.2%, which was similar to the present data. The lower sensitivity of the TMA assay with female urine in the present study compared to earlier data may be explained by a different calculation in the present evaluation based on the number of truly infected persons, which was assessed by including a second amplification for all samples in addition to culture. In two of the studies already published, a second amplification test was used only for discrepant analysis, but not as an alternative comparison test for all positive and negative results of the new technique to evaluate the true number of infected individuals (11, 13).

Furthermore, the detection rate of infected persons was increased by including three sampling sources in the final analysis for women and two sources for men. No single test detected all infected individuals with a single sampling type for either males or females. Excluding LCR data and sampling sources other than urine, the TMA assay of FVU samples would have detected 19 of 21 instead of 25 infected women, increasing the sensitivity of the TMA with urine from 76.0 to 90.5%. This would have also increased the sensitivity of culture for cervical specimens from 52 to 68.4%. Similarly, comparing the TMA results with male urine only with urethral cell culture and restricting LCR evaluation to discrepant analysis, the number of infected men would have decreased from 44 to 39, with a sensitivity of the TMA assay for male urine increasing from 88.6 to 100% and that for culture increasing from 50.0 to 63.3%, respectively. Since cell culture is no longer considered a reliable reference method for nucleic acid amplification assays, the calculation of new diagnostic assays for *C. trachomatis* has to be based on truly infected persons determined by at least two amplification assays (6).

Compared with vulval smears as an alternative noninvasive sample type, the use of urine shows some advantages and disadvantages. The advantage of comparing urine assays with different methods is that the same urine specimen prevents sample-to-sample variation due to collection procedures. However, the volume and the processing of urine differ in the amplification assays, and these variations in procedure could be related to the different outcome of the urine assays (5). In a pilot run of the TMA, positive results were inhibited by a small amount of male or female urine left in the tube after centrifugation of the sediment. Retesting after consequent removal of all urine increased the number of positive results. Since urine seems to harbor a higher number of factors that inhibit amplification, different storage and transport conditions may have a higher impact on the outcome of urine testing (18). It is important for the transport and storage conditions for urine samples to be similar when different test kits are compared with each other during an ongoing study. Furthermore, there seem to be differences between male and female urine. In the present study, the performance of the TMA assay was remarkably better for male urine than that for female urine. A lower sensitivity for female urine has also been observed for other amplifying tests, which may be due to the presence of a larger number of in-

hibitory substances and a smaller load of organisms (8). Recently, it has been shown that vulval swabs can be used as alternative samples for chlamydial diagnosis by LCR (17). This sample type was also included in the present study and compared with other sampling types. The results show that both amplifying techniques performed with an identical high sensitivity of 92%. Vulval samples even showed the highest sensitivity and specificity by the TMA test. Compared with urine, the processing of vulval swabs for the laboratory is uncomplicated and does not appear to be influenced by inhibition problems or by a small load of elementary bodies. Similarly to our previous observation, culture with vulval swabs cannot be recommended due to the small number of viable organisms, even though a second passage might have increased the sensitivity of culture. A low sensitivity of 32.7% for culture was recently reported for patient-obtained vaginal swabs, while the LCR performed with a sensitivity of 91.8%, which was even higher than that for endocervical specimens (7).

In addition to urine and vulval samples, both amplifying methods performed with high sensitivity for urethral and endocervical samples. Neither genital nor urine samples would have detected all infected persons. By using the urethral sample for chlamydial diagnosis only, both amplifying methods detected 41 infected men (93.2%). By using endocervical samples, the TMA assay detected 22 (88%) and LCR detected 23 (92%) infected women. Four and six men, respectively, and five women and one woman would have remained undetected by testing only urine by the TMA assay and LCR. However, three men and two women gave positive results only for urine samples and would have been remained undetected by collecting only invasive specimens. One of the two women suspected of a possibly sexually acquired reactive arthritis was positive by the LCR on urine sediment and confirmed by DFA. The second woman had a *C. trachomatis*-infected sexual partner and had positive urine results by both the LCR and the TMA assays, which were confirmed by DFA on the urine sediment. The results for these two women may be explained by an isolated urethral infection.

Since chlamydial infection is a sexually transmitted disease, a high assay specificity is needed to exclude false-positive results. In the present study, the numbers of unconfirmed and, therefore, false-positive results were small for both assays. For the LCR, three positive results could not be confirmed. One false-positive result was obtained from a sample collected 1 week after a single-dose treatment with azithromycin due to a culture- and LCR-confirmed chlamydial genital infection and was negative when a second specimen was collected 1 week later. Since positive LCR results may occur shortly after treatment, medical history and antibiotic treatment within the 2 weeks prior to specimen collection may be relevant for the interpretation of positive results (20). One nonconfirmed positive LCR result was obtained from an asymptomatic man with a *C. trachomatis*-positive female partner. However, this person was not included in the group of truly infected individuals according to our definition. For the TMA assay, the number of single false-positive results was higher than that for the LCR. All eight false-positive results were not reproducible in a second run. Although the RNA is labile in the laboratory environment, a carryover contamination for these specimens cannot be excluded.

The quantitative evaluation of the amplification assays shows that highly negative results for urine samples may occur in infected individuals and may be due to a small number of EBs or to inhibition. The values for four of six LCR-negative and two of five TMA-negative infected patients were higher than those usually observed for noninfected patients. Negative

results for urine samples with a high RLU or ER may, therefore, indicate a chlamydial infection and the assay may have to be repeated.

In summary, the data of the present study demonstrate a high overall agreement between the LCR and TMA assays and high performance characteristics for both assays, with the exception of urine samples from women, for which the TMA assay performed with a sensitivity significantly lower than that of the LCR. Testing more than one sample type and using an alternative amplification test for all specimens rather than just for discrepant analysis give a better insight into the performance characteristics of new techniques based on the number of truly infected individuals. Vulval specimens can be recommended for chlamydial detection by the TMA assay and seem to be even more suitable than urine.

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