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Development of a Polymerase Chain Reaction-Based Diagnosis of *Trichomonas vaginalis*

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We developed a polymerase chain reaction (PCR)-based test for detecting the protozoan parasite *Trichomonas vaginalis*. Genomic libraries were constructed from two independent clinical isolates of *T. vaginalis*. From these libraries, 12 genomic clones were purified, sequenced, and then screened for uniqueness by computer-assisted sequence comparisons. PCR reactions were performed to evaluate eight PCR-primer pairs, including a primer pair that targeted the *T. vaginalis* ferredoxin gene. All eight primer pairs yielded PCR products of the expected sizes. However, six of the primer pairs amplified their respective target sequences in limited numbers of clinical *T. vaginalis* isolates, suggesting the presence of significant genomic variability among isolates. An exception was a primer pair, termed TVA5-TVA6, that amplified a 102-bp genomic sequence, termed A6p, in all of 24 clinical isolates. The A6p sequence was not detected by PCR in human DNA or in a wide variety of flagellates, ciliates, or bacteria tested. The A6p sequence appears highly selective for a broad range of *T. vaginalis* isolates and holds promise for PCR-based diagnosis of the parasite.

Trichomonas vaginalis is a protozoan parasite that afflicts an estimated 180 million women per year worldwide (17, 26). The parasite is a major cause of vaginitis, cervicitis, and urethritis in women and may cause nongonococcal urethritis, prostatitis, and perhaps other lower genitourinary tract syndromes in men (23–25).

Accurate diagnosis is necessary for specific treatment of trichomoniasis. Routine clinical diagnosis usually depends on microscopic identification of the parasite in wet mount preparations (9, 45). Unfortunately, wet mount examination detects only 60% of culture-positive cases in women (8, 45, 56). Other diagnostic methods include the use of cultures, cytologic smears (37, 54), and direct immunofluorescence with monoclonal antibodies (24). Culture is considered the most reliable diagnostic method followed by direct immunofluorescence (27). Cultures require specialized medium, a trained microbiologist, and 2 to 7 days for diagnosis. For these reasons, accurate culture techniques are currently unavailable to most clinicians. Furthermore, even redundant culture techniques may miss (i) parasites present in low numbers, (ii) defective parasites, or (iii) organisms not surviving the transfer to culture medium. New polymerase chain reaction (PCR)-based methods offer a high degree of selectivity and the ability to amplify target sequences (7, 38). Compared with culture techniques, PCR methods offer advantages of extreme sensitivity, potentially shorter time between initial observations and specific diagnosis, and the ability to detect nonviable or defective microorganisms (7, 38). Further, skills and materials needed to perform PCR tests for a given microorganism are easily adapted to other microorganisms for which PCR tests are available.

An important consideration for the development of PCR-based detection of *T. vaginalis* was the fact that *T. vaginalis* isolates express a high degree of phenotypic variation (1, 2, 24, 50, 52), suggesting differences at the level of expression or genomic sequences or both. Therefore, our strategy was first to evaluate multiple genomic loci as potential targets for PCR-based diagnosis and then to test isolates from a variety of clinical sources.

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MATERIALS AND METHODS

Parasites and cell culture. Microorganisms used in these studies included fresh clinical isolates of *T. vaginalis* from patients attending the Harborview Medical Center Sexually Transmitted Diseases Clinic and the University of Washington Medical Center Prostatitis Clinic (Seattle, Wash.). *T. vaginalis* isolates were obtained from women with signs and symptoms of vaginitis or cervicitis or both (S1114 and S1126) and from asymptomatic women with no clinical signs other than *T. vaginalis*-positive cultures (S996, S975, S1047, S1163, S996, and S975). *T. vaginalis* isolates were also obtained from men with urethritis (S981, S1220, S764, S1138, and S933) whose concomitant cultures for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* were negative and from asymptomatic men (S1190, S1202). We also evaluated isolates from patients in Brooklyn, N.Y. (B34, kindly supplied by William McCormack) and Charlottesville, Va. (C7, kindly supplied by Michael Rein). *T. vaginalis* isolates S13, B34, and C7, maintained in liquid nitrogen, have been described previously (24). Isolate S13 is a laboratory-adapted strain which has undergone repeated passage in culture. Prior to use, each isolate was cloned in agar and then grown to mid-log phase in Diamond's medium at 37°C in 5% CO_2 (25).

DNA isolation. *T. vaginalis* DNA was purified by a recently reported method (42). Briefly, mid-log-phase *T. vaginalis* cultures (75 to 150 ml) were chilled on ice for 10 min and then harvested by centrifugation at 900 × *g* for 5 min at 4°C. The cellular pellet (0.5 ml) was washed twice by centrifugation in 40 volumes of sterile phosphate-buffered saline (PBS, pH 7.4, 4°C), and the pellet was suspended in 1 ml of PBS. After addition of 20 μl of diethyl pyrocarbonate (DEPC; Sigma, St. Louis, Mo.) the cell suspension was shaken and then allowed to sediment on ice for 5 min.

Fresh Triton X-100–reticulocyte standard buffer (RSB) (10 mM Tris, 10 mM NaCl, 5 mM $MgCl_2$ [pH 7.4] containing 1%

[vol/vol] Triton X-100 [Sigma]) was prepared. For each cell suspension to be processed, 40 μl DEPC was added to 100 ml of Triton X-100–RSB (4°C), and the mixture shaken vigorously. The DEPC-treated *T. vaginalis* cells were twice centrifuged (900 × *g*, 3 min, 4°C) in excess DEPC–Triton X-100–RSB. The resulting pellet was heat treated, proteinase K digested, and phenol-chloroform extracted as previously described (42).

The DNA obtained by this method was >12 kb in size, as determined by direct gel analysis (data not shown). All *T. vaginalis* template DNAs were prepared by the same method and used at identical initial concentrations, as determined by multiple A_{260} readings and confirmed by direct gel analysis of stock DNA solutions.

Acanthamoeba castellanii cultures were gifts from Thomas Fritche (Department of Microbiology, University of Washington). *Acanthamoeba* cells were washed twice in sterile PBS, and DNA was prepared as described above for *T. vaginalis* cells. *Giardia lamblia* BE1 DNA was a gift from Mansour Samadpour (Department of Environmental Health, University of Washington). *Tetrahymena thermophila* CU427 DNA was obtained from Meng Chou Yao (Fred Hutchinson Cancer Research Center, Seattle, Wash.). *Paramecium tetraurelia* DNA was a gift from Robert Hinrichsen (Fred Hutchinson Cancer Research Center). *Saccharomyces cerevisiae* was a gift from Stephan Zweifel and Walton Fangman (Department of Genetics, University of Washington). *Trypanosoma* DNAs were gifts from Harvey Eisen (Fred Hutchinson Cancer Research Institute). Human DNA was isolated from a lymphocyte culture obtained from Hans Ochs (Department of Pediatrics, University of Washington). The anaerobic strain, *Gardnerella vaginalis* GV9016B was grown for 1 to 4 days on agar plates, while broth-grown enterococci were prepared as previously described (43, 44). *Peptostreptococcus anaerobius* Clt 17.5, *Peptostreptococcus tetradius* Clt 92.2, and *Streptococcus agalactiae* AES DNAs were prepared as described by LeBlanc and Lee (30). *G. vaginalis*, *Bacteroides intermedius* 25261, *Bacteroides disiens* Clt 75.5, *Fusobacterium nucleatum* 6601 TE10, *Veillonella parvula*, and *Enterococcus faecalis* DS160 DNAs were extracted by the method previously described for anaerobic *Mobiluncus curtisii* (49). Standard DNA extraction protocols were used for *N. gonorrhoeae* F62 (43).

Vaginal swabs were obtained from 12 women attending the Harborview Sexually Transmitted Disease Clinic. Nine of the swabs were positive for *T. vaginalis* by wet mount examination and three were negative. The swabs were vigorously swirled in 0.5 ml of PBS (4°C) in 1.5-ml microcentrifuge tubes (Fisher Scientific, Seattle, Wa.). Cellular material was twice pelleted (1,000 × *g*, 5 min) and washed by resuspension in cold PBS. As described elsewhere (14, 22), the final pellets were suspended in 400 μl of K buffer, and proteinase K (Bethesda Research Laboratories) digestion was carried out.

Strategies for optimizing PCR effectiveness. To maximize the speed and simplicity of our PCR methods, we employed direct, ethidium bromide-stained gel analysis of the PCR products and corresponding restriction fragments (5, 36). Although dot blot hybridizations confirmed the identity of PCR products in this study, hybridizations involving dot blots or Southern blots require significant additional processing and would not be practical for most clinical laboratories. For this reason, we persisted in evaluating a variety of PCR primer pairs until uniform PCR amplification, consistent with direct gel analysis, was seen with a variety of isolates of the parasite.

Our strategy also addressed the characteristic endogenous nuclease activity of *T. vaginalis*. Such endogenous nuclease activity renders *T. vaginalis* DNA unusually susceptible to degradation during isolation (31, 32, 53, 55). Sophisticated procedures for preparation of high-molecular-weight DNA from *T. vaginalis* have been described previously (42, 55). While these procedures are effective, they are technically complex compared with standard methods of sample preparation for PCR (22). Therefore, to develop PCR for potential clinical use in diagnosis of *T. vaginalis*, it was desirable to accommodate less sophisticated but more rapid methods of sample preparation. Since PCR specificity is determined by hybridization specificity of short primer sequences flanking the target sequences (19) and not the length of the target sequences themselves, our strategy was to amplify target sequences whose lengths were minimal. This approach reduced the chance of endonucleolytic cleavage of the target sequence during sample preparation. The largest target sequence in this study was 295 bp for ferredoxin gene PCR.

All of the *T. vaginalis* and *Acanthamoeba* isolates (see Fig. 2 to 4a) represent high-molecular-weight template DNA purified by the DEPC–Triton X-100 method. Further, we treated the vaginal swab specimens by using a standard method of sample preparation for PCR (14, 22) to evaluate both the PCR method and its application to a commonly used method of sample preparation.

PCR reaction conditions. PCR reactions were performed with an automated thermocycler (Perkin-Elmer Cetus, Norwalk, Conn.). Oligonucleotide primers (see Fig. 1) were synthesized at the Howard Hughes Research Institute (Seattle, Wash.). PCR buffer consisted of 10 mM Tris HCl (pH 8.3), 50 mM KCl, and 0.5 to 2.5 mM $MgCl_2$. For each primer pair, $MgCl_2$ concentrations were varied (19, 47) over the range of 0.5 to 5 mM. The primer set TVA5-TVA6 had an optimal $MgCl_2$ concentration of 1.5 mM, while TR5-TR6 was optimized at 1.0 mM $MgCl_2$. Either *Taq* polymerase (Perkin-Elmer Cetus) or Replitherm (Epicenter Technologies, Madison, Wis.) was used as the thermostable enzyme. *Taq* polymerase, Replitherm, and primers were all titrated to ensure optimal concentrations (19, 47). For the primer set TVA5-TVA6, slight differences in PCR product band intensities for repeated determinations were due to differing batches of *Taq* polymerase (see Fig. 4a; compare lanes 2 and 16 or lanes 4 and 17).

Thermal cycles for PCR involving primers TVA5-TVA6 consisted of 7 min at 94°C and then 30 to 45 cycles of sequential variation between 94, 47, and 67°C, 1 min each, followed by a 7-min extension cycle at 67°C. PCR involving primers TR5, TR6, FE1, and FE2 consisted of 7 min at 94°C and then 30 to 50 cycles of sequential variation between 94, 50, and 72°C, 1 min each, followed by a 7-min extension cycle at 72°C. Variation of the annealing temperature between 45 and 55°C did not significantly alter the isolate-specific characteristics of some primer pairs (e.g., TR5-TR6). To avoid product carryover, PCR reactions were initially set up in an area physically separate (28, 35) from all activities involving amplified target sequences, including thermocycling areas, PCR product storage, and the running of gels. A separate set of pipetting devices was devoted to the setup of PCR reactions (28).

DNA from species other than *T. vaginalis* were checked for competence in PCR by specific reactions as described in Results or by universal PCR reactions. Universal PCRs consisted of either the T17 PCR reaction targeting variable repeat DNAs of eukaryotes (42) or a PCR reaction specific

for tetracycline resistance genes (unpublished data) in the case of the prokaryotic DNAs described.

Library construction and nucleotide sequencing. For genomic library construction, high-molecular-weight, total *T. vaginalis* S13 or S981 DNA, prepared by the DEPC–Triton X-100 method, was digested with the restriction enzyme *Sau*3A (Bethesda Research Laboratories). The frequently cutting enzyme *Sau*3A was used since there was no advantage in targeting large-size DNA sequences in an organism that is susceptible to endogenous nuclease activity during DNA purification by standard methods.

The *Sau*3A-cut genomic fragments were ligated to a *Bam*HI-digested (Bethesda Research Laboratories) pUC 18 plasmid vector. The resulting ligation mixture was transformed into *Escherichia coli* JM107 (Bethesda Research Laboratories). Individual transformants were picked and miniprepped (39), and the genomic DNA inserts were sized by restriction enzyme digestion followed by agarose gel electrophoresis. Restriction enzyme digestions were carried out according to the manufacturer's specifications. Further details of ligation, genomic library construction, bacterial transformation, and plasmid purification have been previously reported (39, 41). Nucleotide sequencing was performed by a slight modification of the Sanger method (39).

Computer-assisted homology searches. Each *T. vaginalis* genomic DNA sequence was compared with those in the entire GenBank and European Molecular Biology Laboratory data bases (release 63) by computer-assisted searches. Sequence software consisted of GenePro (Riverside Scientific, Bainbridge Island, Wash.). Homology searches of both nucleotide and peptide sequences were performed. DNA sequences were also compared on the basis of predicted peptides for all open reading frames by using an automatic translating feature of the program. For each sequence, multiple searches were performed by varying the search sequence window size. Sequences were considered as candidate targets for specific *T. vaginalis* detection when the entire data base search yielded no matches in searches that involved a sliding window of twenty nucleotides (attempting to match 18) for nucleotide sequences and a window of 10 (attempting to match 8) for peptides. The peptide searches allowed for conservative amino acid substitution.

RESULTS

Genomic sequences and selection of PCR primer pairs. At the outset of this study, there was no genomic sequence information for *T. vaginalis* in either the GenBank or European Molecular Biology Laboratory data bases. Therefore, we sequenced 12 randomly selected *T. vaginalis* genomic clones. By computer-assisted homology searches, none of the sequences reported in this study exhibited significant similarity (homology) with known sequences. The data base searches were used as an initial, but not definitive, screen for sequences unique to *T. vaginalis*. Eight of the sequences (three are described in Fig. 1) were considered candidates for specific, PCR-directed targeting of *T. vaginalis*. Primer sets were constructed for all eight genomic sequences as well as for the *T. vaginalis* ferredoxin gene reported during the course of our studies (21).

Evaluation of candidate primer pairs. Our criteria for useful PCR detection of *T. vaginalis* were the detection of appropriate PCR products in multiple *T. vaginalis* isolates and absence of PCR products in reactions with a panel of organisms, including other flagellates, ciliates, amoebas, bacteria, genital mycoplasmas, and human DNA.

T17rp sequence:

Targeted sequence includes nucleotides 1 through 146 of the published T17rp sequence (42).

Primers targeting T17rp:

TR 7: CTGTTGTCGACGTTTATCCA

TR 8: GATCACCAGTGGAGGGTGTC

Expected product size =146 bp

T9p sequence:

GATCTGTGAAATATCTGCTTGGTAAATTTGATGCTTGGTGCGCCTTTG
ATAAATTCTGGCATCGTGTGAATTTGCTCTGTTGGTAATTTTTCGTATT
CATTTGCAATATCAGTGAGGCTATGCTCGAGTTTAGAAATATTCTCAC
GCTTGATGTCACTTTCTTTCTGAAGCTGTTGATC*

Primers:

TR5: TTTGATGCTTGGTGCGCCTT

TR6: GATCAACAGCTTCAGAAAGA

Expected product size = 154 bp.

A6p sequence: *Xba* I

GATCATGTTCTATCTTTTCATTGTTTCAAAATCTAAAAAAGACCTCTAG
Hinf I
AAGAAGACTCAGAGTGTAAATAACGTATTTTTTTGTAAACTAAGGTGG
TGATC*

Primers:

TVA5: GATCATGTTCTATCTTTTCA

TVA6: GATCACCACCTTAGTTTACA

Expected product size = 102 bp.

T. vaginalis ferredoxin gene: Targeted sequence includes nucleotides 178 to 452 of Johnson et al. (21).

Primers:

FE1: GGAACAATCACAGCCGTCAA

FE2: TCGAAAACAGCACCATCGTT

Expected size= 295 bp.

FIG. 1. *T. vaginalis* genomic sequences with primer sites underlined. The primers targeting these sequences in PCR reactions and the expected product sizes are also indicated. All sequences are shown in the 5′ to 3′ direction. The T17rp and T9p genomic sequences represent clones isolated from a *T. vaginalis* S13 genomic library. T17rp was briefly described in a previous report (42). Clone A6p was isolated from a *T. vaginalis* S981 genomic library. A *Hin*fI site located 56 bp from one end of the A6p sequence is italicized, as is a nearby *Xba*I site. The ferredoxin gene sequence is not shown since it has been published previously (21).

The first genomic library was constructed from *T. vaginalis* isolate S13. Several PCR primer pairs that detected isolate S13 were synthesized. For example, the primer pair TR7-TR8 (Fig. 1) strongly amplified the 146-bp target in

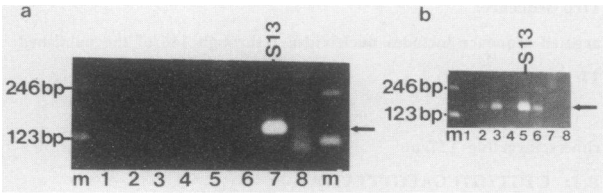

FIG. 2. PCR reactions targeting sequences cloned from *T. vaginalis* S13. (a) Equivalent amounts (determined at A_{260}) of DNA from various *T. vaginalis* isolates were targeted by using primers TR7 and TR8. The expected product size was 146 bp. Lanes: 1, isolate S1190; 2, S981; 3, C7; 4, S1202; 5, S1220; 6, B34; 7, S13; 8, human DNA. Only isolate S13 produced an intense signal of the expected size. The arrow at the right points to the intense 146-bp fragment in lane 7. Lane m, low-molecular-size markers consisting of a 123-bp DNA fragment and integral multiples of 123-bp fragments (e.g., 246 bp, 363 bp, etc.). (b) DNA from *T. vaginalis* isolates targeted by using primers TR5 and TR6. Lanes: 1, isolate S1190; 2, S1220; 3, C7; 4, S981; 5, S13; 6, 1202; 7, *T. herpetomonas* DNA; 8, *T. cruzi* DNA. Primers TR5 and TR6 targeted the correct size sequence in multiple *T. vaginalis* isolates. However, the products exhibit a range of intensity whose magnitude with respect to the isolates consistently was S13 > C7 > 1202 > 1220. S1190 and S981 exhibited weak signals. Lane m, same as for panel a.

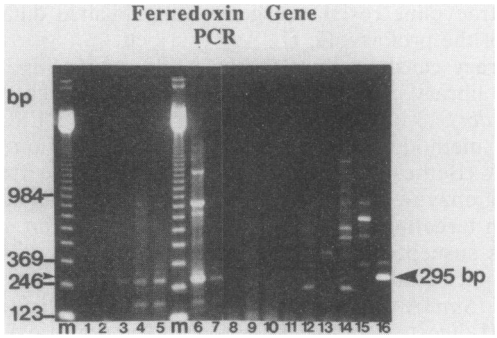

FIG. 3. PCR reactions targeting the *T. vaginalis* ferredoxin gene. Primers FE1 and FE2 were used to target the *T. vaginalis* ferredoxin gene (21). Lanes: 1, *T. vaginalis* isolate S1114; 2, S1138; 3, S981; 4, S1220; 5, C7; 6, 1202; 7, B34; 8 to 10, *T. vaginalis*-positive vaginal swab eluates; 11, no DNA; 12, human DNA; 13, *P. tetraurelia* DNA; 14, *T. equiperdum* DNA; 15, *T. thermophila*; 16, *A. castellanii*; m, the same as for Fig. 2. The arrowheads at 295 bp (right and left sides) show the position of the product expected from *T. vaginalis*. An identical size product was seen with *A. castellanii*.

isolate S13 (Fig. 2a). Unfortunately, this primer pair produced only weak amplification of the same-size product sequence in other isolates (Fig. 2a). Another primer pair, TR5-TR6, was designed to amplify a 154-bp genomic sequence (Fig. 2b). *T. vaginalis* isolates varied widely in the strength of the resulting 154-bp PCR product signals, with consistent relative intensities of the isolates occurring in the following order: S13 > C7 > S1202 > S1220. Reactions involving isolates S1190 and S981 showed the correct-size PCR product but with weak band intensity. No product was seen for human DNA or DNA from other flagellates, such as trypanosomes (Fig. 2b).

We attempted to optimize PCR conditions for this primer set to detect the *T. vaginalis* isolates more uniformly. Initial DNA concentrations for all isolates were identical (see Materials and Methods). Further, complete titrations of $MgCl_2$, *Taq* polymerase, and primers did not alter the signal variation among isolates, nor did increased numbers of thermocycles. We concluded that genomic heterogeneity may contribute to the variation of PCR signal intensities among *T. vaginalis* isolates.

Variation in signal intensity among isolates was also seen (Fig. 3) with primers FE1 and FE2, which were designed to target 295 bp of the coding region of the *T. vaginalis* ferredoxin gene (21). Such variation may reflect isolate-specific primer mismatch at third base or "wobble" positions of codons. In addition to the 295-bp target sequence, the ferredoxin gene PCR reaction produced multiple untargeted products (presumably ferredoxin-related sequences) and products in unrelated microorganisms (Fig. 3). For example, human DNA (Fig. 3, lane 12) produced a PCR product slightly smaller than the 295-bp *T. vaginalis* target sequence, while genomic DNA from *A. castellanii* produced a product, primed by FE1 and FE2, that was indistinguishable in size from the 295-bp *T. vaginalis* sequence (Fig. 3). This product, which may or may not represent a ferredoxin gene in *A. castellanii*, demonstrates limited selectivity of the FE1-FE2 primer pair.

We identified one primer pair, TVA5-TVA6, that did not show significant isolate-specific variation and was also highly selective. This primer pair was designed to amplify the sequence A6p (Fig. 1) cloned from the S981 library. The primer pair TVA5-TVA6 uniformly detected the 102-bp target sequence in 15 clinical isolates of *T. vaginalis*, including some isolates from diverse geographic sources (Fig. 4a). An internal *Hin*fI restriction site confirmed the identity of the 102-bp PCR products (Fig. 5b), as did dot blot hybridizations with the cloned A6p sequence as a probe (data not shown). Two minor, spurious products (see arrowheads between the 123- and 246-bp markers, left side of Fig. 4a) were found to result from excessive PCR cycles and were not seen in subsequent reactions limited to 30 to 45 cycles. We conclude that TVA5-TVA6 uniformly detects a wide variety of *T. vaginalis* isolates, including all isolates tested to date.

Heterologous DNA preparations. Primers TVA5 and TVA6 were tested for cross-reactivity with DNAs from other microorganisms, including the genital mycoplasmas *Ureaplasma urealyticum* and *Mycoplasma hominis*; flagellates, such as *Trypanosoma equiperdum*, *Trypanosoma herpetomonas*, *Trypanosoma cruzi*, and *G. lamblia*; ciliates *P. tetraurelia* and *T. thermophila*; the yeast *S. cerevisiae*; the amoeba *A. castellanii*, and with human DNA (Fig. 4a). No PCR reaction products were seen with any of these heterologous DNAs. In addition, DNAs from a wide assortment of bacteria, including harmless genitourinary flora and some pathogenic bacteria, were tested in PCR reactions for cross-reactivity with primers TVA5 and TVA6. These included *E. coli* DH5α, *B. intermedius* 25261, *P. anaerobius* Clt 17.5, *B. disiens* Clt 75.5, *P. tetradius* Clt 92.2, *F. nucleatum* 6601 TE10, *V. parvula*, *E. faecalis* DS160, *S. agalactiae* AES 14, *G. vaginalis* GV9016B, and *N. gonorrhoeae* F62. No cross-reactivity was seen with DNA prepared from any of these species.

Since the heterologous DNAs were obtained from a variety of sources, we thought it important to determine the template quality and PCR reactivity of each DNA. *Taq* polymerase inhibitors are not uncommon (10, 11, 14) and copurification of such inhibitors with DNA could lead to falsely negative PCR results. To show that the heterologous DNAs which were negative for TVA5-TVA6 recognition sites (Fig. 4a) were, in fact, true negatives, alternative

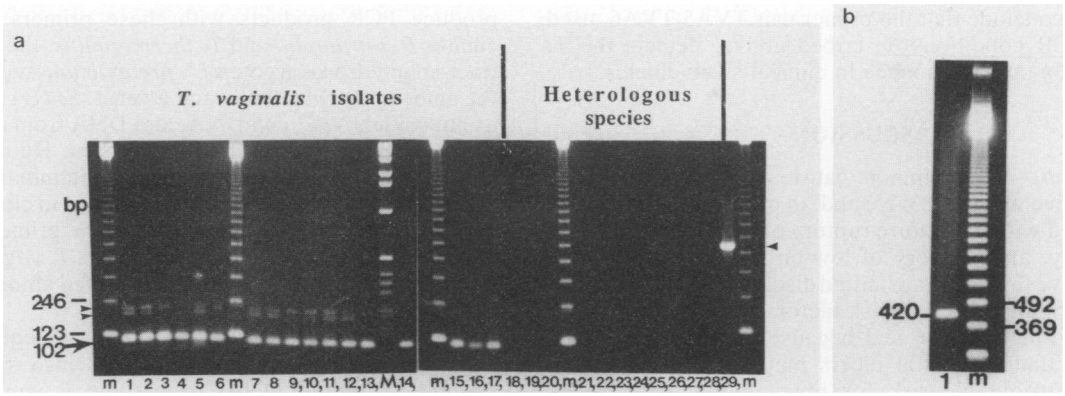

FIG. 4. (a) PCR reactions that involved primers TVA5 and TVA6 to target the A6p sequence originally cloned from *T. vaginalis* S981. The expected product size was 102 bp, as indicated by the arrow at the lower left. The two small arrowheads at the left indicate minor, spurious products, a result of excessive PCR cycles. Equivalent amounts of template DNA were used in each reaction. Lanes: 1, *T. vaginalis* isolate S981; 2, S1220; 3, S1202; 4, C7; 5, B34; 6, S1190; 7, S1047; 8, S764; 9, S1163; 10, S1138; 11, S1114; 12, S933; 13, S1126; 14, S996; 15, S975; 16, S1220 (repetition); 17, C7 (repetition); 18, *U. urealyticum* UU5; 19, *U. urealyticum* UU8; 20, *M. hominis*; 21, *S. cerevisiae*; 22, human DNA; 23, *T. cruzi*; 24, *T. equiperdum*; 25, *T. thermophila*; 26, *P. tetraurelia*; 27, *A. castellanii*; 28, *G. lamblia*; 29, *U. urealyticum* UU5 DNA targeted with primers specific for the urease gene (4). The primers used were UU1 (5′-AGAAGACGTTTAGCTAGAGG-3′) and UU2 (5′-ACGACGTCCATAAGCAACT-3′). The expected product size was 541 bp, in agreement with molecular size standard estimates (see arrowhead at right). Lanes m, low-molecular-size markers, as defined for Fig. 2. (b) Primers targeting the 3′ flanking region of the human phosphoglycerate kinase 1 gene (40) were used in a PCR reaction under conditions identical to those described in panel a. The primer sequences were GK-3 (5′-CTGTGTTAGACTCCTGTTTT-3′) and GK-4 (5′-TTGTCCCTAAAGGTGAAATA-3′). The expected product size was 420 bp (as indicated on the left). Lane m, same as for panel a. The sizes (in base pairs) of two molecular markers are shown on the right.

primer sets were used in PCR reactions with these DNAs. For example, primers directed at the urease gene (4) amplified the correct 541-bp product (Fig. 4a, lane 29) from the same *U. urealyticum* DNA preparation that was negative with the primer pair TVA5-TVA6 (Fig. 4a, lane 19). Thus, the *U. urealyticum* DNA is of adequate template quality for PCR even though it is negative for the primer pair TVA5-TVA6.

Similarly, to show competence of the human DNA preparation, the 3′ flank of the human phosphoglycerate kinase 1 gene (40) was amplified (Fig. 4b) from the same stock human DNA preparation used for Fig. 4a, lane 22. Generic primer sets were used to verify that all of the heterologous DNAs of Fig. 4a, with the exception of *M. hominis*, can serve as templates in the PCR reaction (see Materials and Methods). To date, no PCR reaction for *M. hominis* DNA has been reported, although the DNA preparation reacted well with restriction enzymes. We conclude that the heterologous control DNAs were generally of good quality and that the primer pair TVA5-TVA6 is highly selective for *T. vaginalis*.

Pilot study: PCR detection of *T. vaginalis* in direct clinical samples. To evaluate the potential of PCR-based detection of *T. vaginalis*, we tested the primers TVA5 and TVA6 with direct, uncultured, clinical samples. A 1/400th volume of a vaginal swab eluate was sufficient to give a 102-bp PCR signal in the samples that were *T. vaginalis* positive by wet mount examination (Fig. 5a). One of the wet mount-negative samples that was obtained from a woman at high risk for the parasite was positive by our PCR test (data not shown).

Restriction enzyme analysis of PCR products. PCR primers are complementary to the end-terminal nucleotide bases of the target sequences. Restriction sites in internal portions of PCR product sequences are often used as a convenient, nonradioactive confirmation of PCR products (5, 36). As shown in Fig. 1, the 102-bp A6p genomic sequence that was targeted has internal *Xba*I and *Hin*fI sites. The *Hin*fI site, when cut, should produce two fragments of 56 and 46 bp in size. *Hin*fI digestion of TVA5 and TVA6 PCR products consistently led to the production of the expected fragments (Fig. 5b). The *Hin*fI restriction digests were performed with portions of the same PCR reactions shown in Fig. 5a. Sufficient PCR product was available for multiple restriction digests, suggesting the possibility of routine product confirmation by restriction digests. However, dot blot hybridizations, with as a probe the original A6p plasmid clone, also confirmed the identity of the 102-bp PCR products of Fig. 4a

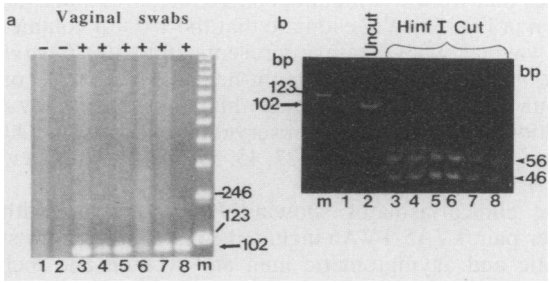

FIG. 5. PCR reactions that involved primers TVA5 and TVA6 to target the *T. vaginalis* A6p sequence in vaginal swab eluate digests. (a) Vaginal swabs both wet mount-positive and -negative (indicated by + and −, respectively, above the lane) for *T. vaginalis* were eluted, washed in PBS, and treated with proteinase K, and a 1/400th volume of each resulting mixture was probed for the 102-bp *T. vaginalis* A6p sequence. The arrow at the right shows the position of the expected 102-bp product just below the 123-bp marker band. DNA from 10 to 100 *T. vaginalis* microorganisms was estimated to be present in each reaction. (b) The 102-bp A6p sequence contains a *Hin*fI restriction site (Fig. 1) that divides the fragment into 56- and 46-bp fragments. The PCR products shown in panel a were digested to completion with *Hin*fI, and the resulting fragments were electrophoresed on a 6% acrylamide gel. Lanes: m, the 123-bp marker fragment; 1, uncut 102-bp A6p product from a vaginal swab eluate PCR reaction; 3 to 8, *Hin*fI-digested vaginal swab PCR products.

and 5a. We conclude that the primer pair TVA5-TVA6, used with the PCR conditions described above, detects the *T. vaginalis* A6p target sequence in clinical swab eluates.

DISCUSSION

T. vaginalis is a common cause of morbidity among sexually active women (23–25) and, in pregnant women, may be associated with premature rupture of membranes, premature delivery, and delivery of low-birth-weight infants (6). Nonulcerative sexually transmitted diseases, including trichomoniasis, are implicated as risk factors for human immunodeficiency virus infection and because of their higher incidence than that of genital ulcers may represent a higher population-attributable risk for human immunodeficiency virus transmission (29). *T. vaginalis* has also been implicated as a cause of nongonococcal urethritis, prostatitis, and other genitourinary tract syndromes in men (23–25). To investigate the role of *T. vaginalis* as a genitourinary pathogen and to improve clinical diagnosis of *T. vaginalis*, we developed methods for PCR-based detection of the parasite.

The exquisite sensitivity of PCR techniques was shown by the ability to detect specific sequences in samples as small as single hairs (15) or even single cells (16), although somewhat larger samples are more amenable to routine analysis. PCR techniques have already been used in preliminary clinical studies. These include the detection of human immunodeficiency virus (12, 18), cytomegalovirus (48), herpesvirus (13, 46), human genital papillomavirus (3, 51), *Mycoplasma genitalium* (20), and a variety of other pathogens. In addition to exquisite sensitivity, PCR methods offer a number of other advantages, including the ability to detect defective microorganisms and target sequences in clinical samples that have undergone fixation or even partial degradation (11, 57).

Our initial goal was to identify a highly selective primer set for PCR-based detection of *T. vaginalis*. Two of eight primer sets exhibited strong selectivity toward *T. vaginalis*. The superior primer pair was TVA5-TVA6 (Fig. 1). With the PCR conditions described, these primers uniformly detected 15 cultured, clinical isolates (Fig. 4a) and nine vaginal swab eluates that were wet mount positive for *T. vaginalis* (six are shown in Fig. 5a). We estimate that the 1/400th volume used from vaginal swab eluates represents 10 to 100 *T. vaginalis* microorganisms. One wet mount-negative swab was positive by our PCR test. We suspect this represents a low-level infection since wet mount observation misses some 40% of culture-positive samples (8, 27, 45, 56) and the woman was at high risk for infection.

The clinical isolates showing PCR reactivity with the primer pair TVA5-TVA6 included isolates from both symptomatic and asymptomatic men and women and included isolates from several geographic locations. In view of the considerable phenotypic variation seen with *T. vaginalis* isolates (1, 2, 24, 50, 52) and the wide range of clinical manifestations associated with the parasite (25, 56), it was encouraging to find a primer pair, TVA5-TVA6, that detected all isolates studied to date, including those from a variety of sources and clinical presentations.

The TVA5-TVA6 primer pair proved highly selective since no products were observed with PCR reactions that involved template DNA from other flagellated protozoans, including *T. cruzi*, *T. equiperdum*, and *G. lamblia*. *G. lamblia* is a flagellated parasite which is frequently compared with *T. vaginalis* since both organisms lack mitochondria, possess extended glycolytic pathways, and are adapted to mucosal surfaces (33, 34). Other organisms that did not produce PCR products with these primers included the ciliates *P. tetraurelia* and *T. thermophilus*, the genitourinary tract-adapted prokaryotes *U. urealyticum* and *M. hominis*, the amoeba *A. castellanii*, and a yeast, *S. cerevisiae*, as well as human DNA, *E. coli* DNA, and DNA from a wide variety of pathogenic bacteria and natural flora. Human DNA was tested since it is an important "contaminant" from the perspective of PCR detection of parasites in clinical samples. The evidence strongly suggests that the primer pair TVA5-TVA6 fulfills the criterion of detecting a *T. vaginalis*-specific target sequence (A6p) which is conserved among the isolates examined.

The primer pair TR5-TR6, targeting a sequence distinct from that of TVA5 and TVA6, was also specific for *T. vaginalis*, although DNAs from some *T. vaginalis* isolates reacted weakly to these primers. This primer pair might be useful in conjunction with radioactive probes, but our primary goal was to develop nonradioactive, direct gel detection methods.

The variation among *T. vaginalis* isolates in PCR product band intensities with the primer pair TR5-TR6 occurred under a wide variety of PCR conditions. Similar variation was associated with the ferredoxin gene PCR reactions (Fig. 3). In fact, three of the four primer pairs presented showed isolate-specific variations in genomic sites targeted by PCR, as did all of four other pairs examined in preliminary studies. One interpretation of these findings is that primer mismatch or mispriming at mutated sites in some isolates led to inefficient PCR reactions. We favor this interpretation, rather than variation in the DNA preparations, since one primer set, TVA5-TVA6, was not subject to such variation with the same DNA preparations (Fig. 4a). Genomic variations analogous to the ones detected in this study may contribute to the significant phenotypic variation seen among *T. vaginalis* isolates (1, 2, 24, 50, 52) although differences in expression may also play a role. Genetic variation or polymorphism may also occur for the genomic locus targeted by TVA5-TVA6 although no evidence for this was seen with the *T. vaginalis* isolates studied.

In summary, we describe a conserved genomic locus, A6p (the target of TVA5-TVA6), that was uniformly detected among *T. vaginalis* isolates and other loci exhibiting heterogeneity among isolates. PCR primer pair TVA5-TVA6 uniformly detected 24 clinical isolates, including isolates from a variety of clinical and geographic sources. This primer pair did not cross-react with DNA from other microorganisms or with human DNA. These methods hold promise for PCR-based diagnosis of *T. vaginalis*.

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