

Colorimetric One-Tube Nested PCR for Detection of *Trichomonas vaginalis* in Vaginal Discharge

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A colorimetric one-tube nested PCR was developed for the detection of *Trichomonas vaginalis* in clinical vaginal discharge specimens. A family of 650-bp specific DNA repeats from the *T. vaginalis* genome was targeted. There was no cross-reaction with human DNA or other infectious agents, including *Pentatrichomonas hominis* and *Giardia lamblia*. The colorimetric assay was applied as an adjunct to nested PCR for semiquantitative determination of *T. vaginalis* DNA at levels corresponding to 1 to 1,000 parasites. PCR of samples prepared by a rapid boiling method was as sensitive and specific as PCR of samples prepared by the standard DNA extraction method: the equivalent of one *T. vaginalis* organism in 20 μ l of vaginal discharge could be detected. The colorimetric nested PCR was compared with wet mount and culture for the detection of *T. vaginalis*. A total of 378 clinical vaginal discharge specimens from symptomatic patients were examined; 31 patients were positive for *T. vaginalis* both by culture and by nested PCR. However, only 17 of these 31 patients were positive by wet mount examination. In addition, of 113 asymptomatic patients, 9 were positive for *T. vaginalis* by nested PCR. Of these nine PCR-positive patients, only two were also positive both by wet mount and by culture, four patients were positive by culture but negative by wet mount, and three patients were negative both by wet mount and by culture. No specimens negative by nested PCR were positive by wet mount or by culture. The three asymptomatic patients with PCR-positive but wet mount- and culture-negative samples were subsequently found to have *T. vaginalis* infection after repeated and prolonged culture was performed. This colorimetric nested PCR was very sensitive compared with culture for the diagnosis of vaginal trichomoniasis, especially asymptomatic *T. vaginalis* infection. It is also simple, specific, rapid, and semiquantitative.

Trichomonas vaginalis is a common pathogen causing vaginitis, exocervicitis, and urethritis in women (4, 16). It has been suggested that *T. vaginalis* infection plays a role in the pathogenesis of preterm birth, preterm rupture of membranes, and posthysterectomy cuff infections (2, 11, 15, 20). More recently, *T. vaginalis* has been implicated as a cofactor in the transmission of human immunodeficiency virus (HIV) (8). Because *T. vaginalis* infections are frequently asymptomatic, an early and accurate diagnosis is necessary for specific treatment (10, 16). Routine clinical diagnosis usually depends on microscopic identification of the parasite in wet mount preparations (7, 21). However, wet mount examination detects only 60% of culture-positive women (21). Although culture is considered the most reliable diagnostic method, with a sensitivity of >90% for detecting *T. vaginalis* (14, 21), it requires complex media and is time-consuming, requiring daily examination for up to 7 days. Moreover, some isolates cannot be cultivated due to either strain requirements, low numbers of parasites, or damaged or nonviable organisms (3). Other diagnostic methods including cytologic smears and immunological assays do not give consistent results (7).

Molecular methods may provide the most sensitive and specific assays for diagnosis. Recently, amplification of target DNA sequences by PCR has been extensively studied for the diagnosis of parasitic infections (23). Although PCR diagnosis of vaginal trichomoniasis has been reported, the analysis of the PCR product was confined to gel electrophoresis and radioisotopic detection (5, 6, 18), which are not suitable methods for

routine diagnostic laboratories. In this study, we targeted a family of 650-bp repeats (Tv-E650) from the *T. vaginalis* genome (13) and designed a novel colorimetric one-tube nested PCR protocol for the detection of *T. vaginalis* in clinical vaginal discharge specimens.

MATERIALS AND METHODS

Collection of vaginal discharge from patients with or without cervicovaginitis. A total of 491 clinical vaginal discharge specimens from women visiting gynecologic clinics (Tri-Service General Hospital, Taipei, Taiwan) were examined in this study. Of these, 378 were from women who were symptomatic with one or more of the following symptoms: irritating discharge, dyspareunia, dysuria, and lower abdominal discomfort. The remaining 113 specimens were from women who were asymptomatic. For each patient, vaginal discharge was carefully collected from the posterior vaginal fornix with a sterile graduated polyethylene transfer pipette. A portion of each vaginal discharge specimen (100 μ l) was combined with an equal volume of normal saline (0.9% NaCl). Immediately, 1 drop of this mixture was applied to a glass slide, covered with a coverslip, and examined at $\times 100$ and $\times 400$ magnifications with a light microscope for the presence of *T. vaginalis*. Negative wet mounts were examined for at least 10 min. At the same time, an aliquot (100 μ l) of vaginal discharge was inoculated onto a culture tube and *T. vaginalis* was identified and quantified by a culture method as described previously (14). Daily examination of the culture was performed for up to 7 days. Subsequently, *T. vaginalis* was isolated and axenically cultivated by a method described previously (9).

Extraction of DNA from cultivated trichomonads and clinical specimens. Portions (100 μ l) of axenically cultivated trichomonads (10^6 parasites/ml) and aliquots (100 μ l) of vaginal discharge from each clinical specimen were processed by each of the extraction methods described below.

(i) Standard DNA extraction method. After two washes in phosphate-buffered saline (pH 7.4) by centrifugation ($1,500 \times g$, 10 min), DNA was extracted from cell pellets by the addition of 500 μ l of lysis buffer and incubation at 65°C for 30 min. The lysis buffer was freshly prepared from concentrated stock solutions (final concentrations, 15 mM sodium citrate, 450 mM NaCl, 0.2% sodium dodecyl sulfate, and 100 μ g of proteinase K per ml). The extracts were then extracted twice with an equal volume of phenol-chloroform (1:1; vol/vol) and once with chloroform. The DNA was precipitated with 2 volumes of 95% (vol/vol) ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2), washed with 70% (vol/vol) ethanol, air dried, and dissolved in 25 μ l of TE buffer (10 mM Tris HCl

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[pH 7.6], 1 mM EDTA). Five microliters of this preparation were used in a 20- μ l PCR mixture.

(ii) **Rapid boiling method.** The cell pellets were resuspended in 500 μ l of 0.5% Tween 20 (Sigma), boiled for 20 min, and pelleted by centrifugation at 12,000 \times g for 5 min. The supernatant was discarded, and the pellet was emulsified by vigorous flicking in 25 μ l of chloroform. Twenty-five microliters of deionized water was then emulsified with the chloroform phase, and the aqueous phase containing water-soluble components including nucleic acids was harvested by centrifugation at 12,000 \times g for 2 min. Five microliters were used in a 20- μ l PCR mixture.

Nested PCR. The four primers used in this study were chosen from within a unique family of 650-bp DNA repeats cloned from the *T. vaginalis* genome and thought to be conserved in all strains (13) (GenBank accession numbers M86482 to M86489). The outer primers (OPs) used were OP₁ (5'-GTGAAAATCTCA TTGGGGTATTAACCT-3') and OP₂ (5'-GTTTTATTTATCACTGAAAAT AACGCTT-3'), both at a concentration of 30 nM, and the inner primers (IPs) used were IP₁ (5'-AACATCCCCAACATCTT-3') and IP₂ (5'-CCATTCCTTT AGACCCCTT-3'), both at a concentration of 500 nM. IP₁ was labeled with biotin at its 5' end and IP₂ was labeled with digoxigenin at its 5' end during the commercial synthesis (R & D Systems, Europe Ltd., Abingdon, England). All PCRs were carried out in a total volume of 20 μ l. In all cases amplification was performed in 50 mM KCl-10 mM Tris-HCl (pH 8.3)-2 mM MgCl₂-200 μ M (each) deoxyribonucleotide triphosphate-0.5 unit of AmpliTaq Polymerase (Roche Molecular Systems, Inc., Perkin-Elmer, Branchburg, N.J.). PCR was performed in two stages, with each stage distinguished by different annealing temperatures. Thirty cycles were performed at an annealing temperature of 62°C (1 min), during which a product of 521 bp was generated by the longer OPs OP₁ (melting temperature [T_m] = 72°C) and OP₂ (T_m = 74°C). IP₁ (T_m = 48°C) and IP₂ (T_m = 50°C) could not anneal during the initial 30 cycles. This was followed by 20 cycles at an annealing temperature of 45°C (1 min), during which the product formed in the first 30 cycles acted as the target for the shorter primers, generating a product of 290 bp. A denaturation temperature of 94°C (45 s) and an extension temperature of 72°C (1 min) were used throughout. A precycle denaturation at 94°C for 2 min was given to reduce nonspecific amplification, and for the last cycle a prolonged extension period of 10 min was used.

A wax cap "hot-start" protocol (1) was used to separate the primers from the other reaction components. This was found to be essential in conjunction with the colorimetric detection procedure which would otherwise generate false-positive results. Briefly, the primers were divided into aliquots and placed into reaction tubes, and the contents of the tubes were overlaid with a plug of wax (BDH, Poole, England). Other reaction components were divided into aliquots and placed onto the wax.

Agarose gel electrophoresis. Ten microliters of each of the PCR products was run in 6-cm 2% agarose gels (Fisher Scientific, Fair Lawn, N.J.) in 1 \times TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA) at 50 V. The gels were then stained with ethidium bromide (0.5 μ g/ml) and viewed under UV illumination.

Colorimetric detection of PCR product. The labeled IPs, incorporated into the PCR product, were captured in an avidin-coated microtiter plate and were detected by an antidigoxigenin-alkaline phosphatase conjugate as described previously (24). Briefly, microtiter plate wells were coated with avidin (10 μ g/ml in 50 mM carbonate buffer [pH 9.6]) at 4°C for 16 h. Ten microliters of each of the PCR products was diluted 1:10 by the addition of 100 μ l of Tris-buffered saline (TBS; pH 7.5)-0.5% (vol/vol) Tween 20 (TBS-Tween 20), and 100 μ l of the diluted product was added to each well. The plates were incubated at 25°C for 30 min. The wells were then washed twice with TBS-Tween 20, and 100 μ l of antidigoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim GmbH, Mannheim, Germany) diluted 1:5,000 in TBS-Tween 20 was then added to each well. After incubation at 25°C for 30 min, the wells were washed three times with TBS-Tween 20 and once with TBS. Finally, 100 μ l of 2.5 mM *para*-nitrophenylphosphate (in 50 mM carbonate buffer [pH 9.6]-1 mM MgCl₂) was added to each well, and the absorbance at 405 nm was read after 30 min.

RESULTS

Specificity and sensitivity of the one-tube nested PCR for axenically cultivated *T. vaginalis*. In our preliminary study we used DNA extracted from axenically cultivated *T. vaginalis*, prepared as described in Materials and Methods, diluted 1:4,000 to give the equivalent of DNA from five *T. vaginalis* organisms in each PCR mixture. The relative concentrations of OPs and IPs were crucial in the one-tube nested PCR. If the concentration of the OPs was too high, products other than the predicted 290-bp band were amplified. Thus, *T. vaginalis* PCR was optimized for maximum synthesis of the 290-bp product by varying the concentrations of OP₁ and OP₂. Preliminary experiments showed that the optimum concentration of the IPs IP₁ and IP₂ was 500 nM, and this concentration was used in all experiments (Fig. 1). The IP product (detected in a colorimet-

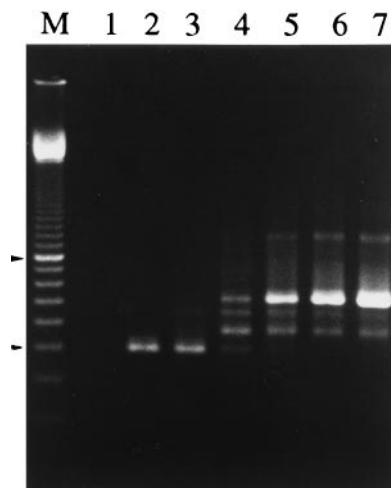


FIG. 1. Effect of OP concentrations on the amplification of 290 bp of DNA from genomic DNA of *T. vaginalis* by nested PCR. Lane M, 100-bp ladder (the upper and lower arrowheads indicate 800- and 300-bp bands, respectively); lane 1, negative control with no DNA (both OPs at 500 nM); lanes 2 to 7, concentrations of both OPs of 15, 30, 60, 125, 250, and 500 nM, respectively (the concentrations of both IPs were 500 nM in all lanes). The predicted 290-bp band is clearly seen in lanes 2 and 3.

ric assay) of 290 bp was seen when the OPs were used at 15 and 30 nM (Fig. 1, lanes 2 and 3, respectively). When the concentration of OPs was increased, the 290-bp band decreased and up to four bands (521, 448, 363, and 290 bp) were seen (Fig. 1, lanes 4 to 7). These four bands are specific for the *T. vaginalis* Tv-E650 repeat and are produced by the four possible pairings (OP₁-OP₂ = 521 bp, IP₁-OP₂ = 448 bp, OP₁-IP₂ = 363 bp, IP₁-IP₂ = 290 bp). Because of minor amplification of the extraneous bands at a concentration of the OPs of 30 nM, we further examined the OPs at concentrations of between 10 and 60 nM. Of the various concentrations (10, 20, 30, 40, 50, and 60 nM) of OPs, it was found that 30 nM of each OP generated the greatest amount of 290-bp product, as determined both by gel electrophoresis and by the colorimetric reaction (data not shown), and extraneous bands were consistently faint. Therefore, this concentration of OPs was used in all subsequent experiments.

Two other flagellates, *Giardia lamblia* and *Pentatrichomonas hominis*, were used to test specificity. No PCR product could be detected when equivalent amounts of genomic DNA extracted by the same methods used to extract *T. vaginalis* DNA were used as the template (data not shown).

To test the sensitivity of this colorimetric nested PCR for the detection of *T. vaginalis*, genomic DNA extracted from axenically cultivated *T. vaginalis* was diluted to give DNA amounts equivalent to the amount of DNA in between 1 and 100,000 *T. vaginalis* organisms per PCR mixture. The one-tube nested PCR was able to detect as few as one trichomonad per assay when analyzed both by agarose gel electrophoresis and by the colorimetric method (Fig. 2). The absorbance of the colorimetric product increased proportionately with the amount of DNA used in the PCR in the range of 1 to 1,000 *T. vaginalis* organisms. This correlation did not extend to absorbances from increasing amounts of DNA (Fig. 2A). Gel electrophoresis showed amplification of the 521-, 448-, and 363-bp products, in addition to the predicted 290-bp band (Fig. 2B). However, only the 290-bp band incorporates both biotin and digoxigenin and can be detected colorimetrically.

To investigate whether host cells could interfere with the

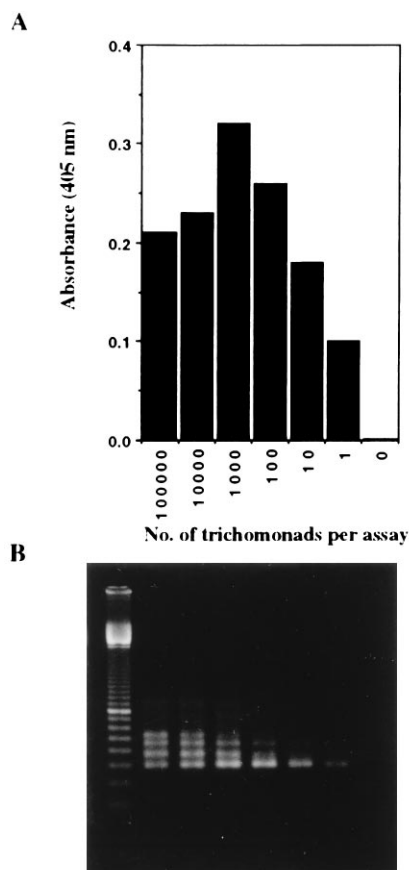


FIG. 2. Sensitivity of the colorimetric one-tube nested PCR for *T. vaginalis*. Genomic DNA was extracted from axenically cultivated trichomonads and diluted to give an amount of DNA equivalent to the DNA content from 100,000 to 1 *T. vaginalis* organism. PCR products were analyzed both by the colorimetric method (A) and by agarose gel electrophoresis (B). (A) A proportional relationship between the absorbance of the colorimetric product and the amount of trichomonad DNA used in the PCR mixture was noted in the range of 1 to 1,000 *T. vaginalis* organisms. (B) A single band of DNA of 290 bp was observed when 1 and 10 trichomonad equivalents of genomic DNA were used. As the amount of trichomonad DNA increased, four bands of DNA with sizes of 521, 448, 363, and 290 bp, respectively, were seen.

sensitivity of the colorimetric PCR, axenically grown *T. vaginalis* organism (5 to 50,000 trichomonads) were mixed with 5×10^4 host cells obtained from vaginal discharge from patients with nontrichomonal vaginitis. DNA was extracted from each mixture by both standard and rapid boiling procedures; both procedures gave identical results, and the results obtained by using DNA extracted by the rapid boiling method are presented in Fig. 3. DNA equivalent to the amount of DNA in between 1 and 10,000 *T. vaginalis* organisms was used in the PCR mixtures. Both the colorimetric assay (Fig. 3A) and gel electrophoresis (Fig. 3B) showed that the equivalent of one trichomonad in $20 \mu\text{l}$ of vaginal discharge containing 10^4 host cells could be detected. Thus, the nested PCR of the 290-bp *T. vaginalis* product was not affected by host cell DNA.

Colorimetric one-tube nested PCR for the detection of *T. vaginalis* in clinical samples. Vaginal discharges were collected from 378 patients with symptomatic vaginitis and 113 women without symptoms. All clinical specimens were processed by wet mount, culture, and colorimetric nested PCR. Each clinical sample was assayed in duplicate, using DNA extracted both by the standard method and by the rapid boiling method, by

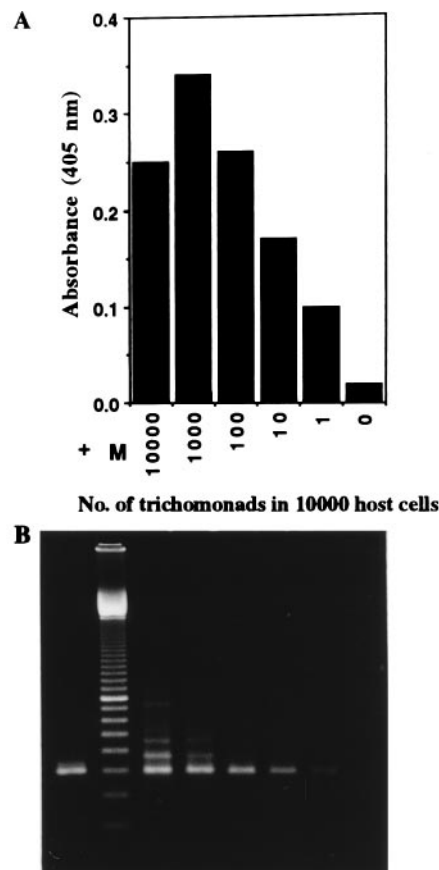


FIG. 3. Influence of host cells on the sensitivity of colorimetric one-tube nested PCR for *T. vaginalis*. DNA was extracted from a mixture of 5×10^4 host cells plus 5 to 50,000 *T. vaginalis* organisms and was used as a template for nested PCR. PCR products were analyzed both by the colorimetric method (A) and by agarose gel electrophoresis (B). (A) Absorbance of PCR mixtures containing DNA from 10,000 to 0 *T. vaginalis* organism. (B) Amplified products from PCR mixtures containing DNA from 10,000 to 0 *T. vaginalis* organism by gel electrophoresis. Lane M, 100-bp ladder marker; lane +, positive control (DNA equivalent to 100 *T. vaginalis* organisms).

colorimetric analysis and gel electrophoresis. The results of colorimetric analysis and agarose gel electrophoresis with DNA extracted by the rapid boiling method are presented in Fig. 4 for 15 of the clinical specimens (standard DNA extraction gave identical results). Negative controls (without DNA) were negative by both colorimetric analysis and gel electrophoresis (data not shown). No samples were positive by colorimetric analysis if no 290-bp product was seen by gel electrophoresis. Similarly, all those samples which yielded product as seen by gel electrophoresis also yielded a color reaction. In the colorimetric assay, the cutoff value (0.045) between positive and negative samples was taken as the mean absorbance plus 3 standard deviations for known negative specimens.

A total of 40 patients (31 symptomatic and 9 asymptomatic) were positive for *T. vaginalis* by nested PCR (Table 1). Among the specimens from symptomatic patients, all PCR-positive specimens were culture positive. However, only 17 of the 31 specimens from symptomatic patients were wet mount positive (Table 1). Among the specimens from asymptomatic patients, of the nine PCR-positive specimens, only two were wet mount and culture positive, another four were culture positive but wet mount negative, and the other three were both wet mount and culture negative (daily examination for 1 week). Those three

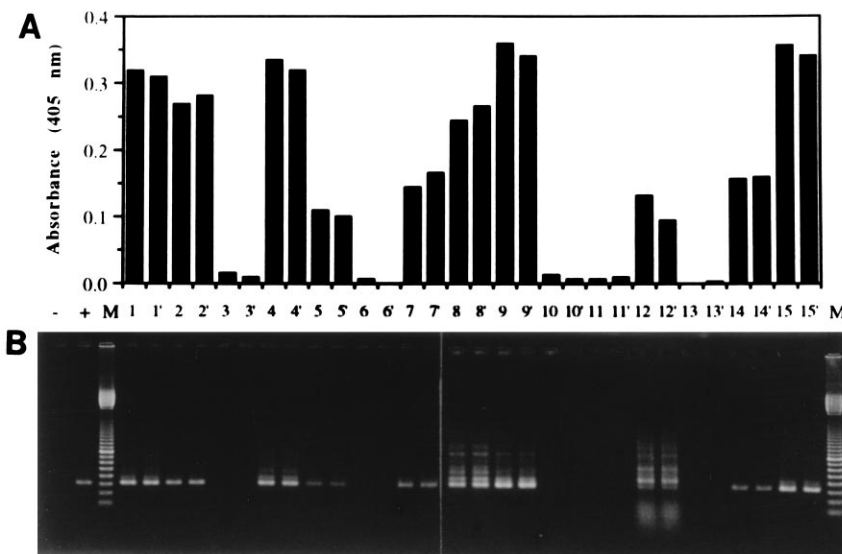


FIG. 4. Detection of *T. vaginalis* in clinical vaginal discharge specimens by colorimetric one-tube nested PCR. DNA was extracted by the rapid boiling method and was processed by the one-tube nested PCR. PCR products were analyzed both by the colorimetric method (A) and by agarose gel electrophoresis (B). Each clinical sample is numbered consecutively, and duplicates are indicated by a prime symbol. Lane M, 100-bp ladder marker; lane -, negative control (deionized water); lane +, positive control (DNA equivalent to 100 *T. vaginalis* organisms).

patients whose samples had a negative culture result but a positive PCR result were asked to submit a second sample for testing; all samples remained negative by the wet mount method but were consistently positive by PCR. However, these three PCR-positive but culture-negative (first sample) patients were subsequently found to have *T. vaginalis* infection after a second and prolonged cultivation of their specimens (up to 10 days). Quantification of *T. vaginalis* in vaginal discharge was obtained by culture on agar (14), and the number of trichomonads in the vaginal discharges from the culture-positive patients (not including the three patients whose first specimens were negative by culture) ranged from 50 to >10⁷ CFU/ml (data not shown). The three asymptomatic patients whose specimens were positive for *T. vaginalis* by a second and prolonged culture had an extremely low parasite burden (less than 5 trichomonads in 100 µl of vaginal discharge [data not shown]). The wet mount examination was consistently positive for patients yielding >10⁵ CFU/ml of vaginal discharge.

TABLE 1. Comparison of nested PCR with culture and wet mount for the detection of *T. vaginalis* in vaginal discharges from symptomatic and asymptomatic patients^a

Detection method	No. of patients					
	Symptomatic patients			Asymptomatic patients		
	Positive	Negative	Total	Positive	Negative	Total
Wet mount	17	361	378	2	111	113
Culture	31	347	378	6 (9) ^b	107 (104)	113
Nested PCR	31	347	378	9	104	113

^a Among the symptomatic patients, 31 patients were positive for *T. vaginalis* both by culture and by nested PCR, while only 17 of these 31 patients were positive by wet mount. Among the asymptomatic patients, two were positive for *T. vaginalis* by wet mount, culture, and nested PCR, four were positive both by culture and by nested PCR but negative by wet mount, and three cases were positive only by nested PCR (negative by wet mount and culture). No PCR-negative patients were positive for *T. vaginalis* by wet mount or by culture.

^b The numbers in parentheses indicate the results for three asymptomatic PCR-positive patients that were negative for *T. vaginalis* by first culture but positive by second culture.

To evaluate the specificity of this nested PCR for the examination of clinical samples from among the 378 symptomatic patients, samples from 96 patients with known infections were analyzed (Table 2). Among these patients, 14 patients had *T. vaginalis* infection alone and 17 patients had mixed infections with other pathogens (bacteria or fungi). The other 65 patients had nontrichomonal vaginitis, and all tested negative for *T. vaginalis* by wet mount, culture, and nested PCR (Table 2).

Semiquantification of *T. vaginalis* in clinical specimens by colorimetric one-tube nested PCR. We also used clinical specimens containing known numbers of *T. vaginalis* parasites (quantification obtained from agar cultures) to extract DNA for use in the colorimetric nested PCR. Nested PCR was performed by using 10-fold serial dilutions of genomic DNA extracted from clinical samples by the rapid boiling method, and the results were assayed colorimetrically. Results for four patients are presented in Fig. 5. By using agar culture, the numbers of *T. vaginalis* organisms in clinical specimens from pa-

TABLE 2. Detection of *T. vaginalis* by wet mount, culture, and nested PCR in vaginal discharges from symptomatic women with cervicovaginitis^a

Infection ^b	No. of patients positive for <i>T. vaginalis</i> by:		
	Wet mount	Culture	Nested PCR
<i>T. vaginalis</i> (14)	9	14	14
<i>T. vaginalis</i> + bacteria (14)	6	14	14
<i>T. vaginalis</i> + <i>Candida</i> (3)	2	3	3
Bacterial vaginosis (28)	0	0	0
<i>Candida</i> (30)	0	0	0
<i>Candida</i> + bacteria (7)	0	0	0

^a Among the 378 symptomatic patients, samples from 96 women with documented infections were analyzed.

^b Numbers in parentheses are the number of patients enrolled.

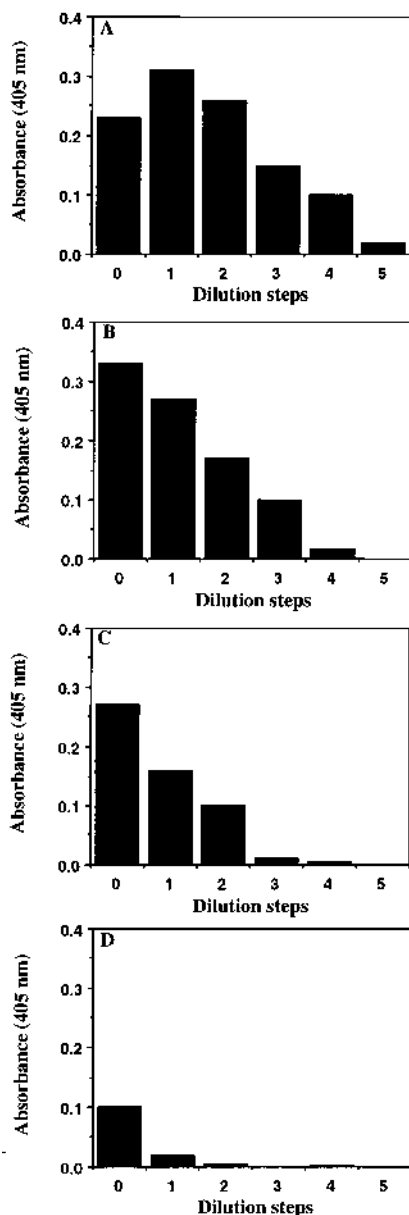


FIG. 5. Semiquantification of *T. vaginalis* in clinical specimens by colorimetric one-tube nested PCR. DNA was extracted from clinical samples containing known numbers of *T. vaginalis* organisms and was used for PCR assay. (A to D) Results for four independent patients. (A) The number of *T. vaginalis* organisms in 20 μ l of vaginal discharge was estimated to be 12,000 by agar culture and colorimetric assay of the PCR products obtained with this DNA is shown at dilution 0. Similarly, DNAs from 1,800 *T. vaginalis* organisms (B), 130 *T. vaginalis* organisms (C), and 1 *T. vaginalis* organism (D) were used in PCR and colorimetric assay and are shown at dilution 0. For the samples for all panels, the DNA was diluted 10-fold five times.

tients A, B, C, and D were estimated to be 60,000, 9,000, 650, and 5 per 100 μ l of vaginal discharge, respectively. DNA from these samples was resuspended in 25 μ l of deionized water as described in Materials and Methods, and 5 μ l was used in the PCR. Thus, DNAs from the equivalent of 12,000 (Fig. 5A), 1,800 (Fig. 5B), 130 (Fig. 5C), and 1 (Fig. 5D) *T. vaginalis* organisms were used undiluted or were serially diluted 10-fold five times for each assay. The results of the colorimetric assay are presented in Fig. 5. In each case the equivalent of one *T.*

vaginalis organism was detected when a cutoff value of the absorbance (405 nm) of 0.045 was used, and the absorbance increased proportionately with increasing amounts of DNA (from up to 1,000 *T. vaginalis* organisms).

Figure 5A shows a cutoff point between dilutions 4 and 5, which represent 1 and 0 parasite per assay, respectively. Moreover, the maximum absorbance was obtained at dilution 1, which represents 10^3 trichomonads in the assay mixture, indicating that patient A was infected with on the order of 10^4 parasites per 100 μ l of vaginal discharge. This result is in good agreement with the number of trichomonad parasites determined by the agar culture method. Similarly, Fig. 5B, C, and D show cutoff points between dilutions 3 and 4, 2 and 3, 0 and 1, respectively. In addition, the maximum absorbances for Fig. 5B, C, and D were all at dilution 0, suggesting that the numbers of parasites in 100 μ l of vaginal discharge for patients B, C, and D are on the order of 10^3 , 10^2 , and 10, respectively.

DISCUSSION

We have developed a colorimetric one-tube nested PCR for the detection of *T. vaginalis* in vaginal discharge. The sensitivity of this nested PCR was for DNA equivalent to one *T. vaginalis* organism in the extract from 20 μ l of vaginal discharge. The unique DNA sequence from the Tv-E650 family of repeats (13) was not detected in human DNA or other common urogenital pathogens. Detection of this nested PCR product has been adapted to a format similar to that used for the enzyme-linked immunosorbent assay, which has found extensive use in both clinical and research applications. This one-tube nested PCR not only reduced the possibility of contamination but also provided a sensitive, rapid (results were obtained in 6 h), and simple method for the detection of *T. vaginalis* in clinical specimens.

DNA from more than 10^3 trichomonads lowered the absorbances obtained by the colorimetric assay. The reduced absorbances may be due to the presence of the 448-bp DNA fragment produced by the biotinylated IP₁ and the OP₂, which would compete with the binding of 290-bp DNA fragment to the avidin coating on the microtiter plates. It is therefore not possible to determine the number of trichomonads in clinical samples from a single PCR assay. For absorbances in the range of 0.1 to 0.3, the presence of either low or very large numbers of *T. vaginalis* is possible. To corroborate estimates of the numbers of *T. vaginalis* organisms, further tests with a series of 10-fold dilution steps for PCR assay or numbers estimated by agar culture may be necessary. However, we feel this method may be semiquantitative. This method may be useful for evaluating changes in trichomonad numbers in vivo and may be particularly sensitive for detecting low-level infections.

Molecular approaches to the diagnosis of *T. vaginalis* have been reported. Rubino et al. (19) used a 2.3-kb *T. vaginalis* DNA fragment as a probe to detect *T. vaginalis* DNA in vaginal discharge by a dot blot hybridization technique. However, the 2.3-kb probe cross-reacted with *P. hominis* and *Trichomonas foetus*, and the detection limit was 200 axenically cultivated flagellates (19). Using the same DNA fragment as a probe, Muresu et al. (12) developed a nonisotopic method for identification of *T. vaginalis* by fluorescent-labeled DNA in situ hybridization. Although this method avoided the use of radioactive materials, it was time-consuming and both its sensitivity and specificity were not fully assessed (12).

The development of a PCR method for detection of *T. vaginalis* in clinical samples was first reported by Riley et al. (18). Using restriction enzyme analysis of PCR products, they

estimated that the sensitivity was from 10 to 100 trichomonads in each reaction mixture. Jeremias et al. (5) adopted the same PCR assay to detect *T. vaginalis* in pregnant and nonpregnant women and concluded that PCR analysis may be useful for women who were negative for *T. vaginalis* by wet mount but whose symptomatic vaginitis remained unexplained. Targeting a different DNA fragment of *T. vaginalis*, Kengne et al. (6) used three primers for a two-round PCR and analyzed products by gel electrophoresis followed by hybridization with a radioisotope-labeled probe. That study demonstrated that a single parasite from axenic cultivation could be detected, but did not show the sensitivity with clinical specimens. All the techniques used in those studies to detect specific PCR products, namely, gel electrophoresis with ethidium bromide staining or hybridization with radioisotope-labeled probes, are not suitable for use in routine diagnostic laboratories.

We examined each clinical sample in duplicate by both the standard DNA extraction method and the rapid boiling method for the DNA extraction and PCR analysis steps. The results obtained by the rapid boiling method were always consistent in duplicate PCR assays. However, by using standard DNA extraction, two samples had discrepant results in the duplicate tests. Repeat PCR of the same extract and the duplicate DNA extraction with material from the same clinical sample resolved the problem.

Although one trichomonad contains only approximately 0.15 pg of DNA, which is 100-fold less DNA/cell than the amount of DNA in host cells (22), our nested colorimetric PCR was positive even when the ratio of host cells to *T. vaginalis* was as high as 10,000:1. The ratio of inflammatory cells (mostly neutrophils) to trichomonads in vaginal discharge is in the range of 10 to 100 in patients with heavy infections (17). This is lower than the ratio that we tested, but it did not affect the sensitivity of our PCR. For symptomatic patients, the nested PCR was at least as sensitive as culture for the detection of *T. vaginalis* in vaginal discharge. Moreover, our nested PCR assay was consistently negative for patients whose symptoms were due to bacterial vaginosis or vaginal candidiasis. For asymptomatic women, nested PCR may be more sensitive than culture for the detection of *T. vaginalis* infection.

Our study by nested PCR demonstrated that less than a single trichomonad from axenic cultivation could be detected (data not shown). This is perhaps not unexpected, since the copy number of the Tv-E650 repeat has been estimated to be about 10^2 to 10^3 per genome (13). Because PCR may detect nonviable microorganisms, our PCR-positive but culture-negative results suggest the possibility of false-positive results, especially for patients treated for *T. vaginalis* infection. Nevertheless, our finding that three patients were culture negative but PCR positive for *T. vaginalis* (first sample) but culture positive upon repeat testing indicates the presence of poorly growing *T. vaginalis* and/or very low numbers of trichomonads in the vaginal discharge from these patients.

Trichomoniasis is not a self-limiting disease in women. Epidemiologic studies of urogenital trichomoniasis have shown that the infective dose of the organism in women is low and that the infection rate is high (10). Untreated asymptomatic vaginal trichomoniasis may result in acute clinical disease, may produce cellular atypia, and may serve as a reservoir for continuing disease transmission (4, 16). It has been shown that *T. vaginalis* carriage at midgestation is associated with preterm delivery, low birth weight, and an increased incidence of postpartum endometritis (2, 15, 20). Moreover, recent epidemiologic evidence points toward a possible predisposition of women with trichomoniasis for HIV infection (8). Therefore, to reduce the risk of adverse conditions during pregnancy and

HIV transmission, early diagnosis of *T. vaginalis* infection by routine screening of women and treatment of infected women and their sexual partners are required. The colorimetric one-tube nested PCR described here is suitable for such screening and is being applied to the detection of low numbers of organisms in asymptomatic subjects and patients with unexplained vaginitis.

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