MOLECULAR TYPING OF TRICHOMONAS VAGINALIS ISOLATES BY HSP70 RESTRICTION FRAGMENT LENGTH POLYMORPHISM

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Abstract. Subtyping isolates of Trichomonas vaginalis is an essential tool for understanding the epidemiology of this common sexually-transmitted disease. Restriction fragment length polymorphism (RFLP) analysis employing a probe from the heat-inducible cytoplasmic HSP70 gene family hybridized with EcoR I-digested genomic DNA was used in the molecular typing of Trichomonas isolates. Analysis of five American Type Culture Collection (ATCC) reference strains and 31 Jackson, Mississippi, isolates from six male and 21 female patients, revealed 10 distinct RFLP pattern subtypes of Trichomonas. The subtypes were temporally stable and cosmopolitan. The RFLP profiles seen in Maryland, Ohio, Massachusetts, and New York ATCC strains were identical to those of some Mississippi isolates, even though the samples were isolated 10–35 years apart. There was no correlation between metronidazole resistance and RFLP subtype with resistant isolates from eight patients distributed among six different subtypes.

INTRODUCTION

Trichomonas vaginalis is the most common non-viral sexually-transmitted disease in the world with at least five million cases occurring annually in the United States alone.1 Trichomonas causes vaginitis, urethritis, and cervicitis,2,3 and is associated with adverse outcomes of pregnancy4,5 and increased risk of human immunodeficiency virus (HIV) acquisition.6 Trichomonas vaginalis is common in some subpopulations with a prevalence of up to 50% of women and 10% of men attending sexually-transmitted–disease clinics.7,8 The disease is frequently asymptomatic in both men and women which makes detection and eradication difficult.9 In addition, there are increasing reports of Trichomonas that is resistant to metronidazole, the only drug currently approved for treatment of the disease in the United States.10

The epidemiology of trichomoniasis is poorly understood as are the precise factors affecting transmission dynamics and pathogenesis. Molecular fingerprinting utilizing intra-specific variation in the microbial genome to identify characteristic subtypes is a valuable epidemiologic tool for many infectious diseases. These methods include restriction fragment length polymorphism (RFLP) of genomic DNA, polymerase chain reaction RFLP (PCR-RFLP), and random amplification of polymorphic DNA (RAPD).11 The RAPD technique has been used to assess interspecies phylogeny and intraspecies polymorphism of Trichomonas.12,13 A potential drawback, however, in its utilization for Trichomonas epidemiology is its inherent variability in response to even slight changes in experimental conditions. The reproducibility of PCR assays can be influenced by the PCR machine used, which affects temperature and ramping parameters, as well as by the quantity and purity of the template DNA, by reagent quality, and even by the placement of individual micro-centrifuge tubes within the PCR block. Utilization of RFLP, which is less sensitive to experimental variation than PCR, would be useful for typing T. vaginalis strains and serve as a basis for evaluating PCR-based typing among different laboratories. The goal of this study was to establish reference RFLP profiles of intraspecies differences for local clinical isolates and American Type Culture Collection (ATCC) standard isolates of T. vaginalis.

MATERIALS AND METHODS

Parasites. Trichomonas vaginalis strains (Table 1) were purchased from the American Type Culture Collection (ATCC, Manassas, VA) or were isolated from a convenience sample of predominantly black patients attending either the Hinds County Sexually Transmitted Disease (STD) Clinic or the University of Mississippi Medical Center Emergency Department (ER). Vaginal, urethral, or urine samples were obtained from patients undergoing examinations in the ER and from clinic patients who were self-referred for unprotected sexual contacts or symptoms of STD. Thirty-one percent of female ER patients15 and eight percent of male ER patients with symptoms of urethritis or dysuria were culture-positive for T. vaginalis. Race/ethnicity data were not available on all patients and were unavailable for donors of T. vaginalis reference strains obtained from the ATCC. Informed consent was obtained from all local patients. These studies were approved by the Institutional Review Board for Human Investigation, University of Mississippi Medical Center.

Parasites were obtained from urethral/penile discharge, posterior vaginal fornix, or urine sediment. Trichomonas trophozoites were isolated in modified Trypticase-Yeast Maltose (TYM) medium supplemented with 10% heat-inactivated adult bovine serum and antibiotics (50 μg/mL gentamicin, 60 μg/mL ciprofloxacin, 25 μg/mL clindamycin, and 50 μg/mL fluconazole), grown and harvested at mid-logarithmic phase (10^6 cells/mL) as described previously.16 Axenic parasites at mid-log phase were archived as frozen isolates in 5% DMSO at −70°C.

Nucleic acid isolation. Genomic DNA for RFLP studies was isolated from log phase (10^6 parasites/mL) cultures using the DEPC-Triton X-100 method of Riley and Krieger17 with two additional chloroform extractions to reduce nuclease contamination. Purity of DNA isolates was determined conventionally by determining OD260/OD280 ratios.

RFLP analysis. Five micrograms of T. vaginalis genomic
DNA was restriction endonuclease digested with EcoRI, HindIII, HindIII, MspI, Sau3A1, PstI, or XbaI, resolved by electrophoresis in a 1% agarose gel, transferred to a nylon membrane, and hybridized with *T. vaginalis* probes from a 2223 nucleotide (nt) cytoplasmatic heat-shock protein 70 (HSP70) cDNA (GenBank U93873, nt 31-2253), a 1210 nt fragment of the sarcoplasmic-endoplasmic reticulum calcium motive ATPase gene (Genbank U65066, nt 704-1913), or a 1557 nt fragment of the 16S ribosomal RNA gene (GenBank U17510, nt 16-1572), which were radiolabelled by random priming. Blots were washed to a final stringency of 0.1x SSPE (15 mM NaCl, 1 mM NaHPO₄, 0.1 mM EDTA), 0.5% sodium dodecyl sulfate at 45°C, and exposed for autoradiography at −70°C.

**Metronidazole resistance.** Determination of the aerobic minimum lethal concentration (MLC) values for metronidazole, expressed in μg/mL, for *T. vaginalis* isolates, followed the protocol of Lushbaugh and others. Minimum lethal concentration was determined by measuring tritiated thymidine incorporation in *Trichomonas* cells after 24 hr exposure to serial dilutions of metronidazole. *Trichomonas* strains with an MLC of less than 10 μg/mL were considered susceptible to metronidazole; moderate metronidazole resistance was defined as an MLC of 10–100 μg/mL; and high level resistance was an MLC ≥ 100 μg/mL.

**RESULTS**

A total of 36 *T. vaginalis* isolates obtained from 27 patients were analyzed by RFLP of restriction endonuclease-digested genomic DNA. RFLP profiles were generated for five ATCC reference strains, all from female patients, and 31 local (Jackson, MS) isolates from 16 female and six male patients (Table 1). The local isolates include sets of paired vaginal and urine isolates (D10, STM, D4, and SPN), male urethral swab and urine isolates (B157), and from a pair of sexual partners (SMP). Different combinations of genomic DNA digested with restriction enzymes containing four nucleotide recognition sites (MspI, Sau3A1) or six nucleotide recognition sites (EcoRI, HincII, HindIII, PstI, XbaI), and radiolabelled probes from a family of dispersed, unlinked heat-inducible cytoplasmic HSP70 genes, a single copy sarcoplasmic-endoplasmic reticulum calcium motive ATPase gene, and from the repeated, linked ribosomal rRNA gene array, were tested in Southern blots for their ability to discriminate *T. vaginalis* strains by RFLP (data not shown). The RNA and SERCA probes failed to adequately differentiate *T. vaginalis* strains, digestion with four nt restriction endonucleases generated complex RFLP patterns, and digestion with most six-nt restriction enzymes was non-discriminating or produced large restriction fragments that were difficult to adequately separate on agarose gels. Digestion with EcoRI and hybridization of the DNA fragments produced with a radiolabelled 2.2 kilobase (kb) *T. vaginalis* HSP70 cDNA yielded the optimal pattern of readily resolvable restriction fragments suitable for RFLP grouping of *T. vaginalis*.

In each of the isolates tested, 6 to 9 distinct DNA fragments ranging from 1.8 kb to > 7.0 kb in size were detected by hybridization of EcoRI digested DNA to the HSP70 cDNA probe. Southern blots of four representative gels illustrating the RFLP patterns produced are shown in Figure 1, Panels A–D. All isolates demonstrated single bands of 2.7 and 3.7 kb, as well as a 5.3/5.5 kb doublet, with the exception of the D6 isolate (Figure 1, Panel C, Lane 10), which had a single 5.3 kb band. The presence or absence of additional hybridizing EcoRI fragments permitted the separation of isolates into 10 distinct groups with unique banding patterns, designated subtypes 1–10. The subtyping is based on the RFLP banding pattern of fragments smaller than the 5.3/5.5 doublet bands since the resolution of bands > 7.0 kb and their transfer to nylon membranes were variable in different experiments. The grouping of individual isolates into subtypes 1–10 is summarized in Table 1, and a pictorial depiction of the subtype banding patterns is shown in Table 2. The same RFLP banding patterns for fragments up to 5.5 kb in size were obtained on multiple gels using different DNA samples for each individual isolate, indicating that the RFLP subtypes identified were consistent and reproducible.

Several of the groups contain only a single or few repre-
FIGURE 1. Restriction fragment length polymorphism (RFLP) profiles of *Trichomonas vaginalis* local (Jackson, MS) and American Type Culture Collection (ATCC) isolates analyzed on Southern blots containing 5 μg EcoRI digested genomic DNA hybridized with a *T. vaginalis* cytoplasmic HSP70 cDNA. Selected band sizes based on molecular weight markers are indicated in the left and right margins. RFLP subtypes 1–10 are indicated above each lane. Subtypes and isolates listed from left to right are: Panel A, subtype 8—SPN (U), SPN; subtype 5—STM (U), STM; subtype 1—SMP (M); subtype 6—SMP (F). Panel B, subtype 7—PA; subtype 5—C6; subtype 6—ATCC 30238; subtype 5—ATCC 50140, subtype 3—ATCC 50143. Panel C, subtype 8—D1, D1F; subtype 2—D2; subtype 9—D3, D3F; subtype 7—D4 (U); subtype 9—D4F; subtype 7—D4; subtype 2—D5; subtype 10—D6, subtype 1—D8. Panel D, subtype 1—ATCC 30235; subtype 2—D10, D10 (U), B157 (U), B157; subtype 3—B161, EWA, EWB; subtype 4—B95.

### DISCUSSION

Effective epidemiological assessment of sexually-transmitted diseases such as trichomoniasis is achieved with tools which identify the causative agent and measure modes of transmission, prevalence, pathogenicity, virulence, and drug resistance. Many studies have described the clinical presentation of trichomoniasis and its diagnosis by microscopic, culture, and antibody-based techniques, and more recently nucleic acid-based diagnostic techniques. However, although trichomoniasis is one of the most common sexually-transmitted diseases in the world, little has been done to study transmission dynamics, incidence and prevalence, virulence factors, mixed infections, and spread of drug resistance—primarily due to the lack of appropriate assays for the detection and typing of *Trichomonas* strains.

Isoenzyme analysis, the generation of electrophoretic protein profiles, serotyping with antibody, and more recently, DNA-based techniques such as restriction fragment length polymorphism (RFLP) and/or nucleic acid amplifications by PCR (RAPD), have become useful tools in molecular typing, epidemiology, phylogenetics, and forensics. The first three approaches however, are limited by their dependence on protein/antigen expression, which can be variable. DNA-based techniques, PCR and RFLP, have the unique advantage of measuring fixed, stable differences present in the genome. PCR-amplification offers the advantages of speed and sensitivity, but results can also be variable and sensitive to experimental conditions. In contrast, RFLP is more time-consuming but less sensitive to experimental variation and has better reproducibility among separate experiments and different laboratories. The ideal protocol for typing *Trichomo-
nas strains would clearly combine the speed and sensitivity of PCR with the reliability of a more conventional technique such as RFLP. As a necessary first step in developing a means of strain typing, we have identified a suitable enzyme/probe combination for RFLP genomic fingerprinting of T. vaginalis isolates. The utility of the assay described here is that it simultaneously measures the polymorphisms present at multiple gene loci in the cytoplasmic HSP 70 gene family, using a single probe.17 This EcoRI-HSP70 RFLP technique thus provides a basis for future epidemiological studies of *Trichomonas*, as well as a standard with which to judge emerging PCR techniques for *Trichomonas* strain typing.

Examination of a group of local (Jackson, MS) isolates and ATCC reference strains by EcoRI-HSP70 RFLP identifies a set of diverse *Trichomonas* subtypes, of which some are cosmopolitan and chronologically stable. The ATCC reference strains originally isolated in 1963 (ATCC 30235 and 30238), 1980 (ATCC 50143), and 1983 (ATCC 50140 and 50142) exhibited the same EcoRI-HSP70 RFLP profile as local isolates obtained in the period from 1993–1998. The RFLP method grouped the isolates from 27 patients into ten subtypes (Tables 1 and 2) on the basis of bands common to all isolates and bands shared among multiple subtypes. Isolates from four different states had RFLP profiles identical with isolates from the state of Mississippi, suggesting a widespread distribution of *Trichomonas* strains within the United States. Due to the limitations that a small sample size and the bias inherent in the self-referral process used to obtain these *Trichomonas* isolates imposed upon the data, we cannot conclude that temporal stability and widespread distribution will be characteristic of all *Trichomonas* subtypes. It is probable, however, that the ten subtypes identified in the current study underestimate the diversity that might be demonstrated by examining a larger sample.

Similar geographical concordance and high levels of strain heterogeneity have been observed in other limited studies of *Trichomonas* isolates.14,27,29 Isoenzyme characterization based on the electrophoretic patterns of four enzymes, classified 32 *Trichomonas* isolates into five groups,29 and 63 isolates into 15 groups.27 The reaction-profiles of 88 isolates to a battery of monoclonal antibodies identified 11 distinct patterns; some groups contained isolates from five disparate geographic sites and one geographic site contained representatives of all 11 groups.27 In the same study, identical profiles obtained for the ATCC reference strains and for isolates collected 25 years later, also suggest a stable antigenic composition of some *Trichomonas* populations over time. A general theory of clonal reproduction has been proposed for parasitic protozoa, and those clones appear to be stable across large geographical areas and over long periods of time.46 An RAPD study of *Trichomonas* strains indicated that some European isolates were more similar to isolates from the United States than they were to other European isolates.14 An observed correlation of metronidazole resistance with RAPD similarities in that study contrasts with the lack of such correlation of RFLP subtypes with metronidazole resistance in our investigation. The reasons for this discrepancy are not clear and point to the need for a more extensive evaluation of *Trichomonas* epidemiology.

Different subtypes were isolated prior to treatment and after failure of therapy (D4, D4F) from one of our patients who strongly denied reexposure, thus raising the question of whether patients may be infected with multiple subtypes. These subtypes might be selected on the basis of drug pressures, immune response, or in vitro culture conditions. The presence of multiple subtypes would complicate studies examining the association of particular strains or subtypes with virulence factors or drug resistance. We have not observed any evidence of mixed subtypes in any of the isolates we have characterized; presumably *in vitro* selective pressures during the axenization of *Trichomonas* isolates result in a single predominant subtype in established cultures. These considerations indicate a need for cloning isolates directly from patient samples prior to selective *in vitro* pressures. In addition, different subtypes were isolated in one couple (SMP) who were sexual partners, which raises the question of whether their own microenvironment selected out different subtypes or whether this was simply a manifestation of selective pressures within individuals in a population is an ideal epidemiologic tool to study sexual networks and the transmission dynamics of STDs.

**Table 2**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Representative isolates</th>
<th>Band size (kilobase)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ATCC-30235</td>
<td>2–3</td>
</tr>
<tr>
<td>2</td>
<td>B157</td>
<td>2–3</td>
</tr>
<tr>
<td>3</td>
<td>ATCC-50143</td>
<td>2–3</td>
</tr>
<tr>
<td>4</td>
<td>B195</td>
<td>2–3</td>
</tr>
<tr>
<td>5</td>
<td>ATCC-50140</td>
<td>2–3</td>
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<tr>
<td>6</td>
<td>ATCC-30238</td>
<td>1–3</td>
</tr>
<tr>
<td>7</td>
<td>ATCC-50142</td>
<td>2–3</td>
</tr>
<tr>
<td>8</td>
<td>D1</td>
<td>2–3</td>
</tr>
<tr>
<td>9</td>
<td>D3</td>
<td>2</td>
</tr>
<tr>
<td>10</td>
<td>D6</td>
<td>3</td>
</tr>
</tbody>
</table>

* Presence of a band of the indicated size is denoted by a filled box.

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REFERENCES


