Real-time PCRs for detection of *Trichomonas vaginalis* β -tubulin and 18S rRNA genes in female genital specimens

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Trichomonas vaginalis is the cause of one of the most common types of vaginitis, trichomoniasis. The incidence of trichomoniasis in developed countries has decreased substantially during the past decade, but high prevalence of this disease can still be found in rural and remote areas of Australia. Clinical manifestations of symptomatic women are generally non-specific, but include vaginal discharge, vaginitis and irritation. *T. vaginalis* infection has also been linked to the increased risk of human immunodeficiency virus transmission. Current diagnosis of *T. vaginalis* relies on the visualization of motile organisms in a wet-mount preparation. Culture is used mainly in reference laboratories. The latter two methods require viable organisms and would not be suitable for use where transportation of specimens can be delayed. Two real-time fluorescence resonance energy transfer (FRET) hybridization probe PCR assays were used in this study to test for *T. vaginalis* DNA, targeting the β -tubulin and 18S rRNA genes. We tested 500 randomly selected female patients, in an STD setting, for *T. vaginalis* DNA. The FRET PCRs targeting the β -tubulin gene and the 18S rRNA gene detected 96 % (85/89) and 100 % (89/89), respectively, of the positive specimens (first-void urine sample or genital swabs). Wet-mount microscopy was performed on 76 of these PCR-positive specimens and showed a sensitivity of 38 % (29/76). The prevalence, by PCR, of trichomoniasis was 18 % in this study. The two real-time PCRs developed in this study, targeting different genetic regions of the organism, provide a rapid, sensitive and specific diagnosis of *T. vaginalis* infection.

Received 11 January 2007

Accepted 20 February 2007

INTRODUCTION

Trichomoniasis is caused by the protozoan parasite *Trichomonas vaginalis*. It is one of the most common causes of vaginitis worldwide, with an estimated 170 million cases occurring annually (WHO, 1995). This disease accounts for 4–35 % of vaginitis diagnosed in symptomatic women presenting in primary care settings. Clinical manifestations of the infection in women are generally non-specific, but may include vaginal discharge, vaginitis and irritation (Smith *et al.*, 2005). Approximately 50–80 % of women infected with *T. vaginalis* are asymptomatic (Schwebke & Burgess, 2004; Smith *et al.*, 2005). The infection in women has also been linked to increased risk of human immunodeficiency virus (HIV) transmission and pregnancy complications such as premature rupture of placental membranes, premature labour and low infant birth weight (Petrin *et al.*, 1998; Soper, 2004). Although generally considered a disease of women, *T. vaginalis* also infects men, but the majority of these cases are asymptomatic

(Petrin *et al.*, 1998). Male patients who show symptoms usually present with a self-limiting mild urethritis, but this is rarely reported.

The prevalence of trichomoniasis in developed countries, such as Australia, has decreased dramatically in recent years due to improved health services and treatment (Bowden & Garnett, 2000). However, rural and remote areas of Australia are an exception to this trend, with hyperendemic rates of *T. vaginalis* infection (Bowden *et al.*, 1999; Knox *et al.*, 2002). A rapid and sensitive diagnostic test using a convenient specimen (e.g. dry genital swab or urine) is needed for both patient and public health management.

Wet-mount microscopy is the most commonly employed laboratory method for diagnosing trichomoniasis (Radonjic *et al.*, 2006). Although this test is rapid and inexpensive, it has a limited sensitivity of 20–60 % (Caliendo *et al.*, 2005; Jordan *et al.*, 2001; Ryu *et al.*, 1999; van der Schee *et al.*, 1999). Culture is the current reference standard in the diagnosis of trichomoniasis, but is rarely used in routine laboratory tests (Schwebke, 2005). This technique is slow, taking up to 7 days of incubation,

Abbreviation: FRET, fluorescence resonance energy transfer.

requires daily microscopy and is relatively expensive. In remote areas of central and northern Australia, the commercially available InPouch culture showed a sensitivity of 63 % (Smith *et al.*, 2005).

Several PCR assays targeting various regions of the *T. vaginalis* genome have been described for diagnosis of this infection (Caliendo *et al.*, 2005; Kengne *et al.* 1994; Lawing *et al.*, 2000; Madico *et al.*, 1998; Mayta *et al.*, 2000). These studies showed a detection sensitivity by PCR in the range of 89–98 %. A recent study showed that *T. vaginalis* DNA is undetectable after 2 weeks of treatment with metronidazole in 85 % of individuals (Van Der Pol *et al.*, 2005).

This study reports the validation of two real-time PCRs with fluorescence resonance energy transfer (FRET) hybridization probes for detection of *T. vaginalis* DNA in genital or urine specimens from female patients. The tests were compared with wet-mount microscopy and discordant results were analysed by nucleotide sequence analysis of the amplified products or a different PCR targeting a highly repetitive DNA region of the *T. vaginalis* genome (Kengne *et al.*, 1994; Paces *et al.*, 1992).

METHODS

Specimens and DNA extraction. First-void urine samples and genital swabs were collected from 500 female patients who were being tested for *Chlamydia trachomatis*, *Neisseria gonorrhoeae* or *T. vaginalis* infections. DNA was extracted from specimens using the automated MagNA Pure LC Instrument (Roche Diagnostics) following the manufacturer's protocols. Following extraction, the DNA samples were stored at -70°C until further use. *C. trachomatis* and *N. gonorrhoeae* controls were provided in the COBAS Amplicor PCR kit (Roche Molecular Diagnostics). The *T. vaginalis* positive control was obtained from a specimen that was positive in both wet-mount and PCR (β -tubulin and 18S rRNA).

PCR primers and probes. Two real-time PCR assays using FRET hybridization probes were designed in this study to detect different genetic regions of *T. vaginalis*. The FRET probe technology has been used and described previously in our laboratory (Burrows *et al.*, 2002; Stone *et al.*, 2004). A pair of FRET hybridization probes consists of two probes, donor and sensor, each labelled with a different fluorophore (fluorescein, LC Red 640 or 705). When the probes are bound to target DNA, the donor fluorophore excites the sensor, which yields an emission fluorescence. The FRET probes and target DNA hybrids can be differentiated at a characteristic temperature (T_m) that is determined using melting-curve analysis.

The first PCR targets the β -tubulin gene of *T. vaginalis*. The primers BTUB3f (5'-TCCAAAGGTTTCCGATACAGT-3') and BTUB_bkmt (5'-GTTGTGCCGACATAATCATG-3') amplify a 195 bp sequence of the *T. vaginalis* β -tubulin gene. The forward primer (BTUB3f) and FRET hybridization probes used were as described previously by Hardick *et al.* (2003), but the reverse primer BTUB_bkmt was a new design that provided increased test sensitivity in preliminary experiments.

In the second PCR, a different primer set was designed to amplify a 323 bp product of the 18S rRNA gene of *T. vaginalis*, TV16Sf-2 (5'-TGAATCAACACGGGGAAAC-3') and TV16Sr-2 (5'-ACCCTCTA-AGGCTCGCAGT-3'). A new set of FRET hybridization probes was designed for the 18S rRNA gene PCR, TV16_FL (5'-CAGGTGG-AAGAGGGTAGCAATAACA-fluorescein-3') and TV16_LC (5'-LC

Red 640-TCCGTGATGCCCTTTAGATGCTCTG-p-3'). All primers and probes were manufactured by TIB MOLBIOL.

β -Tubulin gene PCR. The β -tubulin gene LightCycler PCR master mix contained the following reagents (final concentrations): $1 \times$ LC FastStart DNA Master hybridization probe buffer (Roche Diagnostics), 4 mM MgCl_2 , 0.5 μM of each primer, 0.2 μM BTUB FL and 0.4 μM BTUB LC. Two microlitres of template DNA was added to 18 μl master mix directly before PCR cycling was performed. Cycling conditions were as follows: 95°C for 10 min followed by 50 amplification cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 10 s. Fluorescence was detected throughout the annealing phase of each amplification cycle. After amplification was complete, melting-curve analysis was performed as follows: 95°C for 0 s, 40°C for 60 s followed by a gradual increase in temperature (transition rate of $0.1^{\circ}\text{C s}^{-1}$) to 80°C with continual fluorescence detection. A final cooling step was then performed at 40°C for 30 s. All steps were performed at a temperature transition rate of $20^{\circ}\text{C s}^{-1}$ unless indicated otherwise. Data were analysed in the F2/F1 channel using the LightCycler software (version 3.5).

18S rRNA gene PCR. The 18S rRNA gene LightCycler PCR master mix contained the following final concentrations: $1 \times$ LC FastStart DNA Master hybridization probe buffer, 4 mM MgCl_2 , 0.5 μM of each primer and 0.2 μM of both FRET probes. Two microlitres of template DNA was added to 18 μl master mix directly before PCR cycling was performed. The cycling conditions and fluorescence acquisition were the same as the β -tubulin gene FRET probe PCR, except that the number of amplification cycles was reduced to 45 and the extension phase of each amplification was performed for 13 s.

RESULTS

LightCycler PCR interpretation

A positive result was accepted in the LightCycler PCR when a sample showed amplification fluorescence and a crossing point (calculated by the LightCycler software). The melting-curve analysis was used to confirm positive results. The β -tubulin gene PCR contained a double peak at approximately 59°C and 68°C (Fig. 1a). The 18S rRNA gene PCR contained a single peak at approximately 65°C (Fig. 1b). Both melting-curve analyses were consistent with all positive results obtained in the study.

Sensitivity determination

To determine the sensitivity of the PCR assays, replicate PCRs of the specific gene were prepared and the products were pooled and purified using a PCR purification kit (QIAquick PCR purification kit; Qiagen). The yield of the specific product was estimated by spectrophotometer readings and the FRET PCR sensitivity was then determined. The sensitivity of the β -tubulin gene PCR was determined to be 14 copies of β -tubulin per 20 μl PCR. The 18S rRNA gene PCR was shown to have a sensitivity of 10 copies of the 18S rRNA gene per 20 μl PCR.

Specificity determination

The specificities of both real-time PCR assays were determined by testing nine bacterial species (*Chlamydia*

Fig. 1. Melting-curve analysis of the β -tubulin (a) and 18S rRNA gene (b) FRET hybridization probe PCRs. In (a), two peaks were obtained, one at approximately 59 °C and the other at 68 °C. This double peak is characteristic of a positive *T. vaginalis* β -tubulin gene result. In (b), there is a single peak at 65 °C, which is characteristic of a positive *T. vaginalis* result.

trachomatis, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus* group B and *Streptococcus pyogenes*, seven parasites (*Blastocystis hominis*, *Dientamoeba fragilis*, *Entamoeba hartmanni*, *Enteromonas hominis*, *Giardia lamblia*, *Iodamoeba butchlii* and *Tritrichomonas foetus*), three fungi (*Candida albicans*, *Candida glabrata* and *Candida tropicalis*) and three viruses (herpes simplex virus types 1 and 2 and varicella-zoster virus). All were negative in both PCR assays. Although there was amplification detected with *Tritrichomonas foetus*, the T_m for the β -tubulin gene-specific primers was observed at 54.5 °C, compared with the characteristic double peak at 59 and 68 °C with *T. vaginalis*. The fluorescence signal and T_m of 54.5 °C obtained with *Tritrichomonas foetus* meant that this was not considered a specific reaction consistent with *T. vaginalis*.

Analysis of discordant results

The criteria for a positive result in this study were defined as wet-mount microscopy positive for *T. vaginalis* or positive for *T. vaginalis* DNA by both of the above real-time PCR assays (β -tubulin and 18S rRNA). Specimens that were positive in only one PCR, and wet-mount-negative, were tested in a third PCR (G. Harnett, PathWest LM, Australia,

personal communication) and genomic sequence analysis was used to confirm the result. The third PCR used a minor-groove-binding TaqMan probe labelled with a FAM reporter dye that targeted a highly repeated DNA sequence in the *T. vaginalis* genome (Kengne *et al.*, 1994).

Preliminary tests of *T. vaginalis*-positive patients

Initial testing to optimize the PCR assays was performed on specimens collected from 59 patients who were positive for *T. vaginalis* by wet-mount microscopy. From these 59 patients, 58 genital swabs and 26 urines were collected for PCR testing. The 58 swabs consisted of 24 endocervical, 26 vaginal and 8 genital (sites unspecified) specimens.

Ninety-eight per cent (57/58) of the swabs were positive in both real-time PCR assays, whilst one wet-mount-positive swab was negative in both of the PCRs. The β -tubulin gene PCR detected 77 % (20/26) of the urines, whilst the 18S rRNA gene PCR detected 88 % (23/26) of the urines. All urines that were negative in the 18S rRNA gene PCR were also negative in the β -tubulin gene PCR. There were seven specimens that were negative for *T. vaginalis* DNA by β -tubulin-based PCR, but three of these seven were positive by 18S rRNA gene-based PCR. These did not show PCR inhibition in the COBAS Amplicor PCR (Roche Molecular Diagnostics). The latter PCR is a commercially available kit for simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* DNA with an internal control that is co-amplified to exclude possible test inhibition.

Specimen tests

Following initial testing of specimens collected from wet-mount-positive patients described above, patients with suspected *T. vaginalis* infection were tested to compare the sensitivities of real-time PCR and wet-mount microscopy. In total, 500 randomly selected specimens, submitted to a regional laboratory, from 500 female patients were tested with the real-time PCR assays without prior knowledge of the wet-mount microscopy results. The specimens tested for *T. vaginalis* DNA by real-time PCR consisted of 427 dry swabs and 73 urines. Wet-mount microscopy was performed on 403 of these patients. Wet-mount microscopy was not requested by the attending physicians with the remaining 97 specimens.

There were 29 *T. vaginalis*-positive results detected by wet-mount microscopy, 85 by the β -tubulin gene PCR and 89 by the 18S rRNA gene PCR. Both PCR assays detected all of the wet-mount microscopy-positive results and the 18S rRNA gene PCR detected all of the β -tubulin gene PCR-positive results (Table 1). The 89 positive results obtained by PCR consisted of 74 genital swabs and 15 urines. The genital specimens consisted of 48 low vaginal, 15 endocervical and 14 genital (site unspecified) swabs.

In the group of 500 specimens tested, there were four discordant results between the two real-time PCR assays.

Table 1. Relation between wet-mount microscopy, β -tubulin gene PCR and 18S rRNA gene PCR for detection of *T. vaginalis* in 500 female patients

Wet-mount result	β -Tubulin PCR ⁺ 18S rRNA PCR ⁺	β -Tubulin PCR ⁻ 18S rRNA PCR ⁺	β -Tubulin PCR ⁺ 18S rRNA PCR ⁻	β -Tubulin PCR ⁻ 18S rRNA PCR ⁻	Total
Positive	29	0	0	0	29
Negative	43	4*	0	327	374
Not done	13	0	0	84	97
Total	85	4	0	411	500

*Confirmed positive by a third PCR to a highly repetitive DNA region and/or nucleotide sequence analysis of the amplified product.

All discordant results were positive in the 18S rRNA gene PCR but negative in the β -tubulin gene PCR. Three of these discordant results were confirmed as *T. vaginalis*-positive by a third PCR (G. Harnett, personal communication) and sequence analysis of the 18S rRNA gene. The fourth discordant result was negative in the third PCR, but was confirmed as *T. vaginalis*-positive by sequence analysis of the 18S rRNA gene. All samples sequenced were similar to a *T. vaginalis* sequence in GenBank (accession no. U17510). The 18S rRNA gene PCR was shown to be more sensitive than the β -tubulin gene PCR (10 copies compared with 14 copies per reaction), which may explain the discordant results.

Wet-mount microscopy was performed on 76 of the 89 *T. vaginalis* PCR-positive (β -tubulin or 18S rRNA gene) patients (Table 1). Wet-mount microscopy detected 29 (38%) of these 76 PCR-positive specimens. The FRET hybridization probe PCRs targeting the β -tubulin gene and the 18S rRNA gene showed sensitivities of 96% (85/89) and 100% (89/89), respectively. The prevalence, by PCR detection, of trichomoniasis was 18% (89/500) in this study of 500 female patients. Based on the criteria of a confirmed *T. vaginalis*-positive (wet-mount or both PCR positive) specimen in this study, the specificity of the two FRET probe PCRs was 100% (327/327).

DISCUSSION

Rapid and sensitive diagnosis of *T. vaginalis* infections is important for prompt treatment and to reduce the spread of the disease. Metronidazole has been shown to be a safe and effective drug for the treatment of trichomoniasis in females (Soper, 2004). It is important to reduce the prevalence of this disease, as the infection has been linked to increased risk of HIV transmission and pregnancy complications. The real-time PCR tests in this study provide a rapid diagnostic tool for detection of *T. vaginalis* and the use of FRET hybridization probes increases the specificity of the tests compared with other real-time PCR assays that use TaqMan or molecular beacon probes. The latter are approximately half the length of FRET probes. The use of hybridization probes reduces the need for labour-intensive and time-consuming post-PCR procedures such

as agarose gel electrophoresis. We believe the targeting of two different genetic regions, β -tubulin and 18S rRNA, of *T. vaginalis* provides increased test specificity.

The double peak observed in the β -tubulin PCR (see Fig. 1a) may be caused by the *T. vaginalis* organism containing different alleles of the β -tubulin gene that differ in sequence in the probe-binding region. When this occurs, a double peak may be expected, as one allele is completely homologous to the FRET probe sequences whilst the other allele contains a single mismatch. This mismatch results in a weaker thermodynamic bond between the amplified DNA and the probe, which causes the probe to separate at a lower temperature in the melting-curve analysis. Another reason for the double peak may be back-folding of the amplicon on itself downstream of the sensor probe. This back-folding of the amplicon competes with the FRET probes binding to the amplicon, creating a lowered melting peak.

Trichomonas foetus showed amplification fluorescence in the β -tubulin gene PCR. However, the FRET hybridization melting-curve analysis was able to differentiate between this species and *T. vaginalis* because the T_m differed between the two samples (data not shown). *T. vaginalis* showed a characteristic double peak at 59 and 68 °C, whilst the *Trichomonas foetus* sample showed a single peak at 54.5 °C. These results showed that *Trichomonas foetus* would not be expected to cause false-positive results in the β -tubulin FRET probe PCR. No amplification fluorescence was observed using *Trichomonas foetus* DNA in the 18S rRNA gene PCR for *T. vaginalis*, and no reactions were observed with the two *T. vaginalis* PCR tests against bacteria, viruses or other parasites.

All patients who were wet-mount microscopy-positive, but PCR-negative in urine samples, provided a genital swab. The latter was PCR-positive. This showed that the sensitivity of real-time PCR assays is lower in urine samples than with genital swabs. This is consistent with results obtained from other studies of *T. vaginalis* PCRs (Hardick *et al.*, 2003; Lawing *et al.*, 2000; Smith *et al.*, 2005). The lower sensitivity may be due to low copy numbers present in high-volume urine samples or degradation of the *T. vaginalis* DNA. However, the successful use of urine samples by PCR, although not as sensitive as genital swabs, would facilitate increased diagnosis and reduce

spread of this infection. Centrifuging the urine to increase the concentration of *T. vaginalis* DNA may be a way of increasing the sensitivity of urine testing, but this approach was not tested in this study. The use of spun urine for wet-mount microscopy has a reported sensitivity of 64 %, and 73 % by vaginal fluid, compared with culture (Borchardt *et al.*, 1997) and 51 % compared with PCR (Lawing *et al.*, 2000). It would be expected that a similar approach may increase PCR sensitivity.

Because of the previously published low sensitivity of culture compared with PCR (Smith *et al.*, 2005), culture was not used in this study. The requirement for prompt culture of specimens collected from patients in remote regions precluded the use of this test. Wet-mount microscopy, which is commonly used in routine tests, showed a sensitivity of 38 % compared with PCR. Among the main factors that contribute to low test sensitivity with wet-mount microscopy may be the use of dry swabs or delayed transportation of the specimen to the laboratory. However, in this study, there were no records of the types of swabs used or delay before the specimen was tested. The low sensitivity of wet-mount microscopy makes it unsuitable as the only test for laboratory diagnosis of trichomoniasis.

The real-time PCR assays described in this study showed a high sensitivity (96–100 %) and specificity of 100 % for detection of *T. vaginalis* DNA. The prevalence of trichomoniasis was 18 % (89/500) in this study of 500 female patients. The high prevalence of this sexually transmitted infection probably results from the increased detection sensitivity of PCR compared with wet-mount microscopy. The high sensitivity and specificity of PCR reported in this study would offer a useful rapid screening tool. This could reduce spread and transmission of the infection, in particular from asymptomatic patients.

ACKNOWLEDGEMENTS

The authors would like to thank the staff of the Infectious Diseases Laboratories, IMVS, Adelaide and Alice Springs, Gerry Harnett of PathWest LM (WA, Australia) for his assistance in testing discordant PCR results and J. W. Burgess for the *Trichomonas foetus* sample.

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