A double-labeled fluorescent probe was designed and evaluated for detecting *Trichomonas vaginalis* DNA in a 5'-nuclease (TaqMan) assay. The *T. vaginalis*-specific probe contains a 5'-fluorescein (5'-FAM) and a 3'-rhodamine (TAMRA) derivative. Female genital secretions were collected on Amplicor (Roche Molecular, Indianapolis, Ind.) swabs and by a transport system used for *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* DNA detection by PCR. Five hundred fifty-two female genital specimens, of which 248 (45%) were *T. vaginalis* specimens and 304 (55%) were introital, were tested for both *T. vaginalis* DNA and viable microorganisms using the 5'-nuclease assay and broth culture, respectively. Of these, 304 of 552 (55%) were also evaluated by direct microscopic examination for the characteristic motile organism. After resolving discrepancies, the comparisons produced an analytical sensitivity and specificity for the TaqMan-based PCR assay of 97.8 and 97.4%, respectively. As a result, ΔRQ values (differences in fluorescence due to probe hybridization and resulting 5'-FAM cleavage from the specific PCR product) of ≥2.0 and ≤1.5 were established for *T. vaginalis*-positive and -negative cutoffs, respectively. ΔRQ values between 1.5 and 2.0 were considered indeterminate. Overall findings revealed a high level of agreement between PCR and culture for detecting *T. vaginalis*. Potential benefits of the 5'-nuclease assay include a greater sensitivity compared to direct microscopic examination and the ease of testing large numbers of clinical specimens in a significantly shorter turnaround time compared to culture.

Worldwide, *Trichomonas vaginalis* infection is the most prevalent nonviral, sexually transmitted disease (STD), with an estimated 180 million new infections occurring each year (28). Historically, the presence of *T. vaginalis* has been viewed as a risk marker for other sexually transmitted agents, such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, or bacterial vaginosis (19, 27). *T. vaginalis* is considered also to be a risk factor in transmitting the human immunodeficiency virus (14, 23). Other studies have demonstrated an association with *T. vaginalis* and poor outcome in pregnancy, including premature rupture of membranes, premature delivery, and low-birthweight infants (3–5, 17, 18). *T. vaginalis* infection has been suggested also as a risk factor for developing cervical cancer (25, 29). Therefore, designing a sensitive assay that could rapidly detect the microorganism could have both individual and public health benefits.

Historically, clinicians have diagnosed *T. vaginalis* infections using direct microscopic examination of vaginal discharge material for the presence of the characteristic motile organism. Although highly specific and rapid, direct microscopic examination offers poor sensitivity, detecting only half of all culture-positive cases (7, 24). Subsequently, a selective enrichment broth was developed and introduced into the laboratory that enhanced the growth and detection of this fastidious microorganism (4, 6). However, culturing *T. vaginalis* requires that broth cultures be examined microscopically at least every other day for up to 6 days before a specimen can be reported as negative for trichomonads. Even then, culturing may not detect those organisms that did not survive a lengthy or adverse transport process. Alternatively, the InPouch TV culturing system (Biomed Diagnostics, Inc., San Jose, Calif.) is effective at reducing the loss of organism viability on transport and is a more sensitive method than either Diamond’s or Trichosel medium for detecting *T. vaginalis* (2). Despite this, the InPouch TV system has to rely on the lengthy process of culturing for detection of the microorganism.

To date, numerous *T. vaginalis*-specific PCR assays have been described. Examples of the targets include the ferredoxin gene (10, 20), beta tubulin gene (15), highly repeated DNA sequences (13), 18S ribosomal gene (16) and the family of adhesion proteins (1). The detection systems described for visualizing the amplified products include agarose gel electrophoresis coupled to either Southern blot analyses or restriction enzyme digestion, and microtiter plate-based colorimetric detection assays.

Nucleic acid amplification assays are highly desirable alternatives to culturing, having been found to be both sensitive and specific for detecting *T. vaginalis* DNA. However, most detection systems currently used are time-consuming, adding hours or days onto the turnaround time for the assay. In contrast, homogenous detection systems like the TaqMan-based technology allow target amplification and detection to occur simultaneously and require as little as 30 to 120 min to complete, compared to days for culture methods (11). The TaqMan technology takes advantage of the 5’ exonuclease activity present in the Taq DNA polymerase molecule, which recognizes the double-stranded probe and amplimer hybrid as its substrate, hydrolyzing the 5’ reporter dye during primer-directed DNA amplification (9). This cleavage reaction results in the liberation of the fluorescent reporter dye from the effects of the
quenching dye, with subsequent light emission. The purpose of this study was to evaluate a double-labeled fluorescent probe in a 5’ nuclease assay for detecting T. vaginalis DNA from female genital specimens.

Homogenous detection systems have the potential to rapidly screen large numbers. This technology, coupled to the recent reports describing the successful use of self-collected, introitus-based specimens for detecting STDs, could result in a more convenient means of screening large numbers of women for this important sexually transmitted agent without the need of a pelvic examination (8, 26). Ultimately, this same approach could be used to design an assay that would detect multiple sexually transmitted agents from a single specimen without requiring a supplement-assisted pelvic examination.

MATERIALS AND METHODS

Patient population tested for T. vaginalis. Two different female patient populations were included in this study. One group consisted of 304 women seen at an STD clinic where self-collected, introitus-based samples were obtained for T. vaginalis testing. The other group consisted of 248 pregnant women seen in labor and delivery with evidence of premature rupture of membranes. The specimens from the latter group were collected with the aid of an un lubricated spectrum and tested for T. vaginalis. Approval to obtain two swabs from each patient was granted prior to the start of this study by the Magee-Women’s Hospital Human Investigation Review Board, where informed consent was deemed unnecessary.

Isolation of T. vaginalis in broth culture. Dacron-tipped swabs (catalog no. 22-281-659, Fisher Scientific, Pittsburgh, Pa.) were used to collect the female genital secretions. The specimen-assisted specimens collected from patients in labor were inoculated into 5 ml of Trichosel broth (Becton-Dickinson Microbiology Systems, Cockeysville, Md.) (n = 304). The self-collected introital genital specimens from women attending an STD clinic were inoculated into 5 ml of modified Diamond’s broth (Renel Microbiology Products, Lenexa, Kans.) (n = 248). All inoculated media were transported at ambient temperature to the microbiology laboratory and placed at 37°C for up to 6 days. The broth cultures were examined microbiologically every other day for the presence of motile trichomonads of 15 to 30 μm in length, having a flagellum and four anterior flagella. Direct microscopic examination of genital secretions and vaginal pH measurements were obtained also on the group of 304 women who obtained self-collected introitus-based specimens.

PCR amplification of endogenous β-globin DNA. To assess the quality of the specimen, endogenous β-globin DNA amplification was carried out on every extracted sample prior to T. vaginalis DNA amplification. The previously described primers include BGL-1, 5'-TGTTATTGCTCACCTGGTTGAA-3’ and BGL-2, 5’-TCACCTGGTTGAACTACGCAAGCAGCAGTAC-3’ (22). Five microliters of each prepared specimen was added to 45 μl of the PCR buffer, containing 10 mM Tris- HCl, pH 8.3; 50 mM KCl; 2.5 mM MgCl2; a 200 μM concentration (each) of dATP, dCTP, dGTP, and dUTP; a 0.25 μM concentration of each β-globin-specific primer; 1.25 U of AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.); and 1 μl of uracil N-glycosylase (Perkin-Elmer). Forty cycles of DNA amplification at 94°C for 1 min, 57°C for 2 min, and 72°C for 1 min were performed in a Perkin-Elmer 480 thermocycler.

Synthesis and characteristics of the T. vaginalis-specific TaqMan probe. The T. vaginalis-specific probe was synthesized with a reporter dye, 6-carboxyfluorescein (6-FAM: Applied Biosystems, Foster City, Calif.), at the 5’ end and an amino-modified C6-DT linker arm nucleotide (Glen Research, Sterling, Va.) at the 3’ end, followed by a 5’ phosphate (Phosphalink; Perkin-Elmer Applied Biosystems). The 3’ phosphate was added to prevent extension of the hybridized probe during PCR amplification. Following deprotection, the single-labeled oligonucleotide was ethanol precipitated, resuspended in 0.25 M NaHCO3-Na2CO3 (pH 9.0), and incubated overnight at 37°C with a quencher dye, 6-carboxytetramethylrhodamine (TAMRA NHS ester) (Perkin-Elmer Applied Biosystems). Unreacted dye was removed by passing the reaction mixture over a PD-10 Sephadex column (Pharmacia Biotech Inc., Piscataway, N.J.). The FAM-TAMRA double-labeled hydrolysis probe was purified by reverse-phase high-performance liquid chromatography using a DeltaPak C18 column (Waters, Bedford, Mass.) with a 10 to 80% acetonitrile gradient in 0.1 M triethyl ammomium acetate (pH 7.0). The detailed conditions of high-performance liquid chromatography purification were published previously (12, 21). Individual fractions were collected, ethanol precipitated, resuspended in sterile distilled water, and evaluated for DNA concentration (absorbance at 260 nm) and for low (<1) reporter-to-quencher ratios (about 518 and 580 nm, the approximate maximum emission intensities for FAM and TAMRA, respectively) using an excitation wavelength set at 488 nm.

The DNA sequence of the T. vaginalis probe is 5’-FAM-CTC TGA GTC TTC TTC TAG AGG TC-L(TAMRA)PO4-3’. The probe sequence was designed to hybridize selectively to sequences internal to the 102-bp T. vaginalis DNA amplification product (20). The melting temperature (Tm) of the probe (Tm = 68°C) was designed to be higher than the Tm of each of the two T. vaginalis-specific primers used in the reaction to ensure efficient binding and cleavage of the probe prior to and during the extension phase of the PCR assay.

Detection of the T. vaginalis-specific PCR product using the T. vaginalis-specific TaqMan probe. Fifty microliters of each PCR amplification reaction was transferred into wells of an opaque 96-well microtiter plate (Perkin-Elmer Applied Biosystems). Fluorescent emissions were measured at both 518 and 580 nm, with the excitation wavelength set at 488 nm, on an LS50B fluorometer (Perkin-Elmer Applied Biosystems). At each emission wavelength, the intensity of the PCR buffer blank was subtracted from the intensity measured for each sample and control well. For each reaction, a FAM/TAMRA ratio, RQFAM, was calculated as the fluorescence intensity at 580 nm. The fluorescence emission generated by TAMRA is unaffected by the presence or absence of target and serves to normalize for well-to-well variations in probe concentration, pipetting errors, or microtiter well inconsistencies. Finally, the ΔRQ value was calculated by subtracting RQFAMNT (FAM/TAMRA fluorescence ratio associated with the nontemplate controls) from RQFAM (FAM/TAMRA fluorescence ratio associated with the specimen). Specimens with ΔRQ values of ≥2 were considered positive for T. vaginalis DNA, while those with ΔRQ values of ≤1.5 contained undetectable levels of T. vaginalis DNA and were considered negative.

RESULTS

Analytical sensitivity of the T. vaginalis TaqMan assay. Freshly cultured clinical isolates were used to determine the analytical sensitivity of the T. vaginalis-specific PCR assay. The organisms present in modified Diamond’s broth medium were counted manually, in triplicate, using a hemacytometer. Serial dilutions of clinical isolates of T. vaginalis were made in modified Diamond’s broth and processed in an identical manner to that described for the vaginal secretions. The PCR assay was able to routinely detect as few as five T. vaginalis organisms per milliliter of medium (data not shown).

Comparison of TaqMan-based PCR, broth culture, and direct microscopic examination for detecting T. vaginalis from female genital specimens. In this comparison study, individual female genital specimens were obtained from 552 women for T. vaginalis testing by both culture and PCR. An Amplicor swab (Roche Molecular) was used to collect specimen for PCR amplification, while a Dacron-tipped swab was used to collect material for culturing of the organism. Of the total, 304 of 552
TABLE 1. Comparison of TaqMan-coupled PCR and broth culture for detection of T. vaginalis from female genital specimens

<table>
<thead>
<tr>
<th>PCR result</th>
<th>No. with broth culture result</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Positive</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>493</td>
</tr>
<tr>
<td>Total</td>
<td>45</td>
<td>507</td>
</tr>
</tbody>
</table>

* Sensitivity, 97.8%; specificity, 97.4%; positive predictive value, 77.6%; negative predictive value, 99.8%; prevalence by culture, 8.2%; prevalence by PCR, 10.5%.

* One PCR-positive, culture-negative specimen contained motile, characteristic trichomonads by direct microscopic examination.

(55%) specimens were self-collected introital specimens, with Trichosel being used as the selective enrichment broth for culturing T. vaginalis from these specimens. These 304 self-collected introital specimens were also examined microscopically for the characteristic, motile organism. The remaining 248 of 552 (45%) specimens consisted of vaginal secretions that were cultured using modified Diamond’s broth. The use of a single broth for culturing T. vaginalis was not realized in this study due to the participation of multiple sites whose preferences for T. vaginalis media differed.

Table 1 illustrates the PCR and combined broth culture results from the specimens collected on the 552 women. Broth culture supported the growth of the characteristic, motile trichomonads from 45 of 552 (8.2%) specimens. The majority of the cultures required between 24 and 48 h of incubation before the microorganism could be visualized, with one specimen requiring 4 days of incubation. The overall time to positivity of the broth cultures containing T. vaginalis using the two culture media were equivalent.

The 304 self-collected introital specimens were also examined by direct microscopic analysis. A total of 14 of 304 (4.6%) samples were found to be positive by direct microscopic examination. Thirteen of the fourteen specimens positive by direct examination were also positive by Trichosel broth culture, while all fourteen of these specimens were also positive by PCR (data not shown). Compared to culture and PCR, direct microscopic examination detected T. vaginalis microorganisms in only 50% (14 of 28) and 35% (14 of 40) of positive specimens, respectively.

PCR amplification detected T. vaginalis DNA in a total of 58 of 552 (10.5%) specimens and in 44 of 45 (97.8%) of all culture-positive specimens. The one culture-positive specimen which failed to be detected by the PCR method required 4 days of incubation. The culture-positive, PCR-negative specimen did not contain PCR inhibitors, as exogenous T. vaginalis DNA added into the PCR mixture was amplified successfully (data not shown).

Although 14 of 58 PCR-positive specimens did not grow in culture, one of them revealed motile trichomonads by direct microscopic examination and was considered a true positive. A true-positive result is defined as that being positive by either direct microscopic examination and/or by broth culture. None of the 552 specimens tested for T. vaginalis DNA generated indeterminate ΔRQ values. The overall analytical sensitivity and specificity for the T. vaginalis-specific PCR assay was calculated to be 97.8 and 97.4%, respectively. The positive and negative predictive values were calculated to be 77.6 and 99.8%, respectively.

Performance characteristics of the TaqMan probe in the PCR assay for detection of T. vaginalis DNA. Female genital specimens, both vaginal and introitus-based samples, were tested for T. vaginalis DNA using the TaqMan-coupled PCR assay. Figure 1 illustrates the range of ΔRQ values which resulted from testing the 552 specimens for T. vaginalis DNA by PCR. Of the total number tested, 494 of 552 (89.5%) had ΔRQ values of ≤1.5, ranging from 0.00 to 0.63, with a mean of 0.16 and standard deviation of 0.24, and were considered negative for T. vaginalis DNA. Fifty-eight of the 552 (10.5%) specimens had ΔRQ values of ≥2.0, ranging from 2.23 to 6.63, with a mean of 4.70, and a standard deviation of 1.38, and were considered positive for T. vaginalis DNA. All 552 specimens contained amplifiable β-globin DNA by PCR (data not shown).

DISCUSSION

A previously described set of primers (20) was used in conjunction with a novel, double-labeled fluorescent probe for use with the 5′ nuclease assay to amplify and detect T. vaginalis DNA directly from clinical specimens. The comparison revealed a good level of agreement between PCR and conventional broth culturing for detecting T. vaginalis from female genital specimens. The single false-negative PCR result that occurred in this study could have been due to a sampling error because of very low level of organism present within the sample; i.e., the specimen required 4 days of incubation before the organism was detected in culture. One of the 14 PCR-positive, culture-negative specimens proved to be a true positive, as the organism was present by direct microscopic examination.

The two patient populations studied had different percent positive rates for T. vaginalis. One group—the 248 pregnant women with evidence of premature rupture of membranes—revealed a 6.8% T. vaginalis-positive rate by both culture and PCR. In contrast, the self-collected introital specimens from the 304 women attending an STD clinic had T. vaginalis-positive rates of 9.3 and 13.5% by culture and PCR, respectively. The differences in T. vaginalis-positive rates seen between PCR
and culture in the latter group cannot be elucidated since multiple variables existed in this study, with differences occurring in both the type of specimen collected and the media used to culture T. vaginalis. However, due to participation of two collection sites, these differences could not have been avoided.

In summary, homogenous fluorescent detection systems, like the one described here, have many advantages over conventional nucleic acid amplification and detection methods described in the literature. Eliminating the need for post-PCR processing for PCR product detection would, by its very nature, decrease turnaround time and increase throughput. The closed nature of this system would also dramatically decrease the risk of amplimer contamination in the laboratory. We are taking the next logical step in developing a real-time, quantitative T. vaginalis-specific assay using the ABI 7700 instrument, along with the above-described primers and probe, that will allow us to assess organism burden. This technological development will allow us to ask the question of whether organism load correlates directly with clinical outcome. If a correlation exists, it could be used as a prognostic indicator in pregnant women or women at risk of acquiring other STIs. It would be highly desirable and convenient to the patient if an individual specimen could be analyzed for multiple infectious agents, including T. vaginalis, C. trachomatis, and N. gonorrhoeae. This could be accomplished using a molecular-based, multiplex assay.

ACKNOWLEDGMENTS

This work was supported by a grant from the Magee-Women’s Health Foundation and ERMS grant 00035010.

REFERENCES


