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A New Method for Identification of *Trichomonas vaginalis* by Fluorescent DNA In Situ Hybridization

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The protozoan flagellate *Trichomonas vaginalis* is responsible for human trichomoniasis, one of the most widespread sexually transmitted diseases in the world. Several methods are currently used for laboratory diagnosis, including direct microscopic observation, cell culture, immunological techniques, and more recently, DNA probing and gene amplification. This report describes an in situ hybridization technique with specific DNA probes labeled with either biotin, rhodamine, or fluorescein for detection of *T. vaginalis* with fluorescence microscopy. Repetitive DNA sequences were evident in the nuclei of the protozoa as intensively fluorescent regions, giving a spotted pattern. No cross-reactivity between the probes and the DNAs of mammalian cells, yeasts, or bacteria was noted. This technique is potentially useful for the diagnosis of human trichomoniasis in clinical samples.

The protozoan flagellate *Trichomonas vaginalis* is responsible for human trichomoniasis, one of the most widespread sexually transmitted diseases in the world (7). To achieve the correct laboratory diagnosis necessary for treatment and for the control of the disease, several methods have been employed, including direct microscopic examination of clinical samples (6, 18), in vitro culture (3, 15), and immunological techniques (9). Despite this use of various diagnostic methods, laboratory diagnosis of *T. vaginalis* may be difficult in some cases (1, 8, 10).

Recently, we cloned a 2.3-kb *T. vaginalis* DNA fragment (pROS21) present in strains from diverse geographical areas which has been used as a specific radioactive probe to detect the protozoan DNA in vaginal discharge by a dot blot hybridization technique (19). Furthermore, Riley et al. (17) have used a PCR technique to amplify specific sequences unique to *T. vaginalis*. In this study, we developed an in situ hybridization technique by using a biotin-labeled probe (pROS21) to directly identify *T. vaginalis* in clinical samples with fluorescence microscopy. In order to reduce the time necessary for probe preparation, we amplified and directly labeled with fluorescent nucleotides a 600-bp DNA subclone (pALE1) of the original 2.3-kb pROS21 probe, using PCR (20). This was possible because the sequence of interest is cloned, and primers homologous to the vector sequences that flank the insertion site can be used to amplify inserts (14).

MATERIALS AND METHODS

Sample collection and preparation. *T. vaginalis* (isolates SS-1, SS-7, TO-2, and MPM-3) was isolated from cases of acute trichomoniasis and cultivated as previously described (4). The cells were harvested during the exponential growth phase of the culture, centrifuged, and washed (two times at 800 × *g* for 10 min each) with phosphate-buffered saline, pH 7.4 (PBS).

To test the specificity of the procedure, we added different concentrations of *Trichomonas* cells (2×10^5 to 2×10^6 per ml, final concentration) to a vaginal secretion obtained from a female volunteer with no history of vaginitis due to *T. vaginalis*. Clinical samples were collected from nine women attending the Clinic of Obstetrics and Gynecology (University of Sassari) with symptoms of urogenital disease and from eight asymptomatic women attending the Oncologic Center of Sassari for a routine Pap test.

At least 0.1 ml of the vaginal secretion or discharge was carefully collected from the posterior vaginal fornix with a sterile graduated polyethylene pipette and immediately suspended in 1.0 ml of PBS. Samples were transported at room temperature to the laboratory. Further processing (within 6 h) consisted of dilution with 5 ml of PBS and washing by centrifugation (800 × *g* for 10 min).

For hypotonic treatment, all pellets were gently resuspended for 3 to 5 min at room temperature in 5 ml of 0.075 M KCl preheated at 37°C and centrifuged (800 × *g* for 5 min). When hypotonic treatment is prolonged, cells become fragile and lyse. In some experiments, preparations of nuclei were obtained by suspending pellets in distilled H_2O at room temperature and immediately centrifuging.

Pellets from both KCl-treated cells and nuclear preparations were fixed by gently resuspending them at room temperature in 5 ml of acetic acid-methanol (1:3) for 30 min. After one washing in acetic acid-methanol, pellets were resuspended in a small volume of acetic acid-methanol (0.2 to 1 ml according to pellet size), and a drop of the suspension was placed on cleaned microscope slides, which were air dried.

Probe preparation. Two different probes were prepared. The first probe consisted of recombinant plasmid pROS21 (see Fig. 1, top) which was biotinylated by nick translation with a BRL Bionick Kit (GIBCO-BRL, Uxbridge, United Kingdom) followed by purification through a Sephadex G50 column (Pharmacia, Milwaukee, Wis.) and precipitation with salmon sperm DNA and *Escherichia coli* tRNA. This probe could be visualized indirectly in a system containing fluorescein isothiocyanate-avidin and biotinylated anti-avidin antibodies.

To construct the second probe, the pROS21 recombinant plasmid was cut with *Hin*cII, *Hin*dIII, *Eco*RV, and *Ava*II restriction enzymes (Promega Co., Madison, Wis.) in order to identify suitable sites for insertion into pUC18 (see Fig. 1).

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Two plasmids (pALE1 and pMAR1) were obtained in pUC18, using the same method as previously described (19). PCR amplification and labeling were performed with plasmid pALE1 as a template and two oligonucleotide primers, M13 forward and M13 reverse (Boehringer). The PCR reaction contained 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 4 mM $MgCl_2$, 200 μM (each) dATP, dGTP, and dCTP, 150 μM dTTP, 50 μM fluorescein-12-dUTP (Boehringer, Mannheim, Germany) or 12 μM rhodamine-4-dUTP (Amersham, Buckinghamshire, United Kingdom), 2.5 U of *Taq* polymerase (Perkin-Elmer/Cetus), 0.5 μM (each) primers, and 10 ng of pALE1 DNA. Thirty-two cycles of PCR were carried out, with denaturation at 94°C for 1 min, annealing at 55°C for 45 s, and extension at 68°C for 5 min (last cycle, 7 min). The PCR product was ethanol precipitated in the presence of 2 M ammonium acetate, resuspended in TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]), and used for direct hybridization with the sample.

Hybridization and detection. Hybridization and detection were performed by a modification of the technique described by Pinkel et al. (16). Eighty nanograms of biotinylated probe (or 10 ng of fluorescein- or rhodamine-labeled probe) was mixed with 10 μl of hybridization mix containing 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate [pH 7.0]), 50% formamide, and 10% dextran sulfate); the mixture was denaturated by heating at 80°C for 5 min and quenched on ice to prevent reannealing. *Trichomonas* DNA was denaturated by treating slides with 70% formamide–2× SSC at 70°C for 2 min followed by a series of washes in 70%, then 95%, and then absolute ethanol at 4°C. The probe in the hybridization mix was then placed on the slides under a coverslip (22 by 22 mm). Slides were sealed and placed in a moist chamber at 37°C for 12 h. Posthybridization washes were three 5-min periods at 42°C in 50 ml of 50% formamide–2× SSC followed by three 5-min periods in 50 ml of 1× SSC at 60°C. Biotin-labeled DNA was detected by incubation at 37°C for 30 min in a moist chamber with 100 μl of a mix containing 5 μg of fluorescein-labeled avidin (Vector Lab, Burlingam, Calif.) per ml in 4× SSC–0.1% Tween 20–5% low-fat dried milk (SSCTM). The signal was then amplified by incubation for 30 min at 37°C with 100 μl of biotinylated anti-avidin (Vector Lab) (5 μg/ml in SSCTM) and a second round of 100 μl of fluorescein isothiocyanate-avidin (5 μg/ml in SSCTM) for 30 min at 37°C. After each incubation, the slides were washed three times for 5 min each at 37°C with 50 ml of 4× SSC–0.1% Tween 20. Finally, cells were counterstained with Evans blue solution (Clinical Sciences Inc., Whippany, N.Y.) for 30 min, washed in 2× SSC–0.05% Tween 20, and mounted in antifade solution containing 23 mg of 1,4-diazobicyclo(2,2,2)octane (Sigma) per ml in 80% glycerol–0.02 M Tris-HCl, pH 8.0.

Nuclei were directly hybridized with PCR-labeled probe as described above except that posthybridization washes consisted of three 5-min washes with 50 ml of 50% formamide–4× SSC at 42°C and three washes (5 min each) in 50 ml of 2× SSC at 60°C. Slides were then mounted with an antifade solution containing 0.5 μg of 4,6-diamidino-2-phenylindole-dihydrochloride (Sigma) per ml to visualize chromatin DNA. Evans blue counterstain was omitted in this procedure.

Slides were screened at a magnification of ×250 with a Zeiss AXIOSCOP epifluorescence microscope equipped with Zeiss filter sets 09, 15, and 01. Suitable fields were observed at ×800 magnifications, and color photographs were taken on Fuji color HG-400 color print film.

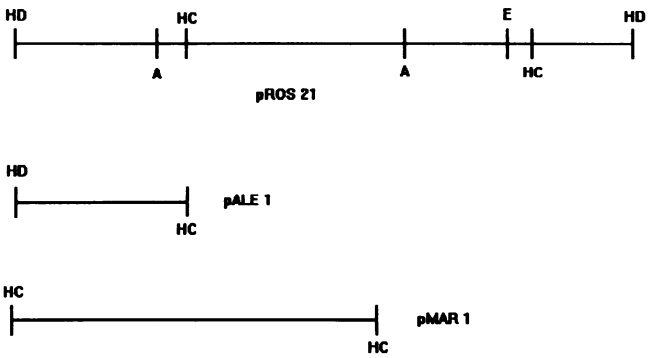

FIG. 1. Restriction maps of the 2.3-kb probe pROS21 and its subclones pALE1 (600 bp) and pMAR1 (1.2 kb). Hc, *Hin*cII; Hd, *Hin*dIII; E, *Eco*RV; A, *Ava*II.

RESULTS

The restriction maps of the two probes used in these experiments are shown in Fig. 1. Plasmid pROS21 was cut and mapped with *Hin*cII, *Hin*dIII, *Eco*RV, and *Ava*II restriction enzymes to identify suitable fragments for subcloning in pUC18. Two subclones containing 600- and 1,200-bp insertions in pUC18 (designated pALE1 and pMAR1, respectively) were obtained and cloned. pALE1 was chosen as a probe for the hybridization experiments. The specificity of this probe was the same as previously described (19) for the pROS21 probe (data not shown).

In situ hybridization with the fluorescent biotinylated probe pROS21 was used in the identification of *Trichomonas* DNA (Fig. 2A to E). Repetitive DNA sequences were evident in the nuclei of *T. vaginalis* as intensely fluorescent regions with characteristic spotted patterns due to hybridization with the probe.

A nonisotopic labeling system for detecting *T. vaginalis* in vaginal secretions was designed. The biotinylated pROS21 probe was hybridized with vaginal secretions to which a given amount of protozoa had been added (Fig. 2C). To confirm these results, vaginal secretions from nine women with symptoms of urogenital disease, including one with trichomoniasis (Fig. 2D), and from eight asymptomatic women (Fig. 2E) were used as samples for hybridization with the pROS21 probe. Under our experimental conditions, we found no cross-reactivity between the 2.3-kb *T. vaginalis* probe and human cells and microbial flora of the vagina (Fig. 2C to E). *T. vaginalis* cells present in the vaginal discharge were clearly marked by the fluorescent probe, and nonspecific hybridization signals that could compromise the visualization of fluorescent signals were notably absent.

In an attempt to simplify the methodology, we labelled the 600-bp DNA probe (pALE1) by PCR with rhodamine-4-dUTP for direct in situ hybridization in interphase nuclei of *T. vaginalis*. The procedure was sensitive enough to permit direct visualization of *T. vaginalis* nuclei (Fig. 2F to H). In comparison to the fluorescein isothiocyanate-avidin-biotin system, nuclei are stained in a more diffuse pattern, although more stained spots may be seen at higher magnification (Fig. 2B). However, the utility of this technique for identification of *Trichomonas* spp. in clinical samples must be tested further.

DISCUSSION

Accurate diagnosis of trichomoniasis in sexually active women is extremely important since *T. vaginalis* may be the

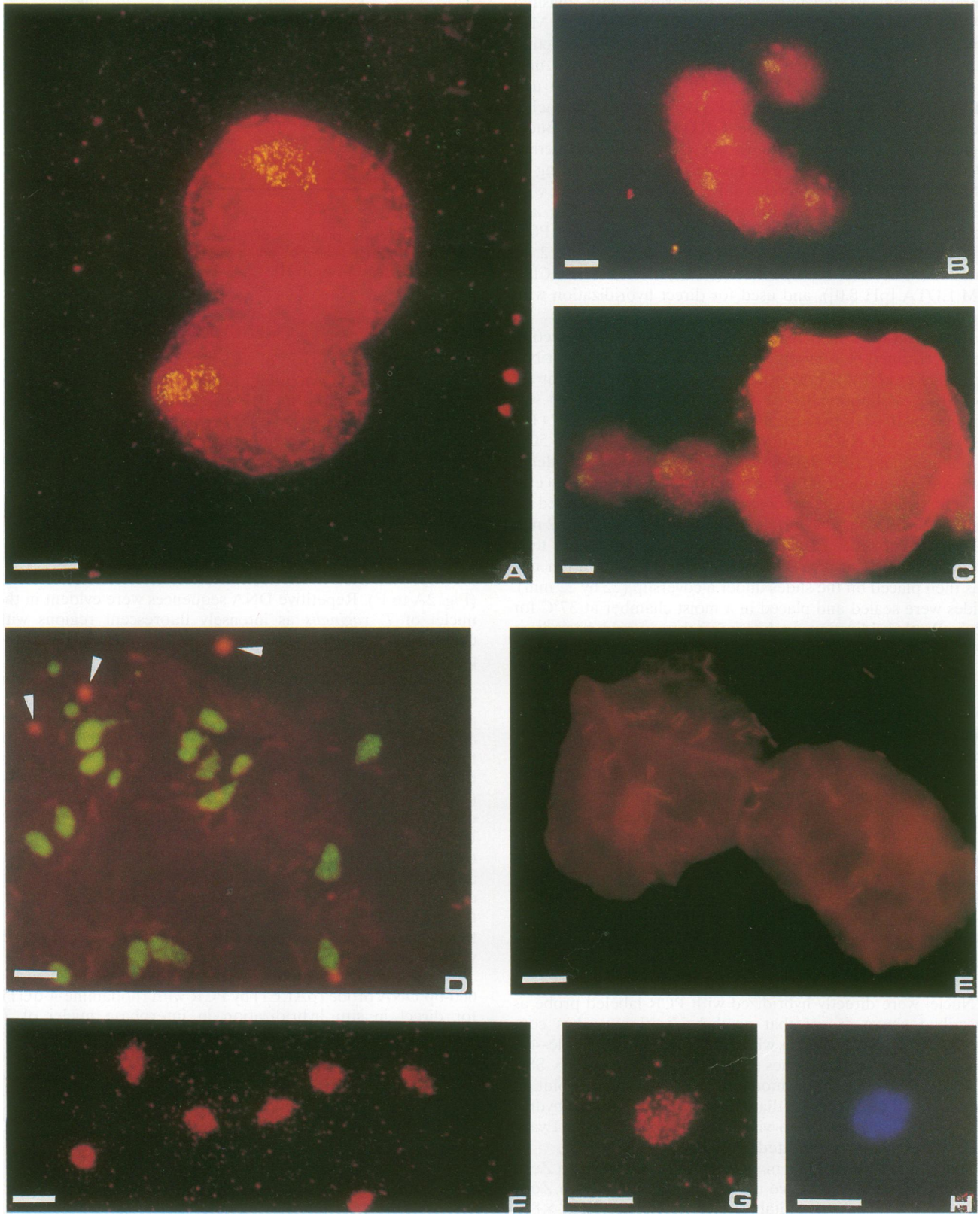
A
B
C
D
E
F
G
H

FIG. 2. Fluorescent in situ hybridization experiments on cells and nuclei of *T. vaginalis* and on vaginal secretions. (A–E) *T. vaginalis* hybridized with the biotinylated plasmid pROS21 containing a 2.3-kb DNA fragment specific for *T. vaginalis* DNA and detected with avidin-fluorescein isothiocyanate. (A and B) Different magnifications of isolated *T. vaginalis* from in vitro culture. (C) *Trichomonas* and vaginal epithelial cells. (D) Vaginal secretion from an infected woman showing *Trichomonas* nuclei (yellow-green), *Candida* cells (red; see arrow), and inflammatory cell debris and bacteria (light red). (E) Vaginal secretion from a healthy control showing epithelial vaginal cells and bacteria. *T. vaginalis* nuclei show a characteristic yellow-green spotted fluorescence due to hybridization of the probe with DNA repetitive sequences. Cell cytoplasm was counterstained in red with Evans blue. (F) Nuclei (red) of *T. vaginalis* hybridized with a 600-bp probe (pALE1) amplified and simultaneously labelled with rhodamine-4-dUTP by PCR. (G and H) Higher magnification of a positive nucleus simultaneously stained with the rhodamine-labeled probe (red) and with 4,6-diamidino-2-phenylindole-dihydrochloride (blue). Bars, 10 μm.

cause of high morbidity and, in common with other nonulcerative sexually transmitted diseases, may be regarded as a risk factor for contraction of human immunodeficiency virus infection (11). For these reasons, several methods for improving diagnosis have been developed. Routine identification based on the wet mount method is inexpensive and easy to perform, but it lacks acceptable sensitivity because a minimal concentration of 10^4 cells per ml of vaginal fluid is necessary for detection of the viable flagellate protozoa (3). With this technique, only 50 to 70% of infected patients show positive results (5, 18, 21). Also, culture techniques require viable protozoans and a minimal inoculum of 10 to 10^4 cells (3). An alternative immunological technique using a single monoclonal antibody may not recognize *T. vaginalis* surface antigens from organisms isolated from diverse geographical areas due to extreme antigenic variability (2). These limitations have been partially overcome by using immunodetection with either polyclonal (2) or pools of monoclonal (1) antibodies.

The dot blot method described by Rubino et al. (19) gave encouraging results, but it has some disadvantages, such as instability of the probe due to radiolysis, time-consuming autoradiographic procedures, and a need for correct preparation of the clinical samples. Furthermore, the use of this method as a routine diagnostic technique requires suitable tools for the handling and disposal of radioactive materials. In the same way, the development of a PCR method (17) for detection of *T. vaginalis* in clinical samples can be very useful but requires the use of the necessary instruments and an adequate laboratory. In an effort to overcome all of these problems, we have applied a nonisotopic method for identification of *T. vaginalis* by DNA in situ hybridization, using a specific 2.3-kb fluorescent labeled DNA probe (pROS21) and a subclone derived from this plasmid. The development of improved in situ hybridization techniques has made this method increasingly applicable to the detection of specific nucleic acid sequences directly within cells or genomes (13).

Our fluorescent probe pROS21, obtained from a repeated sequence of the *Trichomonas* genome (19), is especially suited for in situ hybridization because of the high number of target sequences in the protozoan chromatin. Other advantages of this technique are that fluorescent probes are rather stable and not subject to special disposal requirements and detection is via a fluorescence microscope.

Nonradioactively labeled DNA probes are often produced by nick translation of the appropriate DNA (12) or chemically by covalent linkage of labeled reporter molecules to the DNA (22). As an alternative approach to probe labeling, PCR avoids time-consuming amplification of the cloned template DNA in bacterial cells and the purification of the DNA probe after labeling. The unusually low background and high hybridization efficiency achieved provided easy and precise detection of the *T. vaginalis* genome through microscopic observation. Labeled DNA probe sufficient for in situ hybridization of up to 100 slides can be produced rapidly with 10 ng of DNA template. The direct coupling of fluorophores to probe molecules avoids amplification procedures, enhancing sensitivity. Once the specific oligonucleotide primers have been synthesized, probe generation and fluorescent in situ hybridization can be completed within 17 h. Controls of vaginal secretions with bacteria and human cells without *T. vaginalis* were negative, supporting the specificity of the in situ hybridization procedure. Although the method is rapid, simple, and specific, its use for microbiological diagnosis requires further studies. In particular, the application of these methods to the detection of *Trichomonas* spp. in clinical specimens containing low numbers of the organism, as in patients with chronic infections and in males with trichomoniasis, should be confirmed.

The potential for nonisotopic in situ hybridization procedures is significantly increased by multihybridization protocols, enabling the simultaneous differential delineation of several target sequences. In situ hybridization with multiple fluorescently labeled probes, enabling the simultaneous visualization of three or more DNA regions, could be a powerful tool to detect concomitant infections with virus, bacteria, and yeasts responsible for other sexually transmitted diseases.

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