Rapid Assay for Immunological Detection of *Trichomonas vaginalis*

ROBERT M. WATT,* ABRAHAM PHILIP, SUSAN M. WOS, AND GREGORY J. SAM

Glasgow Research Laboratory, Biomedical Products Department, E. I. du Pont de Nemours and Company, Inc.,
Wilmington, Delaware 19898

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Trichomoniasis is a common sexually transmitted disease with an estimated incidence of 4 million to 8 million cases a year in the United States. The most commonly used method of diagnosis is a direct microscopic observation (wet mount) of vaginal secretions and, although both rapid and inexpensive, the sensitivity of this technique is generally 50 to 70%. We developed an indirect enzyme-linked immunosorbent assay (ELISA) for the detection of *Trichomonas vaginalis* which is both rapid and sensitive (detection limit of approximately 100 trichomonads per ml). This assay, which employs affinity-purified rabbit anti-*T. vaginalis* antibodies in a "sandwich" configuration, is simple to perform and is neither interfered with nor appears to cross-react with other microorganisms which are common inhabitants of the urogenital tract. One hundred and seventy-seven consecutive unselected patients attending a clinic for sexually transmitted diseases were evaluated for trichomoniasis by a broth culture technique monitored for up to 7 days (and considered here to be the standard for positivity), the conventional wet mount method, a solid culture procedure, and the ELISA. Of these, 84 were positive by culture; 33 were positive by the wet mount; and despite the fact that the vaginal specimens were diluted 20-fold during the culture procedures prior to testing in the ELISA, 65 were positive by ELISA.

In addition to exhibiting a sensitivity of 77%, the specificity of the ELISA was 100%. These results demonstrate that the ELISA is a significant improvement over the wet mount method for the diagnosis of trichomoniasis.

Despite increased awareness of the frequency of *Trichomonas vaginalis* infections worldwide, the most popular clinical diagnostic tool remains the wet mount or direct microscopic examination of specimens from patients. Under the best circumstances this technique is only 75% sensitive (25) and more commonly is only 50 to 70% sensitive (9, 27, 28). Despite the modest sensitivity of the wet mount method, the basic reasons for its widespread use are twofold: (i) it is an extremely fast, inexpensive procedure (although its level of sensitivity and specificity is highly dependent on the skill of the technician), and (ii) sequellae accompanying infection are generally regarded as relatively minor compared with other sexually transmitted diseases (24, 25).

Recent observations, however, suggest that the diagnosis of trichomoniasis and its differentiation from other sexually transmitted diseases such as *Chlamydia trachomatis* may become increasingly important (15). Furthermore, respiratory infections in adults, presumably caused by the related trichomonad *T. tenax*, may in part be due to *T. vaginalis* (12). Pharyngeal and respiratory infections in newborns born to mothers with vaginitis caused by *T. vaginalis* may represent yet another important manifestation of trichomoniasis (22). In addition, an increased incidence of endometritis during pregnancy has been implicated with this infection (24). Thus, more sensitive diagnostic procedures may, in fact, need to be implemented.

Several methods have been developed for the detection of the protozoan. These include in vitro culture of the organism, direct microscopic examination of smears both with and without the aid of vital stains (11, 13, 28, 32), immunological staining of fixed specimens (5, 23), assays for antitrichomonad antibodies in either serum or vaginal secretions (2, 20, 21, 29, 30), and indirect immunofluorescence (16). In general, culture methods prove to be inconvenient due to the relatively slow generation time of the organism in vitro, the short shelf life of the culture medium, and the labor-intensive nature of the procedures. Moreover, the staining of specimens prior to microscopy does not provide significant increases in sensitivity (28), and serological methods suffer from the poor correlation between antibody titers and active infection (20, 21, 30). Thus, it appears likely that immunological detection of trichomonad antigens will prove to be the most sensitive approach to the diagnosis of trichomoniasis.

In this report we describe a sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of *T. vaginalis* antigens present in vaginal secretions obtained from 177 consecutive, unselected female patients attending an inner-city clinic for sexually transmitted diseases. This assay, which takes approximately 1 h to perform, is shown to be at least twice as sensitive as the wet mount procedure (compared with culture) and yielded no false-positive results.

**MATERIALS AND METHODS**

**Patient samples.** Vaginal secretions were obtained from 177 consecutive, unselected female patients presenting to the City of Philadelphia Department of Health Sexually Transmitted Disease Clinic No. 1, using a sterile, nonabsorbent pipette. Wet mount examination was performed by standard methods. Briefly, the vaginal secretion was emulsified into 1 to 2 drops of saline on a microscope slide, a cover slip was applied, and then the slide was examined at 400× magnification under a light microscope. A specimen was considered positive if motile trichomonads with an undulating membrane and flagella were seen. After a portion of the sample was prepared for direct microscopic examination, approximately 0.1 ml of the specimen was diluted 20-fold in modified medium described by Diamond (8) and a fraction of the diluted specimen was stored at −70°C until tested in the immunosassay. Specimen dilution was a necessary prerequisite in obtaining sufficient sample volume for subsequent testing. Approximately 0.3 ml of the diluted specimen was inoculated into modified Diamond medium.

* Corresponding author.
supplemented with nystatin, penicillin streptomycin, and 10% heat-inactivated horse serum. Culture and microscopic examinations of patient specimens were completed within 10 to 15 min after collection. Broth cultures were incubated at 37°C in an atmosphere of 7% CO₂ and scored by microscopic examination at 24, 48, and 72 h and at 7 days.

The remaining sample volume was used in a solid culture procedure for the quantitation of viable *T. vaginalis*. The details of this quantitation study have been described more fully by Philip et al. (A. Philip, P. Carter-Scott, and C. Rogers, J. Infect. Dis., in press), so only a terse description will be provided here. Briefly, the solid medium employed was a modification of Diamond medium as described previously by Ivey (14). Agar culture medium (pH 6.0) was prepared daily in 8.5-ml fractions, autoclaved, cooled, and held at 48°C until use. As in the case with the broth medium, horse serum and antibiotics were added immediately prior to the introduction of the specimen. Liquefied agar was poured into plates (100 mm in diameter), and duplicate sets of plates were inoculated with each patient specimen. If the sample was negative for *T. vaginalis* by wet mount, 0.5 ml of the 20-fold-diluted specimen was pipetted onto each plate, and a tube of solid culture medium (held at 48°C) was poured on top of the inoculum, mixed, and allowed to solidify. Wet mount-positive samples were diluted an additional 20-fold (1:400 final dilution) and processed in an identical fashion. Plates were placed in an anaerobic pouch (BBL Microbiology Systems, Cockeysville, Md.), heat-sealed, and incubated at 37°C for 5 days. At the end of this period colonies on both plates were counted; the average was determined; and the concentration of viable organisms, expressed as CFU/ml, in the specimen was calculated. Of the 84 broth-positive specimens obtained in this study, only 3 failed to grow on solid medium and, thus, they could not be quantified.

**Antibody preparation.** Antibodies to *T. vaginalis* were elicited in rabbits by immunization with whole trichomonads. Six adult female New Zealand White rabbits were injected intradermally in multiple sites with a 1-ml inoculum containing 2.5 × 10⁵ organisms emulsified in complete Freund adjuvant (Difco Laboratories, Detroit, Mich.). The animals received three injections within a 1-month period. The sera were pooled, and an immunoglobulin fraction was prepared by ammonium sulfate precipitation. This crude immunoglobulin G (IgG) fraction was applied to a column of sonicated *T. vaginalis* covalently coupled to CNBr-activated Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) (7). Specific antibodies were eluted from the column with 0.1 M acetic acid (pH 2.5) and immediately dialyzed against phosphate-buffered saline (PBS; 120 mM NaCl, 2.7 mM KCl, 10 mM phosphate [pH 7.4]; Sigma Chemical Co., St. Louis, Mo.). Purity of the antibody preparations was verified by electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels (17), and the protein concentration of antibodies was determined by the procedure of Lowry et al. (19).

**Biotinylation of purified antibodies.** Affinity-purified rabbit anti-*T. vaginalis* antibodies were biotinylated by reaction with N-hydroxysuccinimidobiotin (Pierce Chemical Co., Rockford, Ill.). Briefly, antibodies at a concentration of approximately 1 mg/ml were first dialyzed against 0.1 M sodium bicarbonate (pH 8.6) and then reacted with 30-fold molar excess of N-hydroxysuccinimidobiotin (dissolved at 1 mg/ml in dimethyl sulfoxide) for 4 h at room temperature. Free biotin was removed from the preparation by dialysis of the reaction mixture against PBS. Titors of biotinylated antibodies were then determined on *T. vaginalis*-sensitized polystyrene microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Alexandria, Va.) which were prepared by incubating individual wells on the plates with 100 μl of sonicated trichomonads (10 μg/ml) in PBS overnight at 4°C. Horseradish peroxidase-conjugated streptavidin (Bethesda Research Laboratories, Gaithersburg, Md.) was used to detect bound biotinylated antibodies.

**ELISA procedure.** Polystyrene microtiter plates were coated with affinity-purified rabbit anti-*T. vaginalis* antibodies, blocked by incubation with 1% bovine serum albumin in PBS, and stored at 4°C until needed. Assay of the clinical samples, depicted schematically in Fig. 1, involved the addition of 50 μl of the diluted specimens to individual wells in microtiter plates, followed immediately by the addition of 50 μl of biotinylated anti-*T. vaginalis* antibodies in 1% bovine serum albumin in PBS. After a 30-min period of incubation at room temperature, the assay plates were washed nine times (three cycles of three washes each with an automatic plate washer) with PBS containing 0.05% Tween 20, and then 100 μl of horseradish peroxidase-conjugated streptavidin was pipetted into individual wells. Fifteen minutes later, following a second series of three wash cycles with PBS-Tween 20, 100 μl of 2,2'-azino-di(3-ethylbenzthiazoline sulfonate) (ABTS; Kirkegaard and
Perry, Gaithersburg, Md.) was added to each well. After 10 min the resulting green color produced was measured at 410 nm in a microtiter plate reader. Results were also scored by an independent visual inspection of the plates before the absorbance was measured.

All patient samples were run in duplicate, and a series of T. vaginalis standard solutions was assayed in triplicate on each of the five plates used to accommodate all 177 specimens. All samples with an absorbance equal to the mean plus 2 standard deviations of three negative controls run on each plate were considered positive.

RESULTS

Detection of T. vaginalis. A summary of the major findings of this study are given in Table 1. Broth culture, monitored for the presence of motile trichomonads for up to 7 days, revealed that 84 (47.5%) of the 177 samples examined were infected with T. vaginalis. Approximately 37% (31 samples) of these trichomonad-containing specimens were correctly diagnosed by the wet mount method, which was performed immediately after sample collection. However, two specimens diagnosed as being positive for T. vaginalis could not be confirmed by either broth or solid culture and were deemed to be false-positive results. Thus, the specificity of the wet mount method in this setting was 97.8%. However, despite the fact that the samples were prediluted 20-fold with modified Diamond medium prior to testing in the ELISA, the immunoassay correctly identified 65 of the 84 positive samples. Thus, the sensitivity of the assay with respect to culture was 77.4%; the specificity was 100%, as no false-positive results were observed.

Assay response. The response of the 65 samples that were positive for T. vaginalis antigen when assayed by ELISA is presented in Fig. 2. A total of 58 of these samples displayed an optical density at 410 nm (OD_{410}) greater than 0.2, while 7 of the specimens yielded an OD_{410} assay response between 0.1 and 0.2. This is significant when considering the assay response as a function of trichomonad concentration (insert, Fig. 2). The assay standard curve, which is given as the mean response (± standard deviation) of triplicate determinations on each of the five plates (or 15 determinations), shows that the overall assay response of the negative control was 0.091 ± 0.008. Because ABTS appears colorless at an OD_{410} of <0.07 and only faintly blue-green at an OD_{410} of 0.091, those seven samples with absorbance values between 0.1 and 0.2 were easily discerned from the background values with an ELISA plate reader and could be readily distinguished by even a simple visual inspection.

Comparison of the sensitivity of the ELISA with that of the wet mount technique. The number of samples correctly identified by culture, the ELISA, and wet mount methods plotted as a function of the level of trichomonads present in the clinical specimen is shown in Fig. 3. The concentration of organisms presented here represents undiluted, neat samples. For the 81 broth culture-positive samples for which quantitative culture data were obtained, several features of the two assays are apparent. First, in general the wet mount is quite insensitive unless the sample contains on the order of 100,000 trichomonads per ml, as only 2 of 40 (5%) of infected specimens were correctly diagnosed below that level. Second, despite the fact that the actual concentration of ELISA-positive samples was diluted 20-fold from that shown, the ELISA correctly identified positive specimens in the 1,000- to 10,000-CFU/ml range in approximately 50% of the cases; above 10,000 CFU/ml, 58 of the 59 culture-positive samples were correctly identified (98.3%). These data suggest that the ELISA is capable of measuring trichomonads at levels at which the wet mount is demonstrably insensitive.

In total, 19 samples that were culture positive for T. vaginalis were not detected by the ELISA. Quantitative culture data was available for 17 of these specimens, and the number of undetected samples as a function of the concentration of trichomonads in them is presented in Fig. 4. The concentration of organisms given is that in the undiluted sample, as shown in Fig. 3. With the exception of one sample containing >5,000 organisms per ml (i.e., 250 cells per ml as assayed), all the remaining specimens contained the organism at concentrations after dilution below 250 cells per ml.

DISCUSSION

Many efforts to develop sensitive diagnostic tests for trichomoniasis have been reported in recent years. These attempts followed the findings of Fouts and Krauss (9), whose work essentially invalidated the long-held view that clinical symptoms were reliable indicators of trichomoniasis.
Using clinical presentation as the basis for diagnosis, they reported that 88% of women with trichomoniasis went undetected and an additional 29% of their patients were misdiagnosed as having trichomoniasis.

The sensitivity limitation of the wet mount, the most prevalent method of diagnosis, is generally recognized. Various staining methods have been proposed (11, 13, 26, 28, 32) in attempts to increase the sensitivity of microscopic examinations of direct smears. These reports, however, are inconclusive. For example, one study (18) reported a fourfold increase in sensitivity (to approximately 80%) over wet mount when acridine orange staining followed by fluorescence microscopy was used. Yet in a similar study, Greenwood and Kirk-Hillaire (11) observed little difference in sensitivity between the wet mount technique and acridine orange staining. The primary utility of these methods lies in the capability of deferring microscopic examination which adversely affects the sensitivity and specificity of the wet mount due to the decreased motility of the protozoan. Another method often used is the visualization of trichomonads on Papanicolaou smears. This method offers a sensitivity of approximately 70% (28); however, the level of sensitivity and specificity of this technique is also highly dependent on the skill of the cytologist.

Examination of sera or vaginal secretions for antibodies to the parasite offers little promise of being of diagnostic utility (2, 20, 21, 29, 30), primarily because of the lack of correlation between the presence of antibodies and an active infection. Using indirect immunofluorescence microscopy, several investigators (20, 29) reported that while 80 to 90% of patients with trichomoniasis had circulating antibodies to the protozoan, 13 to 17% of a control population of patients did also. Their results in testing vaginal secretions for IgG or IgA antibodies to T. vaginalis were even less encouraging.

Currently, there are no reports in the literature in which an ELISA is used for the detection of T. vaginalis antigens. Here we have reported the development of a rapid, sensitive ELISA for the detection of T. vaginalis antigens in vaginal secretions. This assay was used to diagnose the presence of trichomonads in 177 clinical specimens, in which all of the samples were examined by wet mount and fractions of each specimen were cultured in a broth medium and microscopically examined up to 7 days later for the presence of T. vaginalis. Additionally, the concentration of viable trichomonads in all but one positive sample was measured by a solid culture technique. Despite the fact that fractions of individual samples were prediluted 20-fold prior to testing in the ELISA, this procedure proved to be approximately twice as sensitive (77.4%) as the routinely used wet mount (36.9%). Furthermore, the ELISA was capable of detecting the protozoan at concentrations of the organism that were demonstrably below the level of sensitivity of the wet mount. It is noteworthy that by extrapolating from the assay response as a function of trichomonad concentration plotted in Fig. 2, we estimate that 79 samples would have been correctly identified, if they had not been prediluted, yielding a sensitivity of about 94%. This figure is certainly only an approximation, as assay performance on patient specimens was not observed to be absolutely quantitative. Possible explanations for this phenomenon include fine differences in antigenicity from strain to strain and blocking effects due to the presence of trichomonad-specific secretory IgA.

Although several investigators have generated monoclonal antibodies to T. vaginalis (6, 16), we utilized affinity-purified polyclonal antibodies for several reasons. There is a current lack of information concerning the antigenic relatedness of various strains of T. vaginalis (4). No differences in antigenic composition were observed among several pathogenic strains of T. vaginalis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1, 10; S. M. Wos and R. M. Watt, unpublished data) and fluorography (1), and common reactivity with certain trichomonad antigens has been seen when patient sera is used to probe Western blots of solubil-

![FIG. 3. Detection of T. vaginalis antigens in 177 clinical specimens: a comparison of the sensitivity of broth culture, the wet mount procedure, and the ELISA as a function of trichomonad concentration. The number of patients diagnosed as having trichomoniasis by the culture, ELISA, or wet mount techniques is plotted as a function of the quantitative number of organisms present in the undiluted secretions, as described in the text. Of the 65 ELISA-positive samples, 64 are represented here, one ELISA-positive sample could not be quantitated, as it did not grow in solid culture.](http://jcm.asm.org/)

![FIG. 4. ELISA of 177 clinical specimens: a summary of the trichomonad-positive samples undetected by the assay. Nineteen samples that were culture positive for T. vaginalis were not detected by the ELISA. The undiluted levels of trichomonads in 17 of these specimens for which quantitative culture data were obtained are shown. Aside from one sample with >5,000 organisms per ml (i.e., 250 cells per ml as assayed), all the remaining samples contained <250 cells per ml.](http://jcm.asm.org/)
ized trichomonads (10; S. M. Wos and R. M. Watt, submitted for publication). However, heterogeneity among the organisms has been observed by several serologic-based techniques including ELISA (2, 3), indirect immunofluorescence (16, 31, 33), and radioimmunoprecipitation (3). Although investigators are currently studying the antigenic composition of the organism with monoclonal antibodies (6, 16), data from these reports indicate that obtaining a single monoclonal antibody which recognizes all isolates of the organism could prove to be problematic. In addition, the two-site design of our assay requires antibodies with good affinity both bound to the solid phase and as detectors in solution in contrast to one-site assays developed for detecting the protozoan (e.g., immunofluorescence) (16). Thus, the use of polyclonal antisera eliminates the possible need of a second reagent. Finally, a sufficiently specific ELISA to the flagellated protozoan Giardia lambia has been reported with polyclonal antibodies (34), engendering confidence that an analogous approach to another flagellate could prove fruitful. The data presented here indicate that an ELISA used for antigen detection is rapid, easy to perform, and provides superior sensitivity over the wet mount method. The utility of such a format would be a major step in diagnosing trichomoniasis.

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LITERATURE CITED