Improved Diagnosis of *Trichomonas vaginalis* Infection by PCR Using Vaginal Swabs and Urine Specimens Compared to Diagnosis by Wet Mount Microscopy, Culture, and Fluorescent Staining

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Four vaginal cotton swab specimens were obtained from each of 804 women visiting the outpatient sexually transmitted disease clinic of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands, for validation of various forms of *Trichomonas vaginalis* diagnostic procedures. One swab specimen was immediately examined by wet mount microscopy, a second swab was placed in Kupferberg's Trichosel medium for cultivation, and two swabs were placed in phosphate-buffered saline (PBS), pH 7.2. The resulting PBS suspension was used for direct staining with acridine orange and fluorescence microscopy, inoculation of modified Diamond's culture medium, and a PCR specific for *T. vaginalis*. A total of 70 samples positive in one or more of the tests were identified: 31 (3.8%) infections were detected by wet mount microscopy, and 36 (4.4%) were identified by acridine orange staining, as opposed to 40 (4.9%) and 46 (5.7%) positives in modified Diamond's and Trichosel media, respectively. PCR was positive for 61 (7.5%) samples. Secondly, from each of 200 women were obtained a urine sample and a vaginal cotton swab specimen, and 200 urine samples were obtained from men. For the women, 15 (7.4%) of the samples showed a positive result for either the wet mount ($n = 1$), Trichosel culture ($n = 6$), PCR on the vaginal swab sample ($n = 10$), or PCR on the urine specimen ($n = 11$). Four men (2%) were diagnosed with a *T. vaginalis* infection. Thus, PCR appears to be the method of choice for the detection of genital infections with *T. vaginalis*.

Worldwide, *Trichomonas vaginalis* causes approximately 180 million new infections per year, making it the most prevalent nonviral sexually transmitted disease (STD) agent (14, 17, 21). Infections in women can cause vaginitis, urethritis, and cervicitis (23), and complications include premature labor, low-birth-weight offspring, and postabortion or posthysterectomy infection (27). It has been estimated that 10 to 50% of *T. vaginalis* infections in women are asymptomatic (7), and in men the proportion may even be higher. This parasite has also been implicated as a cofactor in the transmission of the human immunodeficiency virus and other nonulcerative STD agents. However, since the incidence of *T. vaginalis* infection is highest for groups with a high prevalence of other STDs, this latter hypothesis remains to be confirmed (17). In addition, a relationship between *T. vaginalis* infection and cervical cancer has recently been suggested (32).

The most common tool for diagnosis of *T. vaginalis* infection is still microscopic examination of wet mount preparations, which has a sensitivity of approximately 60% (10). Microscopic examination of cultures of the parasite in specialized media improves the sensitivity to 85 to 95% (11, 12, 26). However, the quality of these diagnostic tests is strongly dependent on the skills and experience of the microscopist and also on the quality of the sample. Therefore, improvement of the diagnostic armamentarium is urgently required. Diagnostic improvements have been suggested in past years. Apart from Kupferberg's Trichosel culture medium, other media have been described and evaluated. The most sensitive of these media is thought to be modified Diamonds' medium (11, 12). However, optimal culture conditions vary, and efficacy is influenced by incubation times. An obstacle in the use of culture media is that on-the-spot analysis is difficult to achieve, although the combined use of immediate and culture-based testing has been implemented (5, 8, 16, 26). Acridine orange staining is a nonspecific nucleic acid staining procedure (3) which can be applied for fluorescence-based detection of *T. vaginalis*. Finally, molecular techniques such as fluorescent in situ hybridization, oligonucleotide probing, and PCR have been developed (6, 14, 17, 18, 22–24, 27).

This article describes two prospective clinical studies performed to evaluate various diagnostic methods for the detection of *T. vaginalis* in vaginal swab specimens and in urine samples obtained from female and male patients. All patients presenting for a routine STD checkup were included in both of the studies described below. For the first series of experiments, four cotton swab specimens were obtained from the posterior vaginal fornix of patients attending the STD clinic of the Erasmus University Medical Center Rotterdam in the period between November 1997 and June 1998. This resulted in a total of 846 samples from 804 patients. Two swabs were used for routine microscopy and Trichosel culture. Another two swabs were placed in 1.5 ml of sterile phosphate-buffered saline

(PBS), pH 7.2. The resulting suspension was used for alternative diagnostic techniques. The PBS-immersed material was processed within 4 h after sampling. For the second experimental series (February through May 1999), a urine sample and three vaginal swab specimens were obtained from each of 202 women, whereas a urine sample was obtained from each of 203 men visiting the STD clinic. For three of the men, because of a positive PCR result for the first sample, a second urine sample was obtained approximately 2 weeks after the first. Vaginal swab specimens were used for wet mounts, inoculation of Trichosel culture medium, and PCR, respectively. The swab for PCR was directly immersed in lysis buffer (for this buffer's chemical composition, see below). First-voided urine (volume, 10 to 50 ml) was collected in urine containers. Samples were transported to the microbiology laboratory once a day. After the urine volume was recorded, centrifugation was performed at 37°C for 5 min at 2,000 rpm (tabletop centrifuge; Hettich Rotanta, Tuttlingen, Germany). The sediment was washed once with 800 μl of PBS. For DNA isolation, the sediment was directly immersed in lysis buffer and processed as described below.

Different diagnostic tests were performed by independent investigators in order to prevent bias. None of these individuals was aware of the results obtained with the other techniques. Throughout the different series of experiments, as per the diagnostic routine at the STD clinic, only the results for wet mounts and Trichosel culture were communicated to the physician in charge. During the first series of diagnostic assays, two separate swabs were used for conventional microbiological procedures. One swab was used to produce a wet mount for direct microscopic examination. A second swab specimen was immediately placed in 10 ml of Kupfenberg's Trichosel medium (Becton Dickinson Microbiology Systems, Cockeysville, Md.), incubated at 37°C for 72 h, and examined by wet mount microscopy. Microscopy was performed at a magnification of 400×, and 20 fields were examined. For both series of experiments, wet mount microscopy and Trichosel culture were used as the "gold standard" for studies of the vaginal swab specimens (2). For men, no control assays were performed. Two additional swabs were placed in 1.5 ml of sterile PBS. After transport to the laboratory, the swabs were squeezed against the side of the tube and removed after being vortexed. From this suspension, 25 μl was removed for the preparation of a wet mount. For the culture in modified Diamond's medium (12), 0.5 ml of the suspension was inoculated into 10 ml of medium and incubated at 37°C for 72 h. The cultures were examined by wet mount microscopy. For acridine orange staining, a wet mount was prepared as described above and fixed with methanol. The fixed material was covered with acridine orange (5 mg/ml in water) and left at room temperature for 2 min (22). After being rinsed with distilled water, the slide was examined under a fluorescence microscope (Olympus model BX60 equipped with a 470-to-490-nm filter) at a magnification of 400× (25 microscopic fields).

During the initial phases of the study, 0.5 ml of the PBS suspension was added to 1 ml of guanidinium lysis buffer (4 M guanidinium isothiocyanate, 0.1 M Tris-HCl [pH 6.4], 0.2 M EDTA, 0.1% Triton X-100). After being mixed, the lysate was kept at −20°C prior to processing (4). First, the samples were left at room temperature for 1 h, after which 50 μl of a Celite suspension was added. The samples were kept at room temperature and mixed at regular intervals for 10 min. After vortexing and centrifugation (20 s at 14,000 rpm in an Eppendorf centrifuge), the supernatant was discarded and the pellet was washed twice with a second guanidinium lysis buffer (4 M guanidinium isothiocyanate, 0.1 M Tris-HCl [pH 6.4], twice

with ethanol (70%), and finally once with acetone. The pellet was vacuum dried and emulsified in 100 μl of 10 mM Tris-HCl, pH 8.0. The sample was heated to 56°C for 10 min and centrifuged. The resulting supernatant was used as a template for PCR. During the urine trial, the sediment obtained by centrifugation was dissolved in 1 ml of lysis buffer and further processed as described above.

Initially a single PCR test employing a *T. vaginalis*-specific primer set as described previously (TVK3-TVK7) was used (14). All PCRs were performed in a total volume of 100 μl. The PCR mix contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl$_2$, 0.01% gelatin, 0.1% Triton X-100, a 0.2 mM concentration of each of the deoxyribonucleoside triphosphates, and 0.2 U of Super *Taq* polymerase (HT Biotechnology, Cambridge, United Kingdom). To this master mixture was added 10 μl of template DNA, and the mix was covered with 2 drops of mineral oil. The PCRs were performed in a Thermocycler 60 apparatus (BioMed, Theres, Germany). The PCR consisted of 40 cycles of denaturation at 94°C (1 min), annealing at 60°C (1 min), and extension at 72°C (2 min). A precycling denaturation at 94°C for 4 min was applied. Ten microliters of each of the PCR products was run in a 1% agarose gel containing ethidium bromide. The electrophoresis was performed in 0.5× TBE (50 mM Tris, 50 mM borate, 1 mM EDTA) at a constant current of 100 mA. The gels were examined and photographed under UV illumination. No additional blotting or hybridization procedures were required. During the first series of vaginal sampling, all samples found to be negative in all of the classical procedures but positive in the PCR were reanalyzed by a second *T. vaginalis*-specific PCR. For this purpose, primer set TVA5-TVA6 was used (23). The PCRs were performed in a total volume of 100 μl containing 10 μl of template DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl$_2$, and 2 U of AmpliTaq Gold polymerase (Perkin-Elmer). The samples were each overlaid with 2 drops of mineral oil. These PCRs were also performed in a BioMed Thermocycler 60 apparatus. The PCR consisted of 40 cycles of denaturation at 94°C (1 min), annealing at 47°C (1 min), and extension at 72°C (1 min). A 10-min precycling denaturation period at 94°C was implemented. PCR samples were analyzed as described above. For the urine samples, the confirmatory PCR was not used.

We analyzed a large number of clinical samples by culture, staining, and DNA amplification. The results of the diagnostic assays performed on vaginal swab specimens are presented in the survey shown in Table 1. Of 846 samples tested, 70 (8.3%) were positive by at least one of the techniques used. None of the techniques could detect all positive samples. Only 22 samples (2.6%) were positive by all methods used. In our routine diagnostic procedure (wet mount preparations in combination with Trichosel broth cultures), a total of 48 samples were positive. With Trichosel medium, 17 infections that were not revealed by the wet mount were detected, but also two wet mount positives did not show growth in Trichosel. Only 32 of the Trichosel-positive samples grew in modified Diamond's medium, leaving 14 that were not detected by the latter technique. On the other hand, nine infections which were missed with Trichosel were detected when modified Diamond's medium was used. Direct staining of saline suspension samples with acridine orange led to detection of 36 infections. With culture in modified Diamond's medium, nine extra positives were found. The total number of 46 infections found by examination and culture of the samples in saline did not differ from that of the wet mount-Trichosel combination ($P = 0.72$). PCR of the saline suspension samples led to a significant improvement in the detection of infections, but with PCR only 61

TABLE 1. Survey of the outcome of the *T. vaginalis* diagnostics for 846 samples obtained from 804 consecutive women visiting the STD clinic of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands[a]

Wet mount microscopy	Trichosel culture	Saline	Modified Diamond culture		PCR	No. of positive samples with pattern
		Acridine orange	Wet mount microscopy	Acridine orange fluorescence microscopy		
+[a]	+	+	+	+	+	22
−	−	−	−	−	+	12
−	+	−	−	−	+	8
−	+	+	+	+	+	5
−	−	−	−	+	−	4
−	−	+	+	+	+	3
+	+	+	−	−	+	3
+	+	−	+	+	+	2
−	+	−	+	+	+	2
+	+	−	−	−	+	2
−	−	+	−	−	−	2
−	+	+	+	+	−	1
+	−	−	+	−	+	1
+	−	−	−	−	+	1
−	−	−	+	+	−	1
−	+	−	−	−	−	1
31[b]	46	36	37	40	61	70

[a] +, positive result in the test concerned; −, negative result. In general, and as can be deduced from the totals in the column on the right, samples were positive in more than a single test, indicating the reliability of the test systems applied.

[b] The values in the bottom row are cumulative numbers of positive samples per test.

TABLE 2. Diagnosis of trichomoniasis in women, using urine as opposed to vaginal swab samples as clinical specimens[a]

Wet mount microscopy	Trichosel culture	PCR of:		No. of samples with pattern
		Swab sample	Urine sample	
+	+	+	+	1
−	+	+	+	5
−	−	−	+	5
−	−	+	−	4
1	6	10	11	15

[a] Paired swab and urine samples were obtained from 202 women consecutively attending the STD clinic of the Erasmus University Medical Center Rotterdam, Rotterdam, The Netherlands. The values in the bottom row are cumulative numbers of positive samples per test. +, positivity in the test concerned; −, negative result.

(87%) of the cumulative set of 70 positive samples were detected, 12 by PCR alone. Nine of these samples (75%) could be confirmed for positivity by PCR with TVA5-TVA6; hence, three samples could be considered potential false positives. However, a certain proportion of false positives is certainly acceptable if the sensitivity improves significantly. It has to be emphasized that routine implementation of the PCR test should follow the procedures outlined in Materials and Methods in order for the results to be comparable to those obtained by the strategy described here. Our present data are in line with the conclusions of other PCR-based diagnostic studies of high prevalence groups such as army personnel or STD patients (17, 22), although the prevalence of infection was lower in our study than in the Pennsylvania analysis (20.3%). Both studies document samples that are PCR positive only and a small number of samples that are PCR negative despite a positive score in one of the other tests.

Attempts to diagnose trichomonosis from urine specimens have a longstanding history. In 1980 it was shown that small numbers of parasites could be detected in first-voided urine by using routine technology (31). Culture of urine samples revealed an association between *T. vaginalis* infection and pyuria (25) and identified the number of sexual partners and lack of condom use as significant risk factors for the acquisition of a parasitic infection (1). On the other hand, cultures of urethral swab specimens and discharge were more often positive than those of urine (9, 13, 15). Improvement of the diagnostic strategy was indicated, and prior to our PCR diagnostic trial employing urine, in vitro tests were performed to determine the sensitivity of the amplification. Using a parasite dilution series

in urine obtained from healthy volunteers, it was demonstrated that the sensitivity was on the order of two parasites per PCR. It is interesting that a dilution series for noninfected vaginal swab samples suggested a sensitivity of approximately 50 parasites per PCR (results not shown). This implies that urine may be a more appropriate matrix for *T. vaginalis* detection than vaginal swabs. Of 202 samples derived from female patients, 15 (7.4%) were positive by at least one of the techniques used (see Table 2 for a survey). Using direct wet mount microscopy and culture, six samples were positive, all of which were confirmed by PCR of purified DNA from the swab as well as from urine. An additional nine samples were positive in either the swab PCR (*n* = 5) or the urine PCR (*n* = 4). It is interesting that these PCRs were not mutually confirmatory. It was recently demonstrated that tampons provide a better template for *T. vaginalis* detection than does urine (29), especially when combined with PCR detection (20, 28). Still, these parasites can be detected both in urine and on the vaginal epithelium. Similar discrepancies concerning sampling sites were observed for PCR detection of another sexually transmitted agent, *Chlamydia trachomatis* (19).

As for male patients, 4 of 203 tested positive (2%). Examination of additional urine samples from three of the four men confirmed the initial finding; the PCR was positive again, despite adequate metronidazole therapy. Future studies using genetic typing of the parasites (30) should elucidate whether cases like these represent examples of reinfection, persistent infection, inadequate therapy, detection of dead parasites, or resistance of the parasite to the antimicrobial agent used.

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