

CLINICAL STUDY

Real-time PCR improves detection of *Trichomonas vaginalis* infection compared with culture using self-collected vaginal swabs

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Abstract

Objective. To compare a real-time polymerase chain reaction (PCR) assay with broth culture for the detection of *Trichomonas vaginalis* using self-collected vaginal swabs.

Methods. Self-collected vaginal swabs were obtained from adolescent and young adult African-American women participating in HIV-1 prevention programs. *T. vaginalis* culture was performed using the InPouch TV System. Samples for the real-time PCR assay were collected using the BDProbeTec ET Culturette Direct Dry Swab system and tested in a laboratory-developed assay which targeted a repeated sequence of the genome. Discrepant samples that were culture negative and positive in the real-time PCR assay were tested in a confirmatory PCR which targeted a different region of the *T. vaginalis* genome, the 18S ribosomal DNA gene.

Results. Of the 524 specimens tested by both culture and real-time PCR, 36 were culture positive and 54 were positive in the real-time PCR assay; 16 of the 18 discrepant specimens were also positive in the confirmatory PCR assay. Using a modified gold standard of positive by culture or positive in both PCR assays, the sensitivity of the real-time PCR assay was 100% and the specificity was 99.6%, whereas culture had a sensitivity of 69.2% and a specificity of 100%.

Conclusions. The real-time PCR assay was sensitive and specific for the detection of *T. vaginalis* DNA from self-collected vaginal swab specimens. The ability to use the BDProbeTec dry swab system for the real-time PCR testing allowed for the detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *T. vaginalis* from a single specimen.

Keywords: *Trichomoniasis*, DNA, adolescent women

Introduction

Trichomonas vaginalis is one of the most common sexually transmitted diseases, with an estimated 5 million cases per year in the United States [1] and as many as 200 million cases annually worldwide. In women, common symptoms of *T. vaginalis* infection include vaginal discharge and vulval irritation. Complications of *T. vaginalis* infection can occur in untreated women and include endometritis, infertility and cervical erosion [2,3]. In pregnant women, *T. vaginalis* infections can lead to severe complications including premature rupture of membranes [4], preterm deliveries and low-birth-weight infants [4].

In men, *T. vaginalis* infection is a cause of non-gonococcal urethritis, [5] and if untreated may lead to complications including chronic prostatitis, urethral strictures, epididymitis and/or infertility [2,3]. *T. vaginalis* infection is also associated with an increased risk of acquiring human immunodeficiency virus type 1 (HIV-1) infection [6–9], which is related to local inflammation within the genital tract that occurs with *T. vaginalis* infections. It has been estimated that between 10% and 50% of *T. vaginalis* infections are asymptomatic [10], supporting the need for more intensive screening for *T. vaginalis* infections in sexually active men and women as well as pregnant women.

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Methods used for the diagnosis of *T. vaginalis* infection include microscopic examination of wet-mount preparations, culture and, more recently, polymerase chain reaction (PCR). Wet mount examination of genital discharge material is rapid and inexpensive, but the sensitivity is only 40% to 70% [11]. This poor sensitivity may be due in part to the rapid loss of the characteristic protozoan motility once the organism has been removed from an environment at 37°C. Currently, broth culture is considered to be the gold standard for detecting *T. vaginalis* [12]. Successful growth in culture can be achieved with as few as 300 to 500 TV organisms per milliliter of vaginal fluid, but requires 2 to 7 days of incubation and daily microscopic examination for optimal performance. However, the sensitivity of culture is also low, ranging from 50% to 80%. In addition, culture requires a specialized medium such as Diamond's broth or Tricosal medium. These limitations of wet-mount examination and culture has led to interest in using molecular assays for the detection of *T. vaginalis* DNA from clinical specimens. PCR assays have been developed that are specific and show an improved sensitivity compared with both culture and wet-mount examination [13–17].

The majority of sexually transmitted diseases occur in adolescents and, although screening for sexually transmitted disease has been shown to be cost effective, compliance with testing, particularly among teenagers, remains an important challenge [18]. Self-collected vaginal swabs offer a method of testing adolescent women that could improve compliance with testing, and a recent study has shown that this approach can be easily implemented in a high-school setting, with high acceptability [13]. Thus, self-collection of vaginal swabs improves the diagnosis and treatment of sexually transmitted diseases in adolescents. In this study, we compared the sensitivity of a real-time PCR assay with culture for the diagnosis of *T. vaginalis* infections using self-collected vaginal swabs. Specimens were collected using the BDProbeTec ET Culturette Direct Dry Swab system, allowing for simultaneous testing for *Chlamydia trachomatis*, *Neisseria gonorrhoeae* and *T. vaginalis* from a single specimen.

Subjects and methods

Patients and samples

Samples were collected from African-American adolescent and young adult females enrolled in one of two HIV-1 prevention programs. In the first study, females aged 15 to 21 years were recruited from a county clinic for sexually transmitted diseases. The second study included women between the ages of

18 to 29 years, who were enrolled at Kaiser Permanente in Atlanta, Georgia, USA. As part of the study, the participants provided self-collected vaginal swab samples to be tested for *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis*. Both studies were approved by the Emory University Institutional Review Board (#327–99, #534–2002). Participants collected two vaginal swabs, one for *T. vaginalis* culture and the other for *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* nucleic acid testing. The results of the *C. trachomatis* and *N. gonorrhoeae* testing were not included in this study.

For *T. vaginalis* culture, the specimens were collected using Dacron tip swabs. They were inoculated into the InPouch TV System (BioMed Diagnostics, San Jose, CA, USA) onsite, and were transported to the clinical laboratory where they were immediately incubated at 37°C. For *T. vaginalis* PCR testing, self-collected vaginal swabs were obtained using the BDProbeTec ET Culturette Direct Dry Swab system (Becton Dickinson, Sparks, MD, USA) and transported to the laboratory on icepacks. They were refrigerated until processed, which was carried out within 6 days of collection.

Trichomonas vaginalis culture

T. vaginalis cultures were performed in the Clinical Microbiology Laboratory at Emory University Hospital following standard procedures. The specimens were incubated at 37°C and examined at 72 hours for the presence of *T. vaginalis* trophozoites.

T. vaginalis real-time PCR assay

The vaginal swabs collected in the BDProbeTec ET Culturette Direct Dry Swab system were processed as outlined by the manufacturer for cervical swabs. Briefly, the swabs were placed in a vial of BDProbeTec ET diluent (2 ml); the swab was swirled in the diluent for 5 to 10 seconds and then removed. The tubes were then incubated at 112 °C to 116°C in a dry bath for 30 minutes. An aliquot of the sample was used for *T. vaginalis* PCR testing. The target for the real-time PCR assay was a 67-base pair region of a repeated sequence of the *T. vaginalis* genome [19] (Gene Bank Accession Number L23861). The primers and probes were designed using Primer Express (Applied Biosystems, Foster City, CA, USA). The PCR reaction contained 25 µl of 2X TaqMan® Universal Master Mix (Applied Biosystems, manufactured by Roche, Branchburg, NJ, USA), 5 µl each (final concentration 900 nM) of primer TV forward (5' CAT TGA CCA CAC GGA CAA AAA G 3') and primer TV reverse (5' CGA AGT GCT CGA ATG CGA 3'), 5 µl (final concentration 225 nM) of probe (5'FAM - TCA

TTT CGG ATG GTC AAG CAG CCA - TAMRA 3') and 5 μ l of sample DNA. The samples were amplified in an ABI Prism 7000 Sequence Detector (Applied Biosystems, Foster City, CA, USA) using the following thermal cycling conditions: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, and at 60°C for 1 minute. Each run included a positive and negative control. A positive *T. vaginalis* result was defined as a cycle threshold (Ct) less than 40; samples with a Ct of > 38 and < 40 were repeated to confirm the result. All samples with a Ct > 38 and < 40 on the initial run were confirmed on repeat testing.

Culturing *T. vaginalis* in the InPouch TV System and then counting the organisms using a hemocytometer determined the limit of detection of the assay. The *T. vaginalis* organism was obtained from BioMed Diagnostics (San Jose, CA, USA). The limit of detection was < 0.2 organisms per reaction. The specificity of the assay was assessed by testing a variety of organisms commonly isolated from the female genital tract, including *Escherichia coli*, *Candida albicans*, *Enterococcus faecalis*, *Gardnerella vaginalis*, *Staphylococcus aureus* and *Lactobacillus acidophilus*, and genital pathogens including cytomegalovirus, herpes simplex virus, *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. In addition, cultures of normal vaginal flora grown on sheep blood agar obtained from the Clinical Microbiology Laboratory were also tested in the assay. All of these specimens tested negative for *T. vaginalis* DNA in the real-time PCR assay.

Confirmatory T. vaginalis PCR assay

Discrepant specimens that were culture negative and positive by the real-time PCR assay were also tested in a confirmatory PCR assay that targets a different region of the *T. vaginalis* genome. For this testing an aliquot of the specimen, which had been stored at -70°C, was sent to a second laboratory for confirmatory PCR testing. The confirmatory PCR assay amplified a 312-base pair region of the 18S ribosomal DNA gene [20]. One μ l of the extracted sample was added to a 50 μ l volume of master mix comprising 1X Qiagen Hotstar buffer (Qiagen Inc, Valencia, CA, USA), 0.4 μ M of each primer (TV1: 5' TAA TGG CAG AAT CTT TGG AG 3', TV2: GAA CTT TAA CCG AAG GAC TTC 3'), 200 μ M dNTPs (with UTP), 1.25 U Qiagen Hotstar Taq and 0.5 U AmpErase (Applied Biosystems, Foster City, CA, USA). The reaction was amplified in a Gene Amp 9600 (Applied Biosystems, Foster City, CA, USA) using the following parameters: 10 minutes at room temperature, 15 minutes at 95°C, then 40 cycles of 30 seconds at 95°C, 1 minute at 57°C and 30 seconds at 72°C. The product was

visualized on a 3% agarose gel (Nusieve GTG). Positive and negative controls were included in each run. A visible band in the gel at the appropriate molecular weight was considered a positive result.

Statistical analysis

Differences in Ct values between the culture-positive/real-time PCR-positive specimens and the culture-negative/real-time PCR-positive/confirmatory PCR positive specimens were analyzed using Student's *t*-test.

Results

The real-time PCR assay had a limit of detection of < 0.2 organism per reaction, which was equivalent to approximately 40 copies/ml of specimen. A representative standard curve is shown in Figure 1. The assay was specific for *T. vaginalis*; there was no cross-reactivity with other genital pathogens or organisms found in normal vaginal flora (see Methods for a list of the organisms tested).

A total of 524 pairs of specimens were tested by culture and real-time PCR: 36 specimens were positive for *T. vaginalis* by culture and 54 were positive for *T. vaginalis* DNA by PCR (Table I). Using culture as the gold standard, the real-time PCR assay had a sensitivity of 100% and a specificity of 96.3%. The 18 specimens that were positive by real-time PCR and culture negative, as well as 36 specimens that were negative by real-time PCR and culture, were tested in a confirmatory PCR assay, which targeted a different region of the *T. vaginalis* genome. All 36 of the specimens negative by culture and the real-time PCR assay were also negative by the confirmatory PCR assay; 16 of the 18 specimens

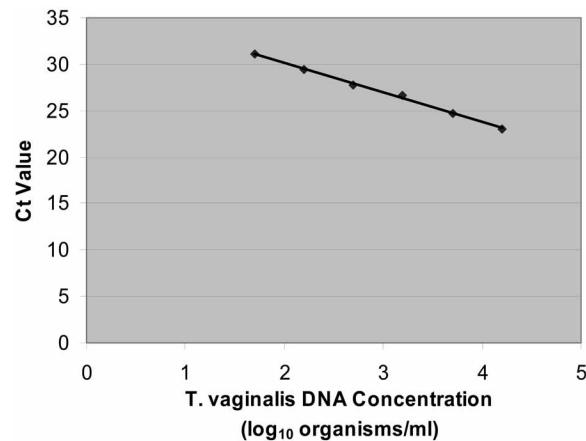


Figure 1. A representative standard curve for the real-time *Trichomonas vaginalis* PCR assay. The \log_{10} concentration of *T. vaginalis* DNA (organisms/ml of specimen) is plotted against the Ct value.

Table I. Comparison of culture and real-time PCR for the detection of *T. vaginalis*.

| Real-time PCR | Culture | |
|---------------|----------|----------|
| | Positive | Negative |
| Positive | 36 | 18 |
| Negative | 0 | 470 |

PCR, polymerase chain reaction; real-time PCR sensitivity = 100%, specificity = 96.3%.

Table II. Comparison of culture and real-time PCR for the detection of *T. vaginalis*.

| Real-time PCR | Culture/confirmatory PCR | |
|---------------|--------------------------|----------|
| | Positive | Negative |
| Positive | 52 | 2 |
| Negative | 0 | 470 |

PCR, polymerase chain reaction; real-time PCR sensitivity = 100%, specificity = 99.6%.

that were culture negative, and positive in the real-time PCR assay, were positive in the confirmatory PCR assay (Table II). These 16 specimens were considered true positive results, whereas the 2 specimens that were not positive in the confirmatory PCR assay were considered false positive results. The Ct values on the real-time PCR assay for the false-positive specimens were 35.1 and 39.4. Using a modified gold standard of positive by culture or positive in both PCR assays, the sensitivity of the real-time PCR assay was 100% and the specificity was 99.6%, whereas culture had a sensitivity of 69.2% and a specificity of 100%. Using the modified gold standard, the prevalence of *T. vaginalis* infection in our population was 10% (52 of 524) and the positive predictive value of the real-time PCR assay was 96.3% with a negative predictive value of 100%.

To assess the organism burden in the various samples, the Ct values were compared between the 36 culture-positive/real-time PCR-positive specimens, the 16 culture-negative/real-time PCR-positive/confirmatory PCR-positive specimens, and the 2 culture-negative/real-time PCR-positive/confirmatory PCR-negative specimens (Figure 2). The range in Ct values for the 36 specimens that were culture positive/real-time PCR positive was 18.3 to 39.1, with a median value of 25.6 and a mean of 26.2. For the 16 specimens that were culture negative/real-time and confirmatory PCR positive, the range in Ct values was 16.1 to 39.8, with a median of 35.1 and a mean of 33.7. The difference ($\Delta 7.5$) was statistically significant ($p < 0.0001$), and on average the concentration of *T. vaginalis* DNA in

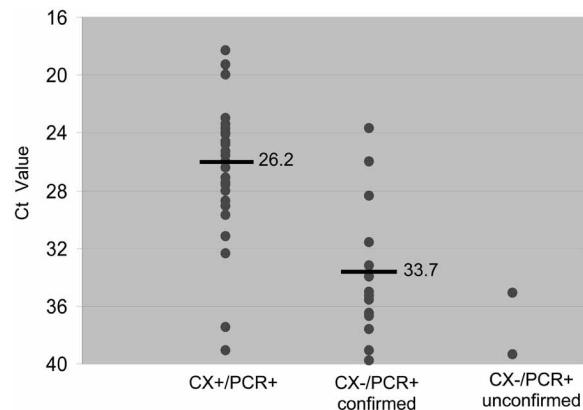


Figure 2. Ct values for culture-positive, real-time PCR-positive (Cx^+ /PCR+), culture-negative, real-time and confirmatory PCR-positive (Cx^- /PCR+ confirmed) and culture-negative, real-time PCR-positive, confirmatory PCR-negative (Cx^- /PCR+ unconfirmed) samples. The line represents the mean Ct value.

the culture-positive/real-time PCR-positive specimens was $> 2 \log_{10}$ higher than that observed in the culture-negative/real-time and confirmatory PCR-positive specimens.

Discussion

We developed a real-time PCR assay for the detection of *T. vaginalis* that was both sensitive and specific. This study confirms the results of others [14–17,21] in showing that PCR testing was more sensitive than culture for the detection of *T. vaginalis*; in this study, the real-time PCR assay detected about 40% more positive samples than culture. This increase in sensitivity was obtained with very little loss of specificity, i.e., 99.6% for the PCR assay compared with 100% for culture. Although the real-time PCR assay was not designed to quantify the amount of *T. vaginalis* DNA in the specimen, the Ct-value was inversely related to the concentration of DNA in the specimen: samples with a low organism burden have a higher Ct value. The comparison of Ct values between the culture-positive, real-time PCR-positive specimens and the culture-negative, real-time and confirmatory PCR-positive specimens, showed that the real-time PCR assay was positive in a greater number of specimens with a low organism burden than was culture. This improvement in detecting infection in samples with a low organism burden should be useful when testing asymptomatic individuals, who might be expected to have a lower burden of organism than those with symptoms. The 2 samples that were not positive in the confirmatory PCR assay had a low amount of *T. vaginalis* DNA as evidenced by the high Ct values (39.4 and 35.1). It is possible that these were false-positive results due to contamination, although the

no-template control was negative in all of the runs. This discrepancy may have also been due to differences in sensitivity between the two PCR assays because of differences in the size of the amplicons, or the number of copies of the target present per genome. Alternatively, there may have been degradation of DNA during storage, before testing in the confirmatory PCR assay.

The BDProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* tests are FDA cleared for detection of these pathogens in urethral, endocervical and urine specimens and are widely used in clinical laboratories. In this study we successfully used the BDProbeTec ET Culturette Direct Dry Swab system for *T. vaginalis* PCR testing. This is important because it allows for the collection of one specimen that is adequate for *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* testing, so that implementation *T. vaginalis* screening becomes convenient for both the clinician and the laboratory. We have also successfully used the NATTM-Nucleic Acid Transport Female Collection Kit (Medical Packaging, Camarillo, CA, USA) System collection devices for *T. vaginalis* PCR testing (data not shown). Other collection devices will need to be assessed to assure that they are compatible with PCR assays and do not contain inhibitors of amplification.

One of the limitations of the real-time PCR assay is that it does not include an internal control to monitor for inhibition. However, an aliquot of the dry swab specimen was also tested for *C. trachomatis* and *N. gonorrhoeae* using the BDProbeTec ET assay, and inhibition of amplification was not observed in any these specimens (data not shown). However, since rates of inhibition vary for different amplification methods, we cannot rule out the possibility of false-negative results with the real-time PCR assay. Another limitation of this study is that not all of the samples were tested in both of the PCR assays. However, the 36 specimens that were negative by culture and real-time PCR were also negative in the confirmatory PCR assay. So it is unlikely that a significant number of additional positive specimens would have been detected if all 470 culture-negative, real-time PCR negative specimens were tested in the confirmatory PCR assay.

Thus, a real-time PCR assay has been designed using self-collected vaginal swabs, that is more sensitive than culture for the detection of *T. vaginalis*, has a high specificity (> 99%), and offers the convenience of using the BDProbeTec ET Culturette Direct Dry Swab system for specimen collection. This real-time PCR assay as designed provides a simplified approach to improving the detection of *T. vaginalis* infection, improving compliance in high-risk groups. Such an improvement,

according to what has been observed for *C. trachomatis* infection, would be predicted to reduce the incidence of *T. vaginalis* infection, possibly to reduce HIV-1 transmission and even to reduce poor pregnancy outcomes.

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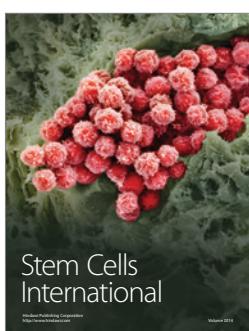
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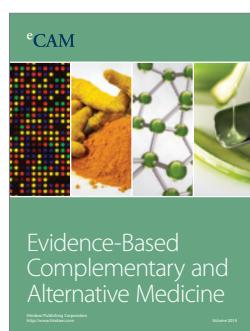
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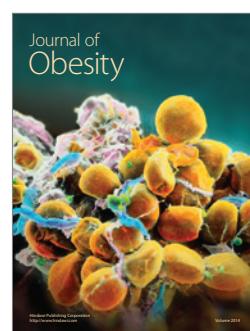
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