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Use of an Adaptation of a Commercially Available PCR Assay Aimed at Diagnosis of Chlamydia and Gonorrhea To Detect *Trichomonas vaginalis* in Urogenital Specimens

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***Trichomonas vaginalis* PCR using reagents from a commercially available assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* was evaluated for detection of infection in women and men attending a sexually transmitted disease clinic. Evaluations included three primer sets, endocervical swabs, vaginal swabs and urine, and various storage conditions. The TVK3/TVK7 primer set was optimal in our hands with sensitivities ranging from 69.5 to 96.8%. In all comparisons, *T. vaginalis* PCR performed better than routine diagnostics using microscopy for women and culture for men ($P > 0.05$). The assay performed well for all sample types tested, and vaginal swabs were stable for up to 7 days at ambient temperature. Using samples prepared for, and reagents from, the *C. trachomatis*-*N. gonorrhoeae* PCR assay allowed incorporation of *T. vaginalis* PCR diagnosis into routine clinical testing.**

Sexually transmitted infections (STI) are among the most common infectious diseases worldwide. The World Health Organization estimated that over 340 million cases of curable STI occurred in 1999 alone (39). Among these curable STI, *Chlamydia trachomatis* remains the most common bacterial cause of infection with an estimated 89 million annual cases while *Neisseria gonorrhoeae* is also common with over 60 million cases estimated to occur annually. However, the most common nonviral STI, worldwide, is caused by *Trichomonas vaginalis* with over 174 million estimated cases in 1999. Potential sequelae of these STI in females include pelvic inflammatory disease (19, 22), ectopic pregnancy, tubal factor infertility (6), adverse pregnancy outcomes (3, 31), and potentially increased risk of both transmission and acquisition of human immunodeficiency virus (HIV) (1, 2, 14, 15, 25, 30, 31, 35). Additionally, investigators have shown epidemiologic associations with chlamydia (21, 28) or trichomonas (5, 34, 36, 40) infection and subsequent cervical neoplasia and carcinoma. While some of these studies attempted to control for human papillomavirus (HPV) infection, the relationships are unclear and the potential role that trichomonas plays has yet to be fully evaluated. The use of *T. vaginalis* PCR diagnostics as a research tool in our laboratory has led to the finding that infection with trichomonas is associated with an increased risk for HPV persistence in adolescent women, which may lead to increased risk of cervical cancer (27).

Screening and control programs for chlamydia and gonorrhea have become increasingly feasible due to the commercial availability of nucleic acid amplification tests (NAAT) and consequent acceptability of less invasively collected specimen types (10, 26, 29, 38). Recommendations for STI control programs strongly support screening of men as well as women,

especially those who may be asymptomatic and therefore not seeking treatment. Such programs have been established in many areas of the world and have resulted in substantial declines in the prevalence of these infections (8, 11).

Screening for trichomonas infection has been performed using direct microscopy or culture of the organism in most settings that include *T. vaginalis* as a component of a sexually transmitted disease (STD) control program. However, many programs do not include trichomonas infection screening and indeed, in the United States, this is not a reportable infection tracked by the Centers for Disease Control and Prevention. Recent reports of the impact of trichomonas infections on the risk of HIV transmission and acquisition suggest that more aggressive trichomonas infection screening measures may be warranted. Laga et al. (15) demonstrated a nearly twofold increase in the odds of HIV infection in women infected with *T. vaginalis* in an African cohort. Although this was not significant at $\alpha = 0.05$, the research group subsequently demonstrated a 1.7-fold increase in relative risk for HIV infection for women infected with *T. vaginalis* (14), suggesting that trichomonas infection may increase women's susceptibility to HIV. Using mathematical modeling, 40% of the new HIV infections in women in the United States may be attributable to increased risk associated with trichomonas infection (2). Studies have also suggested an increase in the risk of transmission during trichomonas infection as a result of increased viral shedding (9, 35). In both men with urethritis (23) and women with vaginal infections (35), HIV viral shedding was quickly reduced following appropriate treatment for *T. vaginalis* infection. These data suggest that incorporating trichomonas infection control into chlamydia and gonorrhea control programs may be useful not only for reducing upper reproductive tract infections and adverse outcomes of pregnancy but also as a prevention measure to reduce the spread of HIV (25).

We have adapted a commercially available CT/NG NAAT to allow amplification and detection of trichomonas DNA.

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Using this assay, we demonstrated the utility of a single sample from women or men for screening for all three organisms and compared the results of the *T. vaginalis* PCR assay to direct microscopy or trichomonas culture to establish the performance characteristics of this test.

MATERIALS AND METHODS

Population and samples. Specimens were collected from men and women attending the Bell Flower clinic, a county health department STD clinic in an urban Midwestern setting. All subjects provided consent for samples to be used in evaluation of novel diagnostic assays. Although not all specimens were collected for each study, samples were collected in the following order: female urine, vaginal swab, cervical or urethral swab for gonorrhea culture, cervical or urethral swab for chlamydia culture, and male urine. Vaginal swabs were placed into sterile, dry tubes or into tubes containing *T. vaginalis* culture medium. Swabs for chlamydia culture were collected in chlamydia transport medium (CTM). First-catch urine with volumes ranging from 15 to 90 ml was collected. For experiments involving archived samples, data were available from original clinical wet preparations, but no vaginal swabs were retained. For studies of freshly obtained samples, female urine was not obtained. For the comparison of TVA and TVK primer sets, female vaginal swabs were used for both microscopy and PCR. For comparison of storage conditions of vaginal swabs, cervical swabs in CTM were also used to define infection.

ATCC *T. vaginalis* isolates 30184, 30185, and 30188 were used as positive *T. vaginalis* controls in titration experiments. *C. trachomatis* strain H-UW₄ and a clinical *N. gonorrhoeae* isolate, 9003-0025, were used in the competition assays.

T. vaginalis wet mount and culture. Women attending the STD clinic were evaluated for the presence of *T. vaginalis* by direct microscopy of a wet preparation. Motile, flagellated trichomonads were identified by clinicians following the routine clinical procedure. Men were not routinely screened for trichomonas infection as a part of clinical exams. Culture was performed in the laboratory using Diamond's medium for vaginal swabs and the In-Pouch culture system for male urine sediment. Urine was held at room temperature prior to transport to the laboratory to maintain the viability of *T. vaginalis* for culture. Ten milliliters of urine was centrifuged at $900 \times g$ for 10 min, the supernatant was discarded, and the sediment was inoculated into the In-Pouch system. Cultures were read using a wet preparation slide on days 0, 1, and 3. Laboratory strains for determination of analytical sensitivity were grown in Diamond's medium and harvested by centrifugation at $900 \times g$ for 10 min.

CT/NG PCR. *C. trachomatis* and *N. gonorrhoeae* testing was performed using the Amplicor *C. trachomatis/N. gonorrhoeae* assay (Roche Diagnostic Corporation, Indianapolis, IN). All testing was performed according to the manufacturer's package insert unless otherwise noted and has been described previously (32). Briefly, specimens were lysed and processed for amplification following protocols specific for urine or swab samples in CTM. Vaginal swabs were transported in dry, sterile tubes. One milliliter of molecular-reagent-grade water was added to the swab followed by vigorous vortexing for at least 10 seconds to elute the sample. Swabs were discarded, and from this point forward, samples were processed as if collected in CTM. Processed samples were added to Amplicor Master Mix (MM) reagent containing polymerase, deoxynucleoside triphosphates, an internal control sequence to verify amplification, and uracil-*N*-glycosylase to reduce potential carryover contamination. This MM was not modified and contained only CT/NG primers. Samples were amplified in a Perkin-Elmer 9600 Thermocycler, and amplified product was detected using oligonucleotide probes specific to an internal sequence of the target DNA. The probes were prebound to polystyrene wells, and the biotinylated primers of amplified sequence were detected using a standard 96-well enzyme-linked immunosorbent assay format (Fig. 1). The optical density was measured at 450 nm, and results were interpreted according to the package insert.

T. vaginalis PCR. Amplification of *T. vaginalis* DNA was performed using biotinylated primers specific for this organism added to the MM reagent. Three sets of primers, TVA (24), TVK (13), and β-Tub (17), and their corresponding oligonucleotide probes were evaluated (Table 1) using the published cycling parameters. Although the MM contained CT/NG primers, the *T. vaginalis* primers required different amplification parameters (for TVK PCR: holding at 95°C for 5 min, followed by 35 cycles of 90°C for 1 min, 60°C for 30 s, and 70°C for 2 min, followed by holding at 70°C for 10 min). *T. vaginalis* DNA amplification was always performed independently of *C. trachomatis/N. gonorrhoeae* amplification (Fig. 1). Amplicor detection kits, in a *C. trachomatis*- or *N. gonorrhoeae*-specific 96-well format, are sold separately from the specimen processing and amplification reagents. In order to detect amplicon from 96 amplified samples, one *C.*
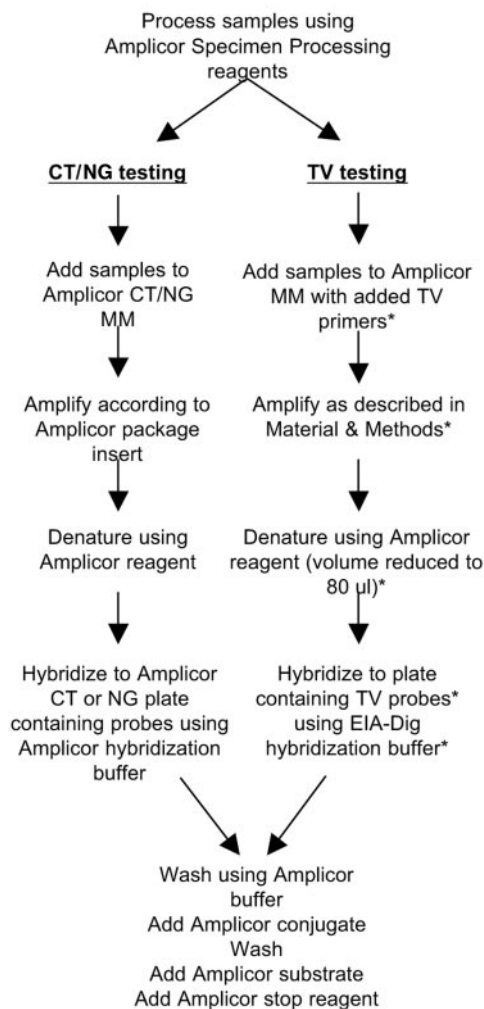

FIG. 1. Procedure for testing 96 samples for *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* using reagents from one set of Amplicor CT/NG reagents. The asterisks indicate *T. vaginalis* PCR deviations from the CT/NG procedure.

trachomatis kit and one *N. gonorrhoeae* detection kit are used. The kits contain identical denaturation, hybridization, avidin-horseradish peroxidase conjugate, substrate, and stop reagents. The only difference between the kits is the 96-well probe-bound plate specific for the *C. trachomatis* or *N. gonorrhoeae* amplicons. The samples amplified for *C. trachomatis/N. gonorrhoeae* are denatured using the reagent from only one of the two kits, leaving the other bottle of reagent available for denaturation of *T. vaginalis* amplification tubes. Each 96-well kit contains sufficient reagent for 144 wells with the least volume in the conjugate reagent. Therefore, using one *C. trachomatis* and one *N. gonorrhoeae* detection kit, 96 samples could be tested for all three pathogens by pooling reagents (Fig. 1). Detection of amplified *T. vaginalis* DNA was performed using the reagents from Amplicor CT/NG kits with the following exceptions. Primer set-specific probes were designed using Oligo (Molecular Biology Insights, Cascade, CO) software and were bound without a capture tag to polystyrene wells (Immulon II; ThermoLabsystems, Franklin, MA) by incubation overnight in freshly prepared 1 M ammonium acetate. Excess probe was removed by rinsing with phosphate-buffered saline with 1 mM EDTA. Plates were blotted, allowed to air dry for 3 h, sealed, and stored at 4°C until use. This method was developed from the probe binding method used for genital ulcer disease multiplex PCR (20). The hybridization buffer included in the CT/NG kit was too stringent to allow binding of the *T. vaginalis* amplified product; therefore, we used a generic PCR-digoxigenin enzyme-linked immunosorbent assay hybridization buffer (catalogue no. 1717472; Roche Applied Science, Indianapolis, IN) instead of the buffer pro-

TABLE 1. Primer and probe sequences evaluated

Primer or probe	TVA5/TVA6	TVK3/TVK7	β-Tub2/β-Tub9
Primer 1	5′ GAT CAT GTT CTA TCT TTT CA	5′ ATT GTC GAA CAT TGG TCT TAC CCT C	5′ GCA TGT TGT GCC GGA CAT AAC CAT
Primer 2	5′ GAT CAC CAC CTT AGT TTA CA	5′ TCT GTG CCG TCT TCA AGT ATG C	5′ CAT TGA TAA CGA AGC TCT TTA CGA T
Probe	5′ AGA CTC AGA GTG TAA ATA ACG	5′ TTC ATG TCC TCT CCA AGC GTA	5′ GCT CAC AAC ACC AAC ATA CGG CGA T

vided with the CT/NG detection kit for detection of *T. vaginalis* amplicons. In response to this change, the denaturation reagent from the CT/NG kit was reduced in volume from 100 to 80 μl/well for denaturation of the *T. vaginalis* amplicons. From this point forward, all reagents were from the CT/NG kit and were used exactly as described in the package insert.

Samples with an A_{450} below 0.300 were interpreted as negative, those above 0.800 were interpreted as positive, and those falling between these values were considered equivocal. Samples with equivocal results were retested in duplicate with at least two/three results over 0.500 required to report the test result as positive. Cutoff points were initially based on recommendations by Roche for the CT/NG assay and the multiplex genital ulcer disease PCR.

Analysis. Patients were classified as infected if the routine diagnostic method (wet preparation or culture) was positive or if two separate PCR results were positive. When multiple samples were available from the same patient, in order to be classified as infected if the routine test was negative, positive PCR results were required from both samples. Similarly, for a comparison of primer sets using the same processed sample, to qualify as an infection when the routine method was negative, both PCR primer results had to be positive. When the routine test was negative and only one PCR result was positive, the PCR was considered a false-positive result. Comparison of multiple assay results was performed using the Cochran Q statistic that tests the hypothesis that there is no difference in dichotomous results between multiple measures on the same individual. McNemar's χ^2 test was used for pairwise comparisons. Pairwise comparisons were performed for the overall results and separately for infected and uninfected participants to evaluate differences in sensitivity and specificity, respectively. Results were considered significant at the $\alpha = 0.05$ level.

RESULTS

Evaluation of *T. vaginalis* PCR using laboratory strains. Laboratory strains of *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis* were loaded onto Dacron swabs to assess both the limit of detection of the *T. vaginalis* PCR assay and the effect of competition for resources in the event of coinfections. To evaluate the sensitivity of the assay, trichomonads, chilled to 4°C to reduce motility, were counted in a wet preparation using a hemacytometer. Since patient samples were eluted in water, all serial dilutions were performed in water to achieve the desired number of organisms. Concentrations tested were 0.54×10^1 to 6.0×10^5 organisms per reaction tube, 25% of which went into the amplification tube. Testing was performed with both the TVA and TVK primer sets. Amplification using the TVA primers was detectable with as few as five trichomonads, and the TVK primer set was positive below the theoretical concentration of one trichomonad per processed sample. These results may overestimate the analytical sensitivity of the *T. vaginalis* PCR since the calculations were based on counted trichomonads and do not account for any lysed organisms from which DNA may have been present in the preparations. The experiment was performed in duplicate with identical results.

The addition of *T. vaginalis* primers to the MM raised the concern that the presence of the *C. trachomatis* and *N. gonorrhoeae* primers, which are included in the commercially avail-

able amplification reagent, might result in a reduction of *T. vaginalis* amplification in the presence of *C. trachomatis* and *N. gonorrhoeae* coinfections. To evaluate this potential effect, swabs were loaded with approximately 35 trichomonads and chlamydial elementary bodies and gonococci were added in concentrations ranging from 0 to 900 inclusion-forming units and 0 to 960 CFU, respectively. Swabs were eluted into 1 ml of water and processed as described for vaginal swabs. After processing, a total of 1.25% of the original sample was loaded into the amplification tube. Samples prepared in this manner were then evaluated using the TVA and TVK primer sets. Both TVA and TVK primers amplified *T. vaginalis* DNA at all concentrations of *C. trachomatis* and *N. gonorrhoeae*. Since the cycling parameters for *T. vaginalis* amplification were different from those used for *C. trachomatis* and *N. gonorrhoeae* amplification, no assays were performed to assess amplification of these organisms' DNA under *T. vaginalis* cycling parameters.

Comparison of TVA and TVK primer sets using vaginal samples. Vaginal swab samples from 174 women were evaluated by *T. vaginalis* PCR using the same processed sample tested by both the TVA and TVK primer sets. The *T. vaginalis* PCR results were compared to clinically obtained wet preparation microscopy results. *T. vaginalis* infection was defined by a positive wet mount or by vaginal swab PCR results positive by both primer sets. Thirty-nine (22.4%) women were classified as infected with *T. vaginalis*. Of these, 18 were positive by both TVA and TVK, only three of which were positive by wet mount. One infection was positive by TVA and not TVK primers, while 13 infections were detected by TVK and not TVA primers. Seven were positive by wet mount alone. The sensitivity of wet mount using this definition of infection was 61.5%, compared to the performance of the TVA and TVK primer sets with sensitivities of 48.7% and 79.5%, respectively (Table 2). Analysis of the agreement of the results, taking multiple comparisons into account, indicated that at least one of the tests performed differently from the others ($P = 0.003$). In pairwise analysis, the sensitivity of the TVK assay was higher than that of the TVA primer set ($P = 0.002$). There was no difference between the specificities of the two primer sets ($P > 0.05$).

Comparison of TVK and β-Tub primer sets using male urine. First-catch urine specimens were collected from 503 men. Urine samples were stored at ambient temperature (AT) prior to transportation to the laboratory to facilitate *T. vaginalis* culture and arrived in the laboratory within 16 h of collection. Immediately following arrival, *T. vaginalis* cultures were prepared from urine sediment and prior to processing for PCR specimens were subsequently stored at 4°C for up to 4 days after collection. All samples were tested for *T. vaginalis*

TABLE 2. Performance of microscopy and two *T. vaginalis* PCR primer sets using vaginal swabs

Type of result (n = 174)	No. of infected women[a] (n = 39; 22.4%[b])	% Sensitivity (95% CI[d])	No. of uninfected women (n = 135)	% Specificity (95% CI)
Microscopy				
+	24	61.5 (46.2, 76.8)	0	100
−	15		135	NA[c]
TVA primers				
+	19	48.7 (33.0, 64.4)	0	100 (100)
−	20		135	
TVK primers				
+	31	79.5 (66.8, 92.2)	3	97.8 (95.3, 100)
−	8		132	

[a] Infection defined by positive wet mount or a sample positive by both PCR assays.
[b] Prevalence of infection.
[c] Not applicable as this test was 100% specific by definition.
[d] CI, confidence interval.

TABLE 3. Performance of culture and two *T. vaginalis* PCR primer sets using male urine

Type of result (n = 503)	No. of infected men[a] (n = 25; 5.0%[b])	% Sensitivity (95% CI[d])	No. of uninfected men (n = 478)	% Specificity (95% CI)
Culture				
+	14	56.0 (36.5, 75.5)	0	100
−	11		478	NA[c]
TVK primers				
+	24	96.0 (88.3, 100)	4	99.2 (98.4, 100)
−	1		474	
β-Tub primers				
+	23	92.0 (81.4, 100)	3	99.4 (98.7, 100)
−	2		475	

[a] Infection defined by positive culture or a urine sample positive by both PCR assays.
[b] Prevalence of infection.
[c] Not applicable as this test was 100% specific by definition.
[d] CI, confidence interval.

using the TVK and β-Tub primer sets, and the TVA primer set was used for discrepant analysis. Infection was defined by a positive culture or two PCR-positive results with independent primer sets. Twenty-five (5.0%) men were classified as infected with *T. vaginalis*. For comparison, in this population 27 (5.4%) were positive for *C. trachomatis* and 26 (5.2%) were infected with *N. gonorrhoeae*. The TVK primer set identified 24 (96.0%) of the infected men, and the β-Tub primer set identified 23 (92.0%) while *T. vaginalis* culture identified only 14 (56.0%) (Table 3). The sensitivities of the three assays were statistically different ($P = 0.001$). In pairwise comparisons, the sensitivity of both PCR assays was statistically different from that of culture (both P values < 0.01). The sensitivity and specificity of the two PCR assays were equivalent (both P values > 0.05). Based on the findings from both male and female samples, all further studies were performed using the TVK primer/probe set as the assay of interest.

Comparison of female specimen types and storage using the TVK primer set. *C. trachomatis* and *N. gonorrhoeae* diagnostic tests are often performed on endocervical or urine samples. Therefore, these specimen types were evaluated for use with *T. vaginalis* PCR. Infection was defined by a positive routine diagnostic test or by positive PCR results from at least two samples (e.g., vaginal and endocervical). In these evaluations, the requirement for duplicate PCR-positive results was intended to control for laboratory error rather than confirm specificity since that had been evaluated using alternate primer sets in the previous experiments.

Samples and wet mount data were collected from 186 women, 41 (22.0%) of whom were classified as infected with *T. vaginalis* (Table 4). Infection was defined by a positive wet mount, culture, or both endocervical and urine PCR results being positive. In this population, *C. trachomatis* was identified in 28 (15.1%) and *N. gonorrhoeae* was found in 17 (9.1%) of the participants. There was no difference in the prevalence of *C. trachomatis* or *N. gonorrhoeae* as a function of the presence of *T. vaginalis* (all P > 0.05; data not shown). Wet mount identified 21 (51.2%) *T. vaginalis* infections while culture detected 25 (61.0%). *T. vaginalis* PCR using vaginal and endo-

cervical swabs identified 37 (90.2%) and 36 (87.8%) of the infected women, respectively. At least one test was significantly different from the others (P < 0.001) with pairwise comparisons indicating that *T. vaginalis* PCR using vaginal or endocervical samples identified more positive samples than did culture ($P = 0.004$ and 0.007, respectively) and wet mount (all P < 0.001). There was no difference in sensitivity or specificity between vaginal and cervical samples for PCR (all P > 0.05).

Residual, archived samples stored at −70°C for approximately 5 years were available from 463 women for comparison of endocervical swabs and urine specimens (Table 4). Infection was defined by a positive wet mount recorded in the clinical chart for that visit or by both endocervical and urine PCR-positive results. Ninety-four (20.3%) women were infected. Endocervical PCR, urine PCR, and wet mount identified 96.8, 94.7, and 51.1% of infected women, respectively. There was a significant difference between wet mount and endocervical or urine PCR (P < 0.001). Sensitivity and specificity of the assay were similar for both endocervical and urine specimens (P > 0.05).

Stability of vaginal swabs. To assess the stability of vaginal swab samples, endocervical swabs in CTM and duplicate vaginal samples were collected. Storage at 4°C was compared to holding samples at AT. Additionally, storage of samples for 3 days was compared to storage for 7 days. Trichomonas infection was defined by a positive wet preparation performed at the clinic or by two separate specimens (e.g., a cervical and a vaginal sample or both vaginal swabs) being positive by *T. vaginalis* PCR. Duplicate vaginal samples were available from 288 women for the evaluation of storage temperature. *T. vaginalis* infection was identified in 67 (23.3%) (Table 4). Storage temperature did not affect the sensitivity or specificity of the assay (all P > 0.05). Duplicate vaginal samples were obtained from 217 women for comparison of storage for 3 or 7 days postcollection (Table 4). Of the 46 *T. vaginalis* cases identified in this cohort, 32 (69.5%) and 35 (76.1%) were positive following specimen storage for 3 or 7 days, respectively. The sensitivity and specificity of the assay were similar for the two storage conditions (all P > 0.05).

TABLE 4. Performance of *T. vaginalis* PCR with various sample types and storage conditions using TVK primers

Comparison	No. of infected women[a]	% Sensitivity (95% CI[b])	No. of uninfected women	% Specificity (95% CI)
Vaginal and cervical swabs (*n* = 186)				
Vaginal				
+	37	90.2 (81.1, 99.3)	2	98.6 (96.7, 100)
−	4		143	
Cervical				
+	36	87.8 (77.8, 97.8)	2	98.6 (96.7, 100)
−	5		143	
n (%)	41 (22.0)		145	
Archived cervical swabs and urine (*n* = 463)				
Cervical				
+	91	96.8 (93.2, 100)	3	99.2 (98.3, 100)
−	3		366	
Urine				
+	89	94.7 (90.2, 99.2)	4	98.9 (97.9, 100)
−	5		365	
n (%)	94 (20.3)		369	
Vaginal swab storage conditions (*n* = 288)				
4°C				
+	56	83.6 (74.7, 92.5)	0	100 (100)
−	11		221	
Ambient temperature				
+	56	83.6 (74.7, 92.5)	3	98.6 (97.1, 100)
−	11		218	
n (%)	67 (23.3)		221	
Vaginal swab holding times (*n* = 217)				
3 days				
+	32	69.5 (56.2, 82.8)	7	95.9 (92.9, 98.9)
−	14		164	
7 days				
+	35	76.1 (63.8, 88.4)	2	98.8 (97.2, 100)
−	11		169	
n (%)	46 (23.3)		171	

[a] Infection defined by positive wet mount or culture or by positive PCR results for both samples.
[b] CI, confidence interval.

DISCUSSION

The ability to integrate this test into routine screening for *C. trachomatis* and *N. gonorrhoeae* using an adaptation of commercially available reagents makes molecular biology-based diagnosis of *T. vaginalis* feasible for many diagnostic laboratories. The use of samples collected by minimally invasive or noninvasive methods that have been stored for several days will allow population-based screening for *T. vaginalis*. In these evaluation studies, the sensitivity and specificity of the *T. vaginalis* PCR using the TVK primer set were high. The assay using the TVK primer set and probe sequence performed better than the TVA primer/probe set and equivalently to the β-Tub assay. These findings were consistent with the primer evaluation performed by Crucitti et al., who found the TVK primer set to be optimal for detection of *T. vaginalis* DNA (4). We chose to use the TVK assay for logistic reasons such as a shorter amplification process while using the β-Tub assay for confirmatory

purposes. The cutoff points used were arbitrarily chosen based on the manufacturer's recommendation. A bimodal distribution of optical density values similar to that described by Kaydos et al. (12) was seen with the results of the TVK assay. Evaluation of over 7,200 samples showed only 1.2% in the equivocal range (data not shown). With repeat testing of equivocals, the proportion of those classified as positive increased from 2.4% to 71.4% as the value of the initial result increased from 0.300 to 0.800, suggesting that the equivocal range is appropriate. It should be noted that, in the absence of a means for determining the true rate of infection with *T. vaginalis*, the performance characteristics described here are only estimates based on the best available techniques. In some analyses (the evaluations of sample types and storage conditions) we used the method under evaluation, requiring two separate samples to be positive, as a means of defining infection with the understanding that this introduces bias by inflat-

ing the apparent sensitivity and specificity of the assay. However, in these comparisons, sensitivity and specificity were similar to the primer set comparisons. In these studies the question of interest was comparability of performance, and this was demonstrated.

The objective of this work was to develop a *T. vaginalis* diagnostic tool that offers improvement over currently used methods such as microscopy and culture. The choice of wet preparation microscopy for women was based on the clinical standard in place during these evaluations. The clinic was not routinely culturing for trichomonas, and men were not being tested at all. Male urine, rather than perhaps more sensitive samples such as urine and urethral swabs combined, was selected as the least invasive addition to the clinical procedures in place. Since cultures were not routinely performed, the In-Pouch package insert recommendation for length of culture was followed. Therefore, the methods for identification of trichomonas infection were either equivalent or superior to the routine clinical practices at the participating STD clinic. Although alternate sample types or variations on comparator testing might have improved the estimates of sensitivity and specificity of the TVK assay, such measures will rarely be available in routine STD control programs and were of less interest than comparison to basic diagnostic procedures.

It was common for *T. vaginalis* to be defined by a positive wet preparation while both PCR samples gave negative results. For example, 17.9% (7/39) of cases shown in Table 3 had positive wet mount and negative PCR results. Women were classified as infected according to the a priori definition, but this may not have been an accurate definition. Wet preparations were read at the clinic site, and the quality of the results was not subject to further analysis, quality review, or proficiency testing. It is possible that wet preparations may have been called positive based on the presence of a species of motile trichomonad that does not contain TVK primer binding sites. If the infection status was more loosely defined as requiring at least two positive results, the number of infections dropped to 31 (17.8%) and the sensitivity of the tests was 54.8, 61.3, and 96.8% for wet mount, TVA PCR, and TVK PCR, respectively. Therefore, the sensitivity of the assay may be underestimated for all comparisons. This approach is justified since we did not attempt to measure inhibition of the PCRs, which might also explain the occurrence of wet-mount-positive/PCR-negative samples. However, even using this potentially conservative approach, the *T. vaginalis* PCR provided improved case finding for both men and women, as has been reported by other investigators (4, 16, 17, 26, 33, 37, 38).

The assay appears to be useful for a variety of sample types in women: endocervical swabs, urine, or vaginal swabs. This was in contrast to the poor performance of female urine as a sample type for *T. vaginalis* PCR described by Lawing et al. (16). We used whole-urine aliquots that had been frozen whereas the Lawing study used urine sediment frozen in water. The difference in storage conditions may explain the difference in urine sensitivity between our results and the previous study (95% and 64%, respectively). Additionally, in studies using fresh urine samples (12, 18, 33) urine performed well as a sample type. Finally, the prevalence of *T. vaginalis* for each comparison cohort was similar, 20.3 to 23.3%, suggesting that the sample types are truly comparable and that the storage

conditions are amenable to sending samples to a reference laboratory. Encouragingly, the prevalence from the repository of cervical and urine samples stored at −70°C for up to 5 years was also within this range, indicating that long-term storage of either of these sample types likely has little effect on the quality of the sample for *T. vaginalis* PCR.

As we demonstrated with mock samples using laboratory strains, in a cohort of women with a high prevalence of *C. trachomatis* (15.1%) and *N. gonorrhoeae* (9.1%), the *T. vaginalis* PCR performed well in the presence of these other STI. This was of particular concern since the Amplicor MM to which *T. vaginalis* primers were added contained *C. trachomatis* and *N. gonorrhoeae* primer pairs as well. Consistent with global data, the prevalence of *T. vaginalis* in these studies in women was substantially higher than that of *C. trachomatis* or *N. gonorrhoeae*. Not surprisingly, the number of cases identified in men was lower than that in women (5.0%), but in this population, the rates of *C. trachomatis* (5.4%) and *N. gonorrhoeae* (5.2%) were also lower. The low STD prevalence rates may be attributable to the handling of the urine in this study. Urine was held at AT until delivery to the laboratory in order to avoid cold shock to any live trichomonads. Approximately two-thirds of urine samples were transported to the lab within 4 h of collection while the remainder were held overnight at the clinic. This delay of up to 16 h between collection and culture with subsequent refrigeration of the urine was possibly responsible for the degradation of both live organisms for culture and DNA for PCR of *T. vaginalis*. However, there was no difference in the frequency of positive results based on overnight storage (data not shown; $P > 0.05$).

Although other methods for amplification detection, such as use of a digoxigenin reporter molecule, may improve the sensitivity of detection of amplified product and be of use in research settings, the assay described here provides an improvement to current routine practice that can be used with very minor modifications to an available diagnostic kit. Kaydos and colleagues (12) saw variability with an assay similar to the one that we have described. This variability prompted them to develop an alternative assay which performed more reliably in their evaluations. Our laboratory has been using the assay described here for more than 6 years with a high degree of probe plate lot-to-lot reproducibility. This may be due in part to the use of the newer version of the Amplicor CT/NG reagents and the substitution of a different hybridization buffer rather than the *C. trachomatis* stand-alone kit used in the study by Kaydos et al. In 66 evaluations of reproducibility performed in our lab, there have only been two (3%) occasions where the reagents did not perform satisfactorily (data not shown). In these two instances the probe-bound plates did not provide adequate binding and the correlation between previous and new lots was less than 0.80. This indicates that the assay reproducibility should be closely monitored, as variations in the plate lot, prior to probe binding, and adherence to the binding procedure can affect assay performance. On all other occasions, the correlations were high, with only three instances of lot testing with correlations below 0.94. This suggests that the assay is stable and reproducible over time.

Several investigators have reported excellent results of screening for *C. trachomatis* and *N. gonorrhoeae* using either patient-obtained or clinician-collected vaginal swabs (10, 16,

29, 38). Many of these studies have also used a NAAT for the detection of *T. vaginalis* to enhance the screening program. Indeed, more recent evaluations of LightCycler assays for detection of *T. vaginalis* suggest improved sensitivity (7). However, the studies published to date rely on DNA isolation procedures, in-house PCR amplification reagents, cumbersome detection methods, or specialized equipment that is restricted to research laboratories. We have described a system which uses commercially available reagents, with the exception of the primers and probe oligonucleotides, which can easily be added to the routine for detection of *C. trachomatis* and *N. gonorrhoeae* in a clinical laboratory. The CT/NG assay can be performed without deviation from the package insert since the *T. vaginalis* primers are not added during the amplification of the *C. trachomatis*/*N. gonorrhoeae* assay. The separate amplification and detection of *T. vaginalis* does not have an effect on the performance of the *C. trachomatis* and *N. gonorrhoeae* testing, making this a suitable assay for laboratories adhering to Clinical Laboratory Improvement Act regulations. The costs associated with this test are minimal since the gross excess reagent provided in the Amplicor CT/NG detection kits can be pooled to allow *T. vaginalis* testing. The major expense is the amplification reagent to which *T. vaginalis* primers are added.

The value of this type of assay for screening programs is improved case ascertainment compared to traditional methods such as microscopy and culture (37). Given the increasing evidence that *T. vaginalis* may play a significant role in the transmission of HIV, both in pandemic regions such as sub-Saharan Africa (1, 14, 15) and in the United States (2, 24), sensitive assays for detection of *T. vaginalis* in a clinical laboratory setting are clearly needed. The assay described here is designed for this type of application. The use of commercially available reagents and samples processed for *C. trachomatis* and *N. gonorrhoeae* testing will allow clinical laboratories to adopt this method and utilize the quality practices of the manufacturers to optimize the quality and reproducibility of the diagnostic results.

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