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Molecular typing of the actin gene of *Trichomonas vaginalis* isolates by PCR–restriction fragment length polymorphism

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ABSTRACT

Human trichomoniasis, caused by the protozoan *Trichomonas vaginalis*, is a highly prevalent sexually transmitted infection. However, little is known about the degree of strain variability of *T. vaginalis*. A reliable classification method for *T. vaginalis* strains would be a useful tool in the study of the epidemiology, pathogenesis and transmission of *T. vaginalis*. A PCR–restriction fragment length polymorphism typing method was designed and evaluated using *T. vaginalis* isolates obtained after culture of vaginal specimens collected in the Democratic Republic of Congo and in Zambia. The variation of the actin gene of *T. vaginalis* was determined for three ATCC reference strains and 151 *T. vaginalis* isolates. Eight different types were identified, on the basis of the digestion patterns of the amplified actin gene, with each of the restriction enzymes *Hin*dII, *Mse*I and *Rsa*I. It was determined that the ATCC reference strains 30001, 30240 and 50141 were of actin genotypes G, H and E, respectively. The actin genotype type E was more common in the Democratic Republic of Congo, whereas type G was the commonest type in Zambia. Translation of the nucleotide sequence showed up to three amino acid substitutions. We developed a reproducible, sensitive and specific typing method for *T. vaginalis*, and were able to distinguish at least eight *T. vaginalis* actin genotypes. Further studies are needed to evaluate the method using clinical specimens and to determine the utility of the typing method for the genotypic characterization of *T. vaginalis.*

Keywords actin gene, genotypes, PCR-RFLP, *T. vaginalis*

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INTRODUCTION

Trichomoniasis is the most common curable sexually transmitted infection (STI) globally [1]. *Trichomonas vaginalis* causes vaginitis, urethritis, and cervicitis, but infection with *T. vaginalis* is frequently asymptomatic in both men and women [2]. Trichomoniasis increases the risk of pelvic inflammatory disease, infertility and adverse pregnancy outcome [3–5]. Trichomoniasis has also been found to be associated with an increased risk of human immunodeficiency virus transmission and acquisition [6–9].

There are still many unresolved questions regarding the epidemiology, pathogenicity and transmission of *T. vaginalis*, as well as drug resistance. Research in order to answer some of these questions would be greatly aided by a reliable classification method for *T. vaginalis*.

Studies of proteins, polysaccharides and isoenzyme profiles of *T. vaginalis* have shown that strain differences do exist, but attempts to develop a classification system based on biological differences, such as virulence, have so far been unsuccessful [10,11]. The development of DNA-based techniques offers new perspectives, and there are already several examples in the literature of the application of molecular methods to the study of the genetic diversity of parasites [12]. These methods include PCR–hybridization, PCR–size polymorphism, PCR–restriction fragment length polymorphism (PCR-RFLP) and random amplification of polymorphic DNA (RAPD) [12]. PCR fingerprinting by RAPD analysis and RFLP analysis are techniques that have

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been used to study variation in *T. vaginalis* [13–16]. These techniques, however, have their limitations. The reproducibility and reliability of RAPD have been found to be poor, and RFLP has low analytical sensitivity, due to the lack of an amplification step [15].

The ideal method for classifying *T. vaginalis* strains should have the sensitivity of PCR and the reliability of RFLP [12]. The PCR-RFLP technique combines PCR and RFLP and can reveal minor variations in a gene where a single base substitution has created or abolished a recognition site for the restriction endonuclease enzyme. The technique has proven its usefulness for the strain typing of different organisms, including *Chlamydia trachomatis*, *Treponema pallidum* and *Neisseria gonorrhoeae* [17–19]. The present article describes the application of this technique in an attempt to develop a reproducible, sensitive and specific molecular typing method for *T. vaginalis*.

MATERIALS AND METHODS

Isolates

Reference trichomonad strains. Fourteen strains that were representative of the family Trichomonadidae were used to assess specificity (Table 1). *T. vaginalis* ATCC 30001 was used in titration experiments to determine the analytical sensitivity of the PCR-RFLP assay.

The trichomonad trophozoites were cultured in modified trypticase yeast maltose medium [20] and incubated at 37°C under aerobic conditions. The pH of the medium was adjusted according to the requirement of the respective trichomonad.

Vaginal specimens. Vaginal specimens for *T. vaginalis* culture were obtained from female sex workers (FSWs) in Kinshasa (Democratic Republic of Congo) and Zambia. In Kinshasa, specimens were collected as part of a study on the prevalence of human immunodeficiency virus and other STIs among women attending a dedicated clinic for sex workers. The methods of this study have been described in detail elsewhere [21]. Samples of vaginal secretions were collected by a health worker during pelvic examination, using a sterile cotton swab. In Zambia, specimens were collected as part of the 2003 behavioural and biological surveillance survey (BBSS) among FSWs, which was carried out by Family Health International (Family Health International. Behavioural and biologic surveillance survey in selected transportation border routes, Zambia. Assessment between 2000 and 2003. Surveillance Studies Among Female Sex Workers. http://www.fhi.org/en/HIVAIDS/pub/index.htm). Participants were recruited at night at their place of work. Consenting FSWs were interviewed and requested to submit samples for testing for STIs. Self-administered vaginal swabs were collected for the diagnosis of gonorrhoea, chlamydial infection and trichomoniasis. Swabs collected in Kinshasa and in Zambia were processed in the same way. They were immediately inoculated in InPouch culture medium, following the manufacturer's instructions (Biomed Diagnostics, San Jose, CA, USA). The culture media were incubated at 37°C for a maximum of 5 days. After final microscopic reading, the pouches were stored at –20°C until shipment on dry ice to the Institute of Tropical Medicine in Antwerp, Belgium. Upon arrival, the culture media were stored at –20°C until tested. Prior to analysis, all participant identifiers were removed from the InPouch samples. An InPouch sample was considered to be positive for *T. vaginalis* if microscopy performed at the study site had been positive or if the sample tested positive on two independent PCR assays using the TVK3/7 and IP1/IP2 primer sets [22,23]. A more detailed description of these PCR assays is given elsewhere [24].

Table 1. Strains included in the analytical specificity tests

Species	Strain and source
Trichomonas vaginalis	ATCC 30001, ATCC 30240, ATCC 50141, ATCC 50144
Trichomonas gallinae	ATCC 30002
Tritrichomonas suis	ATCC 30169
Trichomonas tenax	ATCC 30207
Pentatrichomonas hominis	ATCC 30000, ATCC 30098
Pentatrichomonas hominis	PHKT, received from J. Kulda
Trichomonas vaginalis	Tv 17-48, received from J. Kulda
Tritrichomonas foetus	KVCL, received from J. Kulda
Trichomonas gallinae	TGK, received from J. Kulda
Tetratrichomonas gallinarum gallinarum	1-11 M2, received from J. Kulda

The strains with origin ATCC were obtained from the American Type Culture Collection.

DNA extraction

The genomic DNA from the trichomonads obtained by culture in trypticase yeast maltose or InPouch medium was extracted with the QIAamp DNA minikit (Qiagen, Hilden, Germany), following the manufacturer's instructions. An aliquot of 500 μL of culture medium was extracted, and DNA was eluted with 250 μL of Tris–acetate–EDTA buffer (pH 7.4).

PCR-RFLP

The target of the nested PCR was chosen within the actin gene. The outer primers (OPs) and inner primers (IPs) were chosen within an actin gene sequence belonging to a specific family of at least nine members of actin genes from the *T. vaginalis* genome (GenBank accession number AF237734) [25]. The OPs used were Tv8S (5′-TCTGGAATGGCTGAAGAAGACG-3′) and Tv9R (5′-CAGGGTACATCGTATTGGTC-3′), and the IPs used were Tv10S (5′-CAGACACTCGTTATCG-3′) and Tv11R (5′-CGGTGAACGATGGATG-3′). The primers were synthesized by Eurogentec (Seraing, Belgium). The size of the target was 1100 bp, which is only 28 bp shorter than the full length of the open reading frame of the actin gene.

The PCR mixture consisted of Expand High Fidelity buffer, 3 mmol/L $MgCl_2$, 280 μmol/L deoxyribonucleoside triphosphates (Pharmacia Biotech, St Albans, UK), 0.3 μmol/L of each primer of the primer sets (Tv8S, Tv9R) and (Tv10S, Tv11R), and 1.7 U of Expand High Fidelity DNA polymerase. The Expand High Fidelity was purchased in a kit format, which included Expand High Fidelity 10× buffer, $MgCl_2$ solution, and Expand High Fidelity Enzyme mix containing