Optimization and clinical validation of a Real-Time PCR protocol for direct detection of *Trichomonas vaginalis* in pooled urine samples

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**ABSTRACT**

**Background and Objectives:** A new Real-Time PCR protocol for the detection of *Trichomonas vaginalis* in pooled urine samples has been optimized and validated.

**Materials and Methods:** The amplification protocol, targeting a 2kb repeated gene in the *T. vaginalis* genome, was optimized by varying PCR parameters. As a reference method, a Real-Time PCR protocol targeting the beta-tubulin gene (Y. Versluis et al, 2006, Int J STD AIDS 17:642) was used. Clinical validation was performed with pooled urine samples obtained from patients of the sexually transmitted diseases clinic of a university hospital (n=963; from February – June 2007).

**Results:** Positive samples with the new optimized technique is 1.1% (n=10), while the beta-tubulin real-time PCR method generated four positives (0.3%).

**Conclusion:** The new RT- PCR protocol is a sensitive (1.000) and specific (0.993) procedure to detect and to identify *T. vaginalis* in urine samples.

**Keywords:** Real time PCR, *Trichomonas vaginalis*, molecular diagnosis.

**INTRODUCTION**

*Trichomonas vaginalis*, a parasitic protozoan, is a major causative agent of nonviral sexually transmitted disease (STD) worldwide. The disease is primarily symptomatic in women (1) and most often asymptomatic in men (2), although even in women no more than 50% is asymptomatic (3). Worldwide, *T. vaginalis* accounts for approximately 180 million cases annually (WHO report, 2008). The disease encompasses a broad range of symptoms ranging from a state of severe inflammation and irritation with a frothy malodorous discharge to a relatively asymptomatic carrier state. Infections with *T. vaginalis* have been shown to be a cofactor in the transmission of HIV (4, 5) as well as for adverse pregnancy outcomes (6). Given these data, information about the presence of this etiological agent in clinical specimens is highly relevant. Current diagnosis of *T. vaginalis* includes culture of the parasite in combination with direct microscopy. Molecular-based techniques accelerate the diagnostic procedure and improve the test sensitivity significantly (7-9).

The purpose of this study was to develop a robust and sensitive diagnostic real-time PCR protocol for the detection and identification of *T. vaginalis* in pooled urine samples obtained from women and men, and to validate the test prospectively in 963 STD patients. As target for the *T. vaginalis* specific real-time PCR method, the highly repeated 2-kb DNA sequence ubiquitously present in the *T. vaginalis* genome was selected (7, 8). The PCR protocol as described previously (10) was used as the reference method. All positive results obtained with both methods were confirmed to assess its integrity by amplifying another *T. vaginalis* specific genomic region using TVA primers (1).

**MATERIALS AND METHODS**

**Study population.** The patients (n=963) visiting the STD clinic of a 1000-bed teaching hospital in Rotterdam, the Netherlands, reside in the city. Patients included in this study were divided in two groups; patients clinically suspected of trichomoniasis (n=84) and
patients elected for a routine STD check (n=879) who were previously screened for *Chlamydia trachomatis*. Both men and women were included in this study.

**PCR.** Nucleic acid extraction was performed as described previously (10). Briefly, an RT-PCR protocol was developed targeting the beta-tubulin gene (Genbank accession number L05468) (11) using the LightCycler 1.2 (Roche, Almere, The Netherlands) platform. Primer and probe sequences are outlined in Table 1.

**Optimization of the new Real-Time PCR protocol.** The extraction procedure was performed with the NucliSens EasyMag (bioMérieux, Boxtel, The Netherlands). The performance of this extraction method was validated by using a dilution series of living *T. vaginalis* cells tested with the conventional and the new RT-PCR protocol. The new RT PCR protocol uses a primer and probe set targeting the 2kb repeated gene (Genbank accession number L23861) (7, 8). To optimize the new RT PCR protocol using the MiniOpticon PCR processor (BioRad, Veenendaal, The Netherlands) platform, a positive sample is tested with a varying annealing temperature with an automatic temperature gradient (50-60 °C), the ramprate (1 °C/sec and 2.5 °C/sec), the MgCl₂ concentration (25 mM, 0 μl - 2.5 μl), and primer concentration (15-30 pmol/μl). The optimized RT-PCR showed a temperature profile consisting of 15 min., 95 °C, and 40 cycles of 15 sec 95.0 °C, 1 min, 54.0 °C and 1 min, 72.0°C, and a cooling step of 15 min at 15 °C. Reagents used were 0.15 μl of each primer 0.25 μl of each probe, 2.5 μl MgCl₂ (25 mM) 12.5 μl Hotstar Taq mix (Qiagen), 3.9 μl H₂O and 5 μl nucleic acid extract in a total reaction volume of 25 μl. Primer and probe sequences are outlined in Table 1.

**Confirmation PCR protocol.** The positive results obtained with both the conventional and the new RT-PCR protocol were confirmed with the following procedure. Nucleic acid extraction of the samples was performed with the NucliSens EasyMAG. The MiniOpticon platform was used for amplification of a *T. vaginalis*-specific DNA region using TVA primers (1). Primer sequences are outlined in Table 1. IQ SYBR Green Supermix (25 μl, BioRad, Veenendaal, The Netherlands) was used in a total reaction volume of 50 μl. The amplification programme consisted of 15 min at 94 °C, and a 40 times repeated cycle of 30 sec at 95 °C, 30 sec at 47.0 °C, and 30 sec at 72.0 °C, subsequently followed by a melting curve analysis (45 °C to 95 °C) of the PCR product.

**Prospective study for clinical validation.** The samples used in this study are first void urine samples (n = 963) from patients visiting the STD clinic. Each urine sample (10 ml) obtained from these patients was concentrated by centrifugation (5 min., 2,000 rpm) and 200 μl of the sediment was used for nucleic acid extraction. Only urine samples from patients with a routine STD check (n=897) were pooled (5 samples of 200 μl each in one extraction vessel). Urines from patients suspected of trichomoniasis were analysed.

### Table 1. Primer and probe sequences as categorised per PCR protocol

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>New protocol</td>
<td>(Genbank accession number L23861)</td>
<td>(Riley et al., 1992; Schirm et al., 2007)</td>
</tr>
<tr>
<td>Primer Trich F</td>
<td>5’-AAG.ATG.GGT.TTA.ATA.GGC.TAG.-3’</td>
<td></td>
</tr>
<tr>
<td>Primer Trich R</td>
<td>5’-CGT.TTT.CCA.GTA.TGC.CCC.AGT.-3’</td>
<td></td>
</tr>
<tr>
<td>Probe Trich</td>
<td>5’-fam-CCG.AAG.TTC.ATG.TCC.TCT.CCA.AGC.GT-tamra3’</td>
<td></td>
</tr>
<tr>
<td>Internal control PhHV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer PhHV-267s</td>
<td>5’-GGG.CGA.ATC.ACA.GAT.TGA.ATC.-3’</td>
<td></td>
</tr>
<tr>
<td>Primer PhHV-337as</td>
<td>5’-GCG.GTT.CCA.AAC.GTA.CCA.A-3’</td>
<td></td>
</tr>
<tr>
<td>Probe PhHV-305sq</td>
<td>5’-vic-TTA.TTA.TGT.CCG.CCA.CCA.TCT.GGA.TC-tamra-3’</td>
<td></td>
</tr>
<tr>
<td>Conventional protocol</td>
<td>(Genbank accession number L05468)</td>
<td>(Versluis et al., 2006)</td>
</tr>
<tr>
<td>Primer BTUB 9</td>
<td>5’-CAT.GTA.TAA.CGA.AGC.TCT.TTA.CAT-3’</td>
<td></td>
</tr>
<tr>
<td>Primer BTUB B</td>
<td>5’-CGC.ATG.TTG.TGC.CCG.ACA-3’</td>
<td></td>
</tr>
<tr>
<td>Probe BTUB FL</td>
<td>5’-CCG.TAC.ACT.CAA.GCT.CAC.AAC.ACC.AAC-FL-3’</td>
<td></td>
</tr>
<tr>
<td>Probe BTUB LC</td>
<td>5’-L.Cred640-CGG.CGA.TCT.TAA.CCA.CCT.TGT.TTC.C-PH-3’</td>
<td></td>
</tr>
<tr>
<td>Confirmation PCR</td>
<td>(Genbank accession number AY312361)</td>
<td>(Van Der Schee et al., 1999; Kengne et al., 1994)</td>
</tr>
<tr>
<td>Primer TVA5</td>
<td>5’-GAT.CAT.GTT.CTA.TCT.CCA.C-3’</td>
<td></td>
</tr>
<tr>
<td>Primer TVA6</td>
<td>5’-GAT.CAC.CAC.CTT.AGT.TTA.CA-3’</td>
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</table>
RESULTS

The optimization of the new protocol was done by varying the annealing temperature, the ramp rate, MgCl₂, and primer concentrations. The results have been evaluated by the increase in sensitivity (lower Ct values), until an optimal sensitivity was reached. Accuracy of both the conventional and the new protocol was compared by using a dilution series of _T. vaginalis_ cells. The results of this dilution range in the conventional and the new protocol resulted in a correlation coefficient of 0.955 and 0.997, respectively. The new protocol (10 positives, average Ct-value 24.18, SD 0.04) showed a significant drop in the Ct values of the samples from the dilution series as compared to the conventional protocol (4 positives, average Ct-value 31.48, SD 0.38) with an average decrease of Ct value=6.9, indicating an elevated sensitivity.

In the clinical validation study, urine samples were pooled and tested with both protocols. In samples from patients with a routine STD check (n=879) a total of 10 positive samples from 8 different pools were obtained with the new diagnostic RT-PCR protocol. The conventional RT-PCR protocol generated 4 positive samples from 4 different pools and concordant with the results obtained with the new protocol. The association of the performance of both test protocols were fairly concordant (Cohen’s Kappa, K=0.57). Confirmation of all 10 _T. vaginalis_ positive clinical samples obtained with both methods, using the RT-PCR protocol with TVA primers, resulted in 8 positive samples (Table 2). The remaining two urines were not confirmed.

In patients clinically suspected for _T. vaginalis_ infection (n=84) both RT-PCR tests produced fully concordant results and 5 positive samples were detected (Table 2).

DISCUSSION

This study describes the optimization and validation of a RT-PCR protocol, developed for routine diagnostic testing of _T. vaginalis_ from human urine samples. In comparison with conventional methods, such as microscopy and culture methods, RT-PCR is a sensitive diagnostic test (7, 8). A high sensitivity can be obtained with amplification methods for the detection of _T. vaginalis_ in urine samples (9). This clinical specimen is easy to collect and provides a non-invasive alternative to vaginal or urethral swabbing.

The new RT-PCR protocol demonstrated to be significantly more sensitive than the conventional procedure (Fisher’s exact test, P<0.0001). The detection level of the new optimized protocol is less than 1 parasite/ml, with the conventional protocol detecting 10 parasites/ml. Van der Schee et al used the same 2kb repeat sequence and reached a detection level of 20 parasites/ml (9).

The clinical evaluation of the new protocol was done by testing patients of the STD clinic in the Erasmus Medical Center, Rotterdam. The number of positive urine samples in the patient group for routine STD check varied among the different RT-PCR techniques. The difference between the conventional and the new method can be explained by the higher sensitivity of the latter. The discrepancy between the unconfirmed test results could not be solved since no clinical data were available.

The prevalence of _T. vaginalis_ in the study group asymptomatic for _T. vaginalis_ infection is 0.8% (n=775)
among men and 3.8% (n=104) among women. Severe complications of *T. vaginalis* infection (4-6) warrant routine screening to prevent spread of the parasite.

This protocol establishes a highly sensitive and robust test, which can be used for simple non-invasive routine screening, detection and identification of *T. vaginalis* in pooled human urine samples.

REFERENCES


