Use of the Roche LightCycler Instrument in a Real-Time PCR for *Trichomonas vaginalis* in Urine Samples from Females and Males

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*Trichomonas vaginalis* is the agent of a highly prevalent sexually transmitted infection (STI) that can result in vaginitis, urethritis, and preterm birth. Traditional methods of diagnosis, including wet preparation, can be unreliable. In this study, we describe the adaptation of an existing PCR method for specific detection of *T. vaginalis* DNA into a rapid real-time PCR assay based on fluorescence resonance energy transfer (FRET) probe chemistry. The FRET-based assay described demonstrated high sensitivity with a detection limit of 1.06 organisms, as well as a high specificity. A total of 253 urine samples collected prospectively from both men and women were tested for *T. vaginalis* DNA with both the FRET-based assay and a previously validated PCR assay. When the validated PCR assay was used as the “gold standard” and after discrepancies had been resolved, our FRET-based assay demonstrated an analytical sensitivity and specificity of 90.1 and 100%, respectively. Overall results suggest that FRET-based assays can provide rapid, accurate, and high-throughput detection of *T. vaginalis* and may prove useful in clinical settings and for large-scale screening programs.

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**MATERIALS AND METHODS**

*T. vaginalis* strains for positive controls and standards. The three strains used as positive controls and standards in the real-time PCR assays were collected randomly from women who visited the Druid Hill Health Center in Baltimore, Md., and tested positive for *T. vaginalis* by the Inpouch *T. vaginalis* culture system (Biomed Diagnostics, San Jose, Calif.) with all identifiers removed from patient samples. One milliliter of the Inpouch culture was mixed with 9 ml of Fuji Trichomonas medium (Remel, Lenexa, Kans.) and incubated for 24 h at 37°C. The culture was then centrifuged (800 × g) for 10 min, and 9 ml of supernatant was removed from the culture. Of the remaining 1 ml of the concentrated culture, a 25-μl aliquot was diluted with Evans blue, and *T. vaginalis* organisms were counted in a hemocytometer to determine the organism concentration (number per milliliter). To stabilize the *T. vaginalis* DNA, an additional 50-μl aliquot was subjected to DNA extraction by the Chelex method (16) and was frozen before serial dilution and use as a PCR standard.

Clinical specimens and DNA extractions. Urine samples (*n* = 253) were randomly collected prospectively as part of an ongoing clinical study for *C. trachomatis, N. gonorrhoeae,* and *T. vaginalis* testing. The Johns Hopkins University Institutional Review Board and the Baltimore City Health Department approved this study. The sample group was of young (<25-year-old), sexually active high school students and was presumably a high-risk group for *T. vaginalis* infection. No other demographic information was available, as all patient iden-
tifiers were unlinked from these clinical samples prior to analysis by the different PCR methods. Both male and female urine samples were collected. These samples were subjected to an automated DNA extraction process with a Magna Pure LC instrument (Roche Diagnostics, Indianapolis, Ind.). Reagents from the Magna Pure LC DNA isolation kit (Roche) were used, and DNA extraction was carried out according to the instructions supplied for the Magna Pure LC program. Positive control DNA and PCR standards were thawed, serially diluted, and extracted by using the Magna Pure LC in an identical manner. Negative controls consisting of sterile molecular-grade water or carrier DNA were also extracted in the same manner.

**Design of PCR primers and probes.** The real-time PCR assay design was based on FRET probe chemistry. A previously published T. vaginalis-specific primer set, BTUB9 (5’-CGATGTGTTGCGCGGACATAACTGAT3’) and BTUB2 (5’-GCGAACGCTTCACTGACAT3’), recognizing a 112-bp target within the β-tubulin gene of the T. vaginalis organism was utilized as the basis for development of primers for the BTUB FRET PCR assay (11). The BTUB9/2 primer set used in touchdown enzyme time release (TETR) PCR had a sensitivity of 97% and a specificity of 98% for female vaginal samples (11). The TETR PCR and primer set have been utilized in additional studies involving male urine and female vaginal samples (18, 19).

A modified primer set (BTUB9/B) was utilized in the FRET-based real-time PCR assay. Primer BTUB2 was altered to primer BTUB B (5’-CGCATGTGTCGCGGACACA3’) in order to prevent stem-loop formation between primers BTUB B and BTUB2. The primer set BTUB B and BTUB2 was used in conjunction with two newly designed FRET probes, BTUB FL (5’-CCGATCTCAAGCCTCAACACCAAC-FL3’) and BTUB LC (5’-LCRed640-CGGCGATCTAAACCACCTTGTCTC’). All primers and FRET probes were designed and manufactured by TIB Molbiol LLC (Adelphia, N.J.).

**PCR conditions.** TETR PCR was performed as previously described with PCR products analyzed by gel electrophoresis and was initially considered the “gold standard” (11).

The BTUB FRET PCR was performed with the LC FastStart master hybridization probe kit (Roche) in a LightCycler (Roche). Amplifications reactions were carried out in a total volume of 20 μl at 3 mM MgCl2 (1.6 μM per reaction), 20 pm (each) primers (BTUB9 and BTUB B at 1 μM of each primer per reaction), 20 pm (each) FRET probes (BTUB FL and BTUB LC at 1 μM of each probe per reaction), 2 μl of template DNA, and 1× LC FastStart DNA master hybridization probe buffer (2 μl per reaction). The cycling conditions used were as follows: 95°C for 10 min, followed by 50 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 5 s. A final cooling step of 3 min at 40°C was included for handling of the samples, because the LightCycler has no cooling block like many commercially available thermocyclers. Data collection was performed during the annealing phase of each amplification cycle, and the fluorescence signal was monitored through the F2 channel. Data were analyzed with LightCycler software by the Fit Point method to minimize noise and obtain the best correlation coefficient possible between standards. Positives were defined as samples having a C<sub>T</sub> of less than 40 cycles and significant fluorescent gain when compared to the standard of lowest T. vaginalis concentration.

**Reproducibility studies.** In order to determine the reproducibility of the assay, intra-assay and interassay precision were measured. Six concentrations of T. vaginalis DNA were extracted with the Roche Magna Pure LC robot, and two researchers performed five replicates of the BTUB FRET assay per concentration on different days. Variability is shown as the standard deviation (SD). Statistical and regression analyses were carried out with STATA version 7.0 (Stata Corp., College Station, Tex.) and Sigma Plot software (SPSS Inc., Chicago, Ill.).

**Specificity testing.** DNA from the following organisms were evaluated with the BTUB FRET assay to determine T. vaginalis specificity: Trichomonas tenax (ATCC 30207), Trichomonas foetus (ATCC 30003), C. trachomatis (ATCC VR1477), and N. gonorrhoeae (ATCC FA1090).

**Discrepancy analysis.** Samples that tested positive by the TETR PCR and were initially considered true positives. For the purposes of resolving discrepancies, samples which tested positive by only one of the two PCRs were adjudicated by PCR testing with a third primer set, TVK3 and TVK4, utilizing gel electrophoresis for end-point analysis (9). Samples that were positive by two of the three PCRs were then considered true positives for recalculation of resolved sensitivity and specificity.

**RESULTS**

The detection limit of the BTUB FRET PCR assay was determined by amplifying serial dilutions of T. vaginalis genomic DNA corresponding to 40.8 to 0.255 T. vaginalis organisms per PCR. Runs that contained 40.8, 4.08, 1.02, and 0.255 T. vaginalis organisms per reaction replicated 100% (6 of 6), 100% (6 of 6), 62.5% (10 of 16) and 33% (2 of 6) of the time, respectively. Calculated concentrations of 47.1 ± 7.5 (mean ± SD), 3.3 ± 0.97, 1.2 ± 0.34, and 0.3 ± 0.07 T. vaginalis organisms per PCR were obtained for the standards of 40.8, 4.08, 1.02 and 0.255 T. vaginalis organisms per PCR, respectively. Mean Ct values were 28.4 ± 1.3, 33.5 ± 1.9, 35.2 ± 3.2, and 35.4 ± 2.9 for the 40.8, 4.08, 1.02, and 0.255 standards, respectively.

Two researchers tested five replicates of standards with various concentrations of T. vaginalis organisms. Results for both technicians are shown in Table 1.

To gauge reproducibility, a multiple linear regression was performed to create standard curves corresponding to the two runs. C<sub>T</sub> was regressed on log concentrations and other covariates that allowed the intercepts and slopes of the standard curves corresponding to the two runs to differ. Analysis showed the fitted lines to be statistically different [F(2,50) = 5; P = 0.01]. However, the differences were quite small; for example, the y-intercepts of the regression lines were found to differ by 0.1 cycle, a practically insignificant discrepancy.

The specificity of the primers and probes of the BTUB FRET assay was assessed with genomic DNA extracted from T. tenax, T. foetus, C. trachomatis, and N. gonorrhoeae. No signal was detected for any of these organisms (data not shown).

A total of 253 urine samples were tested with the BTUB FRET PCR over 13 separate runs, all of which had positive (serially diluted standards) and negative controls. The negative control accounted for background fluorescence of the assay, thereby reducing false positives.

Initially, using TETR PCR as the gold standard, there were 15 samples that tested positive by both TETR PCR and BTUB FRET PCR. Five samples were positive by TETR PCR and negative by the BTUB FRET PCR. Four samples were positive by the BTUB FRET PCR and negative by TETR PCR. There were also 229 samples that tested negative by both methods. Thus, the initial sensitivity of the BTUB FRET PCR was found to be 75.0% (15 of 20) (95% confidence interval [CI], 50.9 to 91.3%) and the specificity was 98.3% (229 of 233) (95% CI, 95.7 to 99.5%). The initial positive predictive value (PPV) and negative predictive value (NPV) were calculated to be 78.9% (15 of 19) (95% CI, 54.4 to 94.0%) and 97.8% (229 of 234) (95% CI, 95.1 to 99.3%), respectively (Table 2). After discrepancy resolution of discordant samples with primer set TVK3 and TVK4, all four samples that were positive by BTUB FRET and negative by TETR PCR were confirmed to be true positives. Of the five samples that were positive by TETR PCR and negative by BTUB FRET PCR, three were confirmed to be true negatives, while two confirmed as true positives. Thus, the resolved sensitivity of the BTUB FRET PCR was 90.5% (19 of 21) (95% CI, 69.6 to 98.8%); the resolved specificity was 100% (232 of 232) (95% CI, 98.4 to 100%). The resolved PPV and NPV were 100% (19 of 19) (95% CI, 82.4 to 100%) and 99.1% (232 of 234) (95% CI, 96.9, 99.9%), respectively (Table 2). Under the same new standard, TETR PCR was found to be 81% sensitive (17 of 21) (95% CI, 58.1 to 94.6%) and 98.7% (232 of 235) (95% CI, 96.3 to 99.7%) specific with a PPV of...
The BTUB FRET PCR system represents an improvement over other reported PCR systems tested with urine. With true positives defined as samples having two detectable amplifications from three sets of PCR primers, our study demonstrated the BTUB FRET PCR system to be comparable, in terms of sensitivity and specificity, to TETR PCR, which used a similar set of primers and traditional gel-based methods for amplification detection (11). A recent study utilizing TETR PCR for analysis of male urine specimens found that the sensitivity of TETR PCR was superior to that of culture, but confirmation by another PCR occurred in a limited number of cases, indicating that TETR PCR could be improved upon (19).

Some reports suggest that using discrepancy analysis to resolve sensitivity and specificity incorporates positive bias into these calculations (12, 13). However, another report suggests that the bias incorporated into these calculations is minimal, particularly when the culture specificity approaches 100% (4). Due to the labor and time involved, the BTUB FRET PCR assay was not compared to culture, which is a potential limitation of that study. Both amplified tests utilized as dual gold standards in this study have specificities approaching 100% and have been previously compared to T. vaginalis culture (9, 11).

The BTUB FRET PCR system shows marked improvement in assay time over other methods, including TETR PCR. Given that amplification and detection occur concomitantly, real-time PCR assays require less time, and labor, than methods in which post-PCR processing is necessary. However, even among real-time systems, the BTUB FRET PCR is markedly faster. For example, Jordan et al. reported a real-time PCR assay tested with vaginal samples (6). At 40 cycles, their TaqMan PE Thermocycler-based system took about 2.5 h to complete. By using LightCycler technology, runs of the BTUB FRET PCR assay were completed in under 30 min at 50 cycles. Additionally, assay time is also improved by utilizing the Roche Magna Pure LC robot for DNA extraction. The robot is capable of extracting 32 samples, including standards and negative controls, in 90 min, yielding a total assay time of approximately 2 h, a vast improvement over traditional methods.

Even with the extremely short cycle time, the LightCycler-based system was found to be robust. Reproducibility experiments involving the separate work of two different researchers showed that although derived standard curves were statistically different, the discrepancies were trivially small. Moreover, with standard numbers of replicates, the detection limit of the system was found to be consistently between one and four T. vaginalis organisms per PCR, which is comparable to the limit of another reported real-time method for T. vaginalis detection (6). Although not directly comparable, the sensitivity and specificity of the BTUB FRET PCR in urine approach the sensitivity (97.8%) and specificity (97.4%) of the other real-time assay utilizing vaginal swabs for sample collection (6). Although the sensitivity of the BTUB FRET PCR was lower (90.4%), the specificity was higher (100%).

Ultimately, a quick, accurate assay for urine samples is needed to make high-throughput screening for T. vaginalis possible. Culture may be the gold standard, but it is inherently limited because it relies on the organism to be viable for proper detection. Additionally, culture results can be subject to interpretation by the viewer, whereas PCR offers a more definitive result. Traditional gel-based PCR assays have been found to be less accurate with urine samples than with vaginal swabs (10). Employment of an enzyme-linked immunosorbent assay as the post-PCR amplification detection method has been reported to improve the sensitivity and specificity in urine to 90.8 and 93.4%, respectively, and the sensitivity and specificity of the

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TABLE 1. Reproducibility of the BTUB FRET PCR assay

<table>
<thead>
<tr>
<th>Technician</th>
<th>Standard (known concn)</th>
<th>Mean calculated concn (SD)</th>
<th>Mean C&lt;sub&gt;t&lt;/sub&gt; (SD)&lt;sup&gt;a&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>1</td>
<td>1 (84)</td>
<td>88.1 (21.4)</td>
<td>30.2 (0.40)</td>
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<tr>
<td></td>
<td>2 (42)</td>
<td>38.8 (5.65)</td>
<td>31.5 (0.23)</td>
</tr>
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<td></td>
<td>3 (21)</td>
<td>19.4 (3.81)</td>
<td>32.6 (0.35)</td>
</tr>
<tr>
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<td>4 (10.5)</td>
<td>12.2 (1.50)</td>
<td>33.3 (0.21)</td>
</tr>
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<td></td>
<td>5 (5.25)</td>
<td>6.50 (3.30)</td>
<td>34.5 (0.78)</td>
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<td></td>
<td>6 (1.06)</td>
<td>0.84 (0.03)</td>
<td>37.6 (0.05)</td>
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<td>2</td>
<td>1 (84)</td>
<td>91.1 (21.6)</td>
<td>31.0 (0.45)</td>
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<tr>
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<td>2 (42)</td>
<td>45.6 (10.5)</td>
<td>32.4 (0.44)</td>
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<td></td>
<td>3 (21)</td>
<td>22.3 (3.80)</td>
<td>33.5 (0.33)</td>
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<td></td>
<td>4 (10.5)</td>
<td>10.6 (2.47)</td>
<td>34.7 (0.40)</td>
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<td>5 (5.25)</td>
<td>4.48 (0.95)</td>
<td>36.4 (0.39)</td>
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<td></td>
<td>6 (1.06)</td>
<td>1.62 (1.10)</td>
<td>38.4 (1.30)</td>
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<tr>
<td>Pooled means</td>
<td>1 (84)</td>
<td>89.6 (21.5)</td>
<td>30.6 (0.43)</td>
</tr>
<tr>
<td></td>
<td>2 (42)</td>
<td>40.7 (8.10)</td>
<td>31.9 (0.33)</td>
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<tr>
<td></td>
<td>3 (21)</td>
<td>20.8 (3.80)</td>
<td>33.1 (0.34)</td>
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<tr>
<td></td>
<td>4 (10.5)</td>
<td>11.4 (1.90)</td>
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<tr>
<td></td>
<td>6 (1.06)</td>
<td>1.23 (0.60)</td>
<td>38.0 (0.70)</td>
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</table>

<sup>a</sup> All values are means based on five replicates analyzed for each technician and 10 replicates analyzed for the pooled means.

<sup>b</sup> T. vaginalis organisms per PCR generated by the LightCycler utilizing Ct and the known concentration of the standards.

<sup>c</sup> Ct was generated based on the cycle number where the LightCycler software interpreted a logarithmic increase in fluorescence of the given sample.

85% (17 of 20) (95% CI, 62.1 to 96.8%) and an NPV of 98.3% (229 of 233) (95% CI, 95.7 to 99.5%).

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TABLE 2. Initial and resolved sensitivity and specificity of the BTUB FRET assay

<table>
<thead>
<tr>
<th>Data type</th>
<th>PCR</th>
<th>Sensitivity (%)</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
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<tr>
<td>Initial&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TETR</td>
<td>100 (20/20)</td>
<td>100 (233/233)</td>
<td>100 (20/20)</td>
<td>100 (233/233)</td>
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<td></td>
<td>BTUB FRET</td>
<td>75.0 (15/20)</td>
<td>98.3 (229/233)</td>
<td>78.9 (15/19)</td>
<td>97.8 (229/234)</td>
</tr>
<tr>
<td>Revolved&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TETR</td>
<td>81 (17/21)</td>
<td>98.7 (232/235)</td>
<td>85.0 (17/20)</td>
<td>98.3 (232/236)</td>
</tr>
<tr>
<td></td>
<td>BTUB FRET</td>
<td>90.4 (19/21)</td>
<td>100 (232/232)</td>
<td>100 (19/19)</td>
<td>99.1 (232/234)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Sensitivity and specificity of BTUB FRET compared to TETR PCR.

<sup>b</sup> Sensitivity and specificity where infection status is determined by two positive PCR results, BTUB FRET and TVK3/4 PCR (14).
BTUB FRET PCR are comparable to those obtained by enzyme-linked immunosorbent assay (8). The BTUB FRET PCR offers improved accuracy over traditional PCR assays in urine samples. It has the potential for accuracy comparable to those assays for vaginal swabs, and these features are coupled with decreased assay time due to the elimination of post-PCR processing. Although future studies comparing the accuracy of the BTUB FRET PCR system to the true gold standard, i.e., culture, are required, the BTUB FRET PCR system has the potential to be an accurate, high-throughput means of testing for T. vaginalis infection.

REFERENCES


