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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

Taq DNA polymerase and *Tgo* DNA polymerase (Roche Applied Science, Penzberg, Germany). The volume was adjusted to 40 μL with Milli-Q water, and 10 μL of culture DNA extract was added to each reaction mixture for the amplification with the OPs. The same reaction mixture was prepared for amplification with the IPs, the volume was adjusted to 49 μL with Milli-Q water, and 1 μL of amplified product was added to each reaction mixture.

PCR amplification was performed in two stages in a thermocycler (Perkin-Elmer, Cetus, Norwalk, CT, USA). The first stage consisted of a total of ten cycles. Each cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and a 3 min extension at 72°C. The first cycle was preceded by 5 min of denaturation at 95°C. The second stage consisted of 25 cycles with the same denaturation and annealing steps. The extension step was extended by 5 s per cycle.

The last cycle was followed by a 7 min final extension at 72°C. Upon completion of PCR, 15 μL of each amplified specimen was analyzed by electrophoresis in a 2% agarose gel in Tris–acetate–EDTA buffer (pH 8.5). The gel was stained with ethidium bromide (0.5 mg/L; Sigma, Bornem, Belgium) and was photographed under short-UV light. The size of the amplified products was assessed by comparing them with a commercial weight marker, Smartladder (Eurogentec).

After visualization of the amplified product, 5 μL was digested for 4 h at 37°C with restriction endonucleases *Hin*dII, *Mse*I and *Rsa*I, respectively. The restriction endonucleases *Hin*dII and *Rsa*I were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and *Mse*I from New England BioLabs Inc. (Ipswich, MA, USA). The fragments were separated using 3% agarose gel in Tris–acetate–EDTA buffer (pH 8.5). The gel was stained with ethidium bromide (0.5 mg/L; Sigma) and was photographed under short-UV light. The size of the amplified products was assessed using a 100-bp commercial weight marker, Smartladder SF (Eurogentec).

All assays were performed according to standard quality assurance guidelines for molecular diagnosis [26,27]. In addition, each test run included one negative and one positive control. The negative control consisted of the reaction mixture with water instead of DNA extract, and the positive control was a previously typed *T. vaginalis* ATCC reference strain, randomly selected from among the ATCC reference strains at each run. Testing was considered valid if, after nested PCR, no amplified product was detected in the negative control and if, after digestion, the reference ATCC strain gave the previously determined actin genotype.

Sequencing

Amplicons from the *T. vaginalis* reference strains ATCC 30001, ATCC 30240 and ATCC 50141 and six clinical isolates (four from Zambia and two from Kinshasa) were sequenced to determine the size of the fragments and to confirm the banding patterns. Sequencing was performed by VIB Genetic Service Facility (Wilrijk, Belgium) using capillary sequencers (Applied Biosystems 3730 DNA Analyzer) combined with ABI PRISM BigDye Terminator cycle sequencing kits. In addition, the use of the ABI Genetic Analyzer for the determination of fragment size and banding patterns ensured that the results were unbiased.

The nucleotide sequences were deposited at GenBank under accession numbers EU076578–EU076586.

Assessment of analytical sensitivity

In order to determine the analytical sensitivity of the nested PCR technique, a ten-fold serial dilution of *T. vaginalis* trophozoites in Tris–acetate–EDTA buffer (pH 8.5) was extracted, as described above, and tested. The *T. vaginalis* trophozoites were counted in a Neubauer counting chamber.

Assessment of clinical sensitivity

An InPouch sample was considered to be truly positive for *T. vaginalis* if motile trichomonad trophozoites had been observed using microscopy or if the sample tested positive using PCR with two different primer sets (TVK3/7 and IP1/IP2). The sensitivity of the nested PCR assay performed on the InPouch media was calculated using this definition of positivity as an expanded reference standard. Estimates of the sensitivity with 95% CIs were made using standard methods.

Assessment of robustness and reproducibility

A panel of 14 DNA extracts of *T. vaginalis* isolates was amplified by nested PCR and digested twice by the restriction enzymes at two different points in time with an interval of 4 h.

The same panel of DNA extracts was amplified by nested PCR, 2 days after the first amplification run, and again digested by the restriction enzymes at two separate time points with an interval of 4 h.

RESULTS

Species specificity of the nested PCR

The actin gene nested PCR assay amplified all *T. vaginalis* reference strains but not trichomonad species other than *T. vaginalis*. There was also no amplification of sterile InPouch medium.

Sensitivity of the nested PCR

The analytical sensitivity of the nested PCR assay was determined to be equivalent to ten trichomonad trophozoites per reaction.

In total, 160 InPouch extracts that were positive according to the expanded reference standard were analyzed using the actin gene nested PCR assay. Five of these InPouch media were initially considered to be negative for *T. vaginalis* at the study site but were found to be positive on the two independent PCR assays. The actin gene nested PCR amplified 151/160 of the isolates, giving a clinical sensitivity of the nested PCR of 94.4% (95% CI 91.0–99.3). Of the nine isolates from which no amplified product was obtained with the actin gene nested PCR assay, five were also negative on the two primer set PCRs.

RFLP patterns

Digestion of the amplified product with *Hin*dII yielded three distinct DNA fragments of 827, 213 and 60 bp or four DNA fragments of 426, 401, 213 and 60 bp.

All isolates showed the 60 bp and 213 bp single bands. The presence of the 426 bp and 401 bp or 827 bp single fragments allowed classification of the isolates into one of two groups.

The restriction enzyme *Mse*I digested the amplified products into two or three fragments. Three groups could be distinguished on the basis of the presence of one fragment band of 519 bp or two fragment bands of 333 and 186 bp or 315 and 204 bp, besides the 581 bp fragment present in all isolates.

Four different DNA fragment patterns were obtained after digestion of the amplified products with the restriction enzyme *Rsa*I. Each pattern consisted of a minimum of four fragments. All of them showed bands of 236 and 106 bp. Differences among the patterns were based on the presence or absence of the 568 bp vs. 452 bp and 116 bp fragments, and of the 190 bp vs. 103 bp and 87 bp fragments.

By combining the DNA fragment patterns, eight distinct types could be identified. An isolate was considered to be a mixture of *T. vaginalis* strains if the total sum of the different fragments obtained with one specific restriction enzyme was superior to the target size of 1100 bp. However, simultaneous amplification of another member of the actin gene family with a different pattern cannot be ruled out. The exact size of the DNA fragments was determined by sequencing the amplicons of the *T. vaginalis* reference strains and by sequencing six clinical isolates as described above under Methods.

In conclusion, digestion of the amplified products with *Hin*dII, *Mse*I and *Rsa*I yielded two, three and four different digestion patterns, respectively. Genotypes were designated according to the combination of digestion patterns. Genotype A was defined as showing pattern 1 after *Hin*dII digestion, pattern 1 after *Mse*I digestion, and pattern 1 after *Rsa*I digestion, whereas genotype E was defined as showing pattern 1 after *Hin*dII digestion and pattern 2 after *Mse*I and *Rsa*I digestion. Table 2 presents the size of the different DNA fragments, pattern groups and *T. vaginalis* actin genotypes of the *T. vaginalis* isolates. Isolates with similar DNA patterns were considered to be a single *T. vaginalis* actin type.

Table 2. Size of fragments, pattern groups and actin genotypes of the *Trichomonas vaginalis* reference strains and clinical isolates from Kinshasa and from Zambia

	Restriction with *Hin*dII						Restriction with *Mse*I						Restriction with *Rsa*I							
Genotype	827 bp	426 bp	401 bp	213 bp	60 bp	*Hin*dII pattern	581 bp	519 bp	333 bp	315 bp	204 bp	186 bp	*Mse*I pattern	568 bp	452 bp	236 bp	190 bp	116 bp	106 bp	103 bp	87 bp	*Rsa*I pattern
A	+	–	–	+	+	1	+	+	–	–	–	–	1	+	–	+	+	–	+	–	–	1
E	+	–	–	+	+	1	+	–	–	+	+	–	2	+	–	+	–	–	+	+	+	2
G	–	+	+	+	+	2	+	+	–	–	–	–	1	+	–	+	+	–	+	+	–	1
H	–	+	+	+	+	2	+	+	–	–	–	–	1	+	–	+	–	–	+	+	+	2
I	–	+	+	+	+	2	+	+	–	–	–	–	1	–	+	+	+	+	+	–	–	3
M	–	+	+	+	+	2	+	–	+	–	–	+	3	+	–	+	+	–	+	–	–	1
N	–	+	+	+	+	2	+	–	+	–	–	+	3	+	–	+	–	–	+	+	+	2
P	–	+	+	+	+	2	+	–	+	–	–	+	3	–	+	+	–	+	+	+	+	4

+, fragment present; –, fragment absent.

The *T. vaginalis* actin genotypes of the reference strains were determined to be as follows: ATCC 30001 = genotype G; ATCC 30240 = genotype H; ATCC 50141 = genotype E; ATCC 50144 = genotype G; and Tv 17–48 = genotype E.

Separation of the DNA fragments by gel electrophoresis is shown in Fig. 1 for the *T. vaginalis* actin genotypes of the ATCC reference strains 30001, 30240 and 50141, genotypes G, H and E, respectively. Fig. 2 shows the gel electrophoresis patterns of the DNA fragments of the isolates Kinshasa K2143, Zambia N133, and Zambia N74, which represent actin genotypes M, E and H, respectively.

Assessment of robustness and reproducibility of the PCR-RFLP

Amplification and digestion of the amplicons on different days gave identical DNA fragments.

Nucleotide sequences

Supplementary Fig. S1 shows the alignment of the sequences of the actin gene target of the strains ATCC 30001 (GenBank accession number EU076578), ATCC 30240 (GenBank accession number EU076579), ATCC 50141 (GenBank accession number EU076580), strain K2143* (GenBank accession number EU076583) and strain K1086 (GenBank accession number EU076584) from Kinshasa, and strains N78 (GenBank accession number EU076585) and N113 (GenBank accession number EU076586) from Zambia, against the sequences described by Espinosa *et al.* (GenBank accession number AF237734) and by Bricheux *et al.* (GenBank accession number U63122) [25,28].

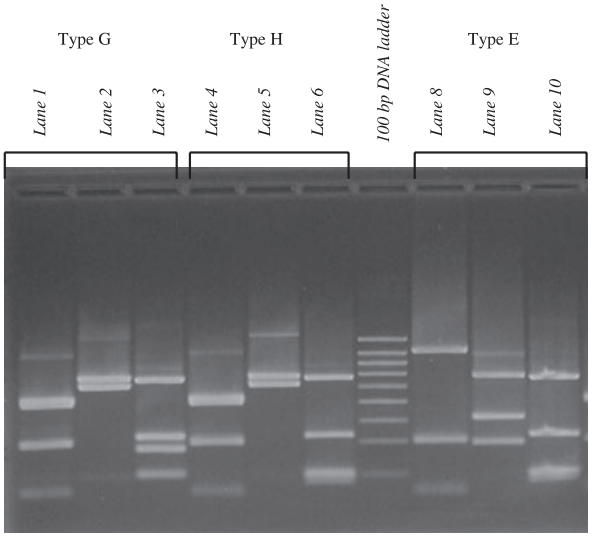

Fig. 1. Gel electrophoresis of DNA fragments obtained after digestion of the Tv actin genotypes G, H, and E. Lanes 1, 2 and 3 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of ATCC 30001, genotype G; lanes 4, 5 and 6 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of ATCC 30240, genotype H; lane 7 is a 100-bp DNA ladder; lanes 8, 9 and 10 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of ATCC 50141, genotype E. The faint, less intensive, bands of higher sizes were not scored.

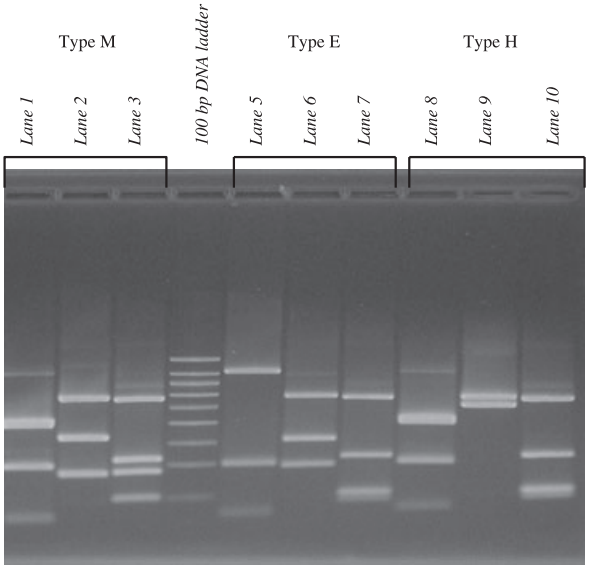

Fig. 2. Gel electrophoresis of DNA fragments obtained after digestion of the Tv actin genotypes M, E, and H. Lanes 1, 2 and 3 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of specimen K2143, genotype M; lane 4 is a 100-bp DNA ladder; lanes 5, 6 and 7 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of specimen N133, genotype E; lanes 8, 9 and 10 show the banding pattern after digestion with *Hin*dII, *Mse*I, and *Rsa*I, respectively, of specimen N74, genotype H. The faint, less intensive, bands of higher sizes were not scored.

In total, 15 different single nucleotide changes in the open reading frame of the actin gene were detected. Three of them, nucleotides 371, 575, and 904, resulted in an amino acid substitution.

Geographical distribution of the Tv actin genotypes

The *T. vaginalis* RFLP types were determined for 61 isolates from Kinshasa, and for 90 isolates from

Table 3. Prevalence of the *Trichomonas vaginalis* actin genotypes in Kinshasa and Zambia

	No. of *T. vaginalis* actin genotype	
Tv actin genotype	Kinshasa, *n* (%), *N* = 61	Zambia, *n* (%), *N* = 90
A	1 (1.6)	1 (1.1)
E	34 (55.7)	5 (5.5)
G	14 (23.0)	42 (46.7)
H	6 (9.8)	15 (16.7)
I	1 (1.6)	6 (6.7)
M	2 (3.2)	0
N	2 (3.2)	6 (6.7)
P	0	5 (5.6)
Mixed	0	10 (11.0)
Not typeable	1 (1.6)	

Zambia. Eight different types were identified among the isolates from Kinshasa and Zambia. One isolate from Kinshasa could not be typed. The distribution of the types is presented in Table 3. In Kinshasa, the most common type was type E (57% of isolates), whereas in Zambia the most common type was type G (47% of isolates). Patterns that were suggestive of mixed infection were found in Zambia (11% of isolates) but not in Kinshasa.

DISCUSSION

Strain typing techniques are useful tools to study the epidemiology of infectious organisms. They provide information on the nature and extent of genetic diversity of the organism in a specific population [12]. For *T. vaginalis* in particular, the technique can be used to study modes of transmission, pathogenicity and drug resistance.

So far, only two promising molecular typing techniques for *T. vaginalis* have been described in the literature, including an RAPD technique described by Vanacova *et al.* [13], and an RFLP technique followed by hybridization with a heat shock protein 70 (HSP70) probe described by Stiles *et al.* [14]. Vanacova *et al.* [13] used five random primers (OPD1–OPD5) and two primers complementary to the *T. vaginalis* repetitive fragment TV E-650, and included ATCC reference strains. The same technique was applied by two other research groups using four (OPD1, OPD2, OPD3, OPD5) and five (OPD1–OPD5) random primers, respectively [29,30]. It is not clear whether the same reference strains were used, and if identical RAPD patterns were obtained, which would have confirmed the validity of the results. Kaul and colleagues found that isolates from symptomatic women appeared to form two distinct clusters, whereas Snipes *et al.* found certain RADP patterns to be associated with metronidazole resistance. Fraga *et al.* [16] optimized the RAPD technique by designing ten random primers (Tv1–Tv10). Using this method, an association was found between the genetic variability of *T. vaginalis* and clinical presentation of infection. A genetic marker of 490 bp appeared to be present only in isolates from symptomatic patients [31]. Stiles *et al.* published an RFLP technique in which *T. vaginalis* DNA was digested with *Eco*RI. Fragments were transferred to a nylon membrane, and this was followed by hybridization with a HSP70 probe. For each of the five ATCC reference strains, they obtained a unique *Eco*RI HSP70 RFLP profile. They could not find an association between metronidazole resistance and RFLP subtypes [14].

In this study, an PCR-RFLP technique was applied, combining the higher sensitivity of the PCR with the reliability of the RFLP. The actin gene was selected as the target, on the basis of the role of actin in the morphological changes of the parasite. Actin is a ubiquitous protein involved in the formation of filaments, which constitute a major component of the cytoskeleton. It provides the basis for muscular contraction and many aspects of cell motility. *T. vaginalis* changes from the flagellate to the amoeboid form when it adheres to the host cells, and pseudopods are formed at the site of contact [28]. The ability to undergo morphological changes is presumed to be related to virulence, and the importance of cytoskeletal integrity for *T. vaginalis* cytopathogenicity has been highlighted [32–34]. Actin might thus play a role in the pathogenesis of the parasite. The actin protein of *T. vaginalis* is encoded within a family of at least nine genes [25,28]. The internal transcribed spacer region of the rDNA was not considered to be the target for the PCR-RFLP, as Snipes *et al.* [29] concluded that the internal transcribed spacer region of *T. vaginalis*, in contrast to those of other organisms, lacked variability. Recently, a PCR-RFLP technique for *T. vaginalis* that included amplification of the ribosomal intergenic spacer regions and digestion with eight restriction enzymes was published. However, the observed polymorphism was not significant, and sequence information on the amplified intergenic spacer rDNA was lacking [35].

The PCR-RFLP technique described here enabled identification of a total of eight different *T. vaginalis* types. In addition, different fragment patterns obtained for types E, G, H, I, M, N and P were confirmed with the determination of their respective nucleotide sequences. Different distributions of types were found in Kinshasa (Democratic Republic of Congo) and Zambia. Patterns suggestive of mixed infections were found only in Zambia. The absence of mixed infections in Kinshasa could be explained by the much lower prevalence of trichomoniasis among women in Kinshasa than among women in Zambia (8.6% vs. 30.1%) [21]. The *T. vaginalis* actin genotypes of the reference strains were not different from the types detected in Kinshasa and Zambia, confirming the findings of Stiles *et al.* [14], who describe the stability of *T. vaginalis* strains. The stability of the genotypes is in line with the clonal reproduction of parasitic protozoa, resulting in the production of clones that are stable over time and across geographical areas [14].

The discriminative ability of our method is comparable to that of the RFLP technique targeting HSP70 described by Stiles *et al.* [14]. With this technique, ten different types were identified. However, the overall discriminative power of the designed method was low. The actin gene may not be a suitable marker for strain discrimination if it is used on its own, but the overall discriminative power can be improved if a combination of markers for strain discrimination is used [36].

There was 100% similarity in this study between the nucleotide sequences of reference strain ATCC 50141, clinical isolate N133, both actin genotypes E, and the actin clone sequence reported by Espinosa *et al.* [25]. Among the other determined nucleotide sequences, 15 different single nucleotide changes were observed in the open reading frame of the actin gene. Twelve were silent mutations, and three were missense mutations resulting in an amino acid substitution in the primary protein structure of the actin. The simultaneous three amino acid substitutions occurred in genotype G; nucleotide 371 substituted alanine for valine, nucleotide 575 substituted glycine for aspartic acid, and nucleotide 904 substituted lysine for glutamine. Genotypes H, M, I and P all showed the same two amino acid substitutions; alanine was substituted for valine, and lysine for glutamine. The only missense mutation detected in the nucleotide sequence of genotype N resulted in substitution of alanine for valine. The nucleotide sequence of genotype A was not determined.

In this study, at least ten trophozoites were needed per reaction to have an adequate concentration of amplicons for the restriction assays. The longer target size was selected to determine the maximal numbers of mutations. However, on the basis of these results, shortening of the target size could be considered, which may increase the sensitivity of the nested PCR, and make it eventually more suitable for the application of the PCR-RFLP technique for clinical specimens.

In conclusion, a sensitive, specific and easy-to-perform strain typing method for *T. vaginalis* was developed. The technique was evaluated using *T. vaginalis* isolates obtained by *in vitro* culture of clinical isolates, which may have led to selective growth and selection of genotypes [12]. Further studies should focus on the validation and application of this typing technique using clinical specimens. Additional studies are needed to determine the technique's particular application in molecular epidemiological studies of *T. vaginalis*.

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TRANSPARENCY DECLARATION

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SUPPORTING INFORMATION

The following Supporting Information may be found in the online version of this article:

Fig. S1. Alignment of the nucleotide sequences of *Trichomonas vaginalis* actin gene of different *T. vaginalis* isolates.

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