JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 702–706
0095-1137/91/040702-05$02.00/0

Vol. 29, No. 4

Molecular Probe for Identification of *Trichomonas vaginalis* DNA

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Received 23 July 1990/Accepted 3 January 1991

Trichomoniasis is one of the most widespread sexually transmitted diseases in the world. Diagnosis can be achieved by several methods, such as direct microscopic observation of vaginal discharge, cell culture, and immunological techniques. A 2.3-kb *Trichomonas vaginalis* DNA fragment present in strains from diverse geographic areas was cloned and used as a probe to detect *T. vaginalis* DNA in vaginal discharge by a dot blot hybridization technique. This probe was specific for *T. vaginalis* DNA. It recognized strains from two regions in Italy (Sardinia, Piemonte) and from Mozambique (Africa). In addition, our probe did not cross-react with bacterial (*Escherichia coli*, *Enterococcus* spp., group B streptococci, *Gardnerella vaginalis*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and *Lactobacillus* spp.), viral (herpes simplex virus type 2), fungal (*Candida albicans*), protozoan (*Entamoeba histolytica*, *Giardia lamblia*, *Plasmodium falciparum*, *Leishmania major*, and *Leishmania infantum*), or human nucleic acids. The probe reacted with *Pentatrichomonas hominis* and *Trichomonas foetus*. The limit signal recognized by our probe corresponded to the DNA of 200 *T. vaginalis* isolates. The 2.3-kb probe was used in a clinical analysis of 98 samples. Of these, 20 samples were found to be positive both with the probe and by cell culture, and only 14 of these were positive by a standard wet mount method.

Trichomonas vaginalis is responsible for human trichomoniasis (for a review, see reference 11), one of the most widespread sexually transmitted diseases in the world, with an incidence of 10 to 50% in sexually transmitted disease clinics (26). Accurate diagnosis is necessary for specific treatment and for the control of the infection. One method of diagnosis is identification of the motile flagellate protozoan in a vaginal discharge by direct microscope examination (wet mount). By this method it is possible to identify *T. vaginalis* in 50 to 70% of infected patients (26). Several methods have been developed for improving detection of *T. vaginalis* in clinical samples. These include in vitro culture (5, 21) and direct microscopic examination by cytological staining procedures (9, 10). There are also numerous immunological techniques with polyclonal and monoclonal antibodies, such as immunofluorescence, enzyme-linked immunosorbent assay, and latex agglutination (1, 4, 14, 16, 24, 27, 29, 33). In addition, assays for antitrichomonas antibodies in either serum or vaginal secretions have been described (19, 27, 28). Despite this, laboratory diagnosis of trichomoniasis may be difficult when a low number of *T. vaginalis* is present, for example, in males with prostatitis and urethritis (15), asymptomatic females (12), those with acute infections with lytic antibodies (2), and some patients with chronic infections.

In recent years, recombinant DNA techniques have increasingly been used in clinical laboratories to improve the specificity and sensitivity of the diagnostic methods. Specific DNA probes that are able to detect bacteria, viruses, fungi, and parasites in clinical samples have been produced (13, 18, 22). Some commercial kits are now available for these purposes.

In this study, we cloned a 2.3-kb *T. vaginalis* DNA fragment that was used as a probe for the detection of the protozoans in vaginal secretions by a dot blot hybridization technique.

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MATERIALS AND METHODS

Reagents. Agarose and low-melting-temperature agarose were purchased from Sigma Chemical Co. (St. Louis, Mo.). Restriction enzymes, lambda *Hin*dIII markers, DNase-free RNase A, and T4 ligase were purchased from Promega Co. (Madison, Wis.). [α-^{32}P]dCTP was obtained from Dupont de Nemours (Bad Homburg, Federal Republic of Germany). Proteinase K was purchased from International Biotechnologies Inc. (New Haven, Conn.). The random priming labeling kit came from Boehringer (Mannheim, Federal Republic of Germany). All other chemicals were of the highest purity commercially available.

Culture. *T. vaginalis* was isolated from female patients with clinical symptoms of urogenital disease in two different regions of Italy, Sardinia (isolates SS-1, SS-2, SS-6, SS-7, SS-9, and SS-11) and Piemonte (isolates TO-1 and TO-2), and in Mozambique, Africa (isolates MPM-1 and MPM-3). Clinical samples were routinely incubated in Vagicult (Orion Diagnostica, Espoo, Finland) for 3 to 5 days, and positive samples were axenically subcultivated in Diamond medium (6) without agar supplemented with 10% heat-inactivated bovine serum and containing penicillin G (100 IU/ml) and streptomycin (100 μg/ml).

Specimen collection. Vaginal secretions or discharges were collected from 25 women attending the Clinic of Obstetrics and Gynecology (University of Sassari) with clinical signs of acute trichomoniasis and 73 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 transfer pipette.

Wet mount. One drop of the clinical samples was applied to a glass slide, covered with a cover slip, and examined at $\times$100 and $\times$400 magnifications with a light microscope. The wet mount was examined for the presence of motile protozoans suggestive of *T. vaginalis*.

DNA isolation. The *T. vaginalis* culture was harvested by

centrifugation while the organism was in the logarithmic phase of growth at a concentration of 1.5×10^6/ml. The cell pellet (a total of 10^8 cells) was washed with phosphate-buffered saline; was immediately used for DNA isolation by adding 10 ml of lysis buffer (LBT) containing 1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 300 mM NaCl, 0.2% sodium dodecyl sulfate (SDS), and 100 μg of proteinase K per ml; and was incubated at 65°C for 20 min. It was then extracted twice with an equal volume of phenol-chloroform (1:1; vol/vol) (17). To the aqueous phase was added 2.5 volumes of ethanol (17). The pellet obtained from centrifugation (10,000 × *g* for 20 min) was resuspended in 500 μl of TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA [pH 8]) with 20 μg of DNase-free RNase per ml and was incubated at 37°C for 30 min. After further extraction with phenol-chloroform, DNA was precipitated with LiCl (final concentration, 0.8 M) and 2.5 volumes of ethanol. The centrifuged pellet was finally resuspended in 400 μl of TE buffer. This method gave a total DNA yield of about 300 to 400 μg.

A DNA minipreparation was set up with 10 ml of *T. vaginalis* culture (10^6 cells per ml) by the same method described above with proportional amounts of reagents.

Total DNA was purified from *Escherichia coli* (four isolates), *Enterococcus* spp. (three isolates), group B streptococci (three strains), *Neisseria gonorrhoeae* (four strains), *Lactobacillus* spp. (two strains), *Gardnerella vaginalis* (three strains), and *Candida albicans* (five strains) isolated from vaginal swabs in our diagnostic laboratory. DNA was purified by the method of Meade et al. (20). Human lymphocyte DNA was a gift of M. Siniscalco (Porto Conte Research Laboratories). Herpes simplex virus type 2 DNA was obtained from infected human fibroblasts (a gift of A. Dolei). *Chlamydia trachomatis* DNA (three strains) was obtained from infected McCoy fibroblasts (kindly provided by S. Zanetti). *Entamoeba histolytica* DNA was a gift of P. Sansonetti (Institut Pasteur, Paris, France). *Giardia lamblia* (two strains) was a gift of L. Gradoni (Istituto Superiore di Sanità, Rome, Italy). *Plasmodium falciparum* FCR/3 was cultured as described previously (30). *Leishmania major* (one strain) and *Leishmania infantum* (one strain) were cultured in RPMI 1640 medium supplemented with inactivated 10% fetal bovine serum. *Tritrichomonas foetus* 3741 and *Pentatrichomonas hominis* 30098 were a gift of J. Ackers (London School of Tropical Medicine and Hygiene).

All protozoal DNAs were purified by adding lysis buffer (LTB), kept at 65°C for 15 min, phenol extracted twice, and ethanol precipitated. DNA was resuspended in TE buffer.

Cloning. *T. vaginalis* DNA was cut with the enzyme *Hin*dIII according to the directions of the manufacturer and was size fractionated on a low-melting-temperature 0.8% agarose gel. A fragment of 2.3 kb, which was present in all *T. vaginalis* isolates from the diverse geographical areas tested (data not shown), was purified from the gel and ligated into the unique *Hin*dIII site of the pUC18 polylinker by standard protocols (17). Recombinant plasmids (pROS21, pPAT22) were isolated from $CaCl_2$-competent *E. coli* MC1061 selected on Luria broth (LB) agar plates containing ampicillin (50 μg/ml). In order to show that the 2.3-kb fragment was identical to the 2.3-kb *T. vaginalis* fragment, the pROS21 plasmid was digested with *Hin*dIII and tested by Southern blotting hybridization (25).

Labeling of DNA probe and hybridization. A total of 50 ng of the 2.3-kb fragment was radioactively labeled with a random priming labeling kit, as described in the protocol of the manufacturer. Southern hybridization was performed at 65°C in 6× SSC–10 mM EDTA–5× Denhardt solution (consisting of 0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin)–0.5% SDS–100 μg of denatured herring sperm DNA per ml. After hybridization, the filters were washed twice at room temperature in 2× SSC–0.5% SDS for 15 min, followed by washing in 1× SSC–0.5% SDS for 30 min at 65°C and then 0.5× SSC–0.5% SDS for 30 min at 65°C. The autoradiographs were prepared with Kodak diagnostic films (X-OMAT AR) with intensifying screens. The films were exposed at −70°C for various lengths of time (6 to 24 h).

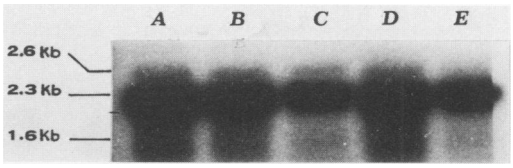

FIG. 1. Autoradiograph of a Southern blot of DNA cut with *Hin*dIII from five representative strains of *T. vaginalis* probed with the 2.3-kb probe. Lanes: A, strain SS-1; B, strain SS-2; C, strain TO-1; D, strain TO-2; E, strain MPM-3.

Dot blot hybridization. To test the sensitivity of the probe, we added different amounts of cultivated *T. vaginalis* (between 10^3 and 10^6) to vaginal secretions obtained by washing the posterior fornix of healthy female volunteers with no history of *T. vaginalis* vaginitis with 1 ml of sterile saline buffer.

These DNA samples and the DNAs of clinical specimens were extracted as described below and were used for hybridization with the DNA probe. DNA was extracted from 200-μl samples with equal volumes of lysis buffer (LBT), kept at 65°C for 15 min, phenol extracted, and ethanol precipitated. DNA was resuspended in 10 μl of TE buffer, and samples of 1 or 5 μl were spotted onto a Biotrans nylon membrane (ICN, Irvine, Calif.). Negative controls without *T. vaginalis* were treated in the same way. DNAs from bacteria, viruses, fungi, and protozoans were spotted in a similar way, but with a 20-fold greater concentration compared with the concentration of *T. vaginalis* DNA used and were hybridized with the [α-^{32}P]dCTP radioactively labeled 2.3-kb fragment purified from plasmid pROS21 as described above.

RESULTS

We developed a rapid protocol for the preparation of DNA in which DNase activity was inhibited by adding proteinase K in the lysis buffer. This method yielded a well-preserved DNA that can be used for enzyme restriction analysis and gene cloning.

As a candidate for a molecular DNA probe, we selected a 2.3-kb fragment that was present in all our *T. vaginalis* strains that were digested with *Hin*dIII (data not shown). The fragment was purified from a low-melting-temperature agarose gel and cloned into a pUC18 plasmid DNA vector.

Figure 1 shows an autoradiograph of a Southern blot of DNA extracted from five representative *T. vaginalis* isolates from diverse geographic areas (Sardinia SS-1 and SS-2, Turin TO-1 and TO-2, Maputo MPM-3). DNA was digested with the endonuclease *Hin*dIII and probed with the 2.3-kb *Trichomonas* probe. A band of the expected size was visible in all *T. vaginalis* isolates examined.

The reactivity of the probe with DNA minipreparations from 10 *T. vaginalis* isolates isolated from two regions in

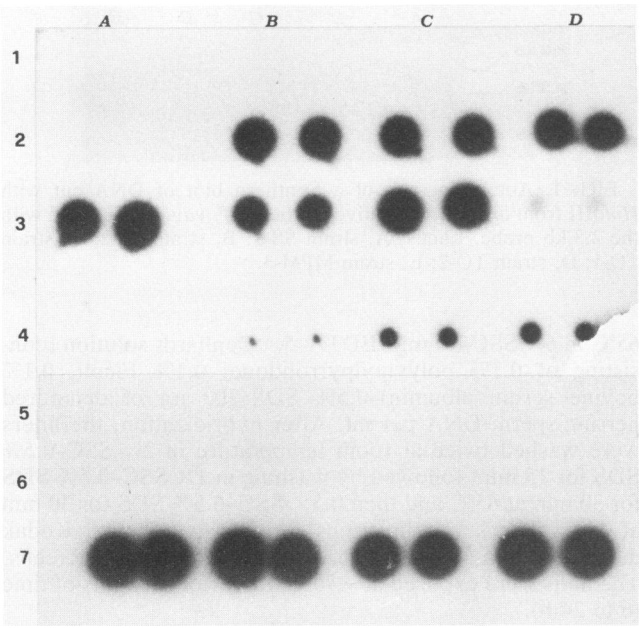

FIG. 2. Autoradiograph of a dot blot hybridization of *T. vaginalis* DNA probes with DNAs from different sources. A1, *E. coli*; B1, *C. albicans*; C1, *Streptococcus* spp.; D1, *Lactobacillus* spp.; A2, human lymphocytes; B2, *T. vaginalis* TO-1; C2, *T. vaginalis* TO-2; D2, *T. vaginalis* MPM-3; A3, *T. vaginalis* SS-1; B3, *T. vaginalis* SS-2; C3, *T. vaginalis* SS-6; D3, DNA from a vaginal secretion of a patient with acute trichomoniasis positive on cultural examination; A4, DNA from a vaginal secretion of a healthy woman negative on cultural examination; B4, DNA corresponding to 200 *T. vaginalis* cells; C4, DNA corresponding to 2,000 *T. vaginalis* cells; D4, DNA corresponding to 20,000 *T. vaginalis* cells; A5, *C. trachomatis*; B5, *N. gonorrhoeae*; C5, *Entamoeba histolytica*; D5, *G. vaginalis*; A6, *G. lamblia*; B6, *P. falciparum*; C6, *L. major*; D6, herpes simplex virus type 2; A7, *T. vaginalis* SS-7; B7, *T. vaginalis* SS-9; C7, *T. vaginalis* SS-11; D7, *T. vaginalis* MPM-1.

Italy (Sardinia, Piemonte) and from Mozambique is evident in a dot blot hybridization autoradiogram of two different experiments (Fig. 2).

In order to evaluate the specificity of the probe, we prepared DNA obtained from bacteria (*E. coli*, *Enterococcus* spp., group B streptococci, *N. gonorrhoeae*, *Lactobacillus* spp., *G. vaginalis*, *C. trachomatis*), protozoans (*E. histolytica*, *G. lamblia*, *P. falciparum*, *L. infantum*, *L. major*), a virus (herpes simplex virus type 2), and a fungus (*C. albicans*) and human DNA. Under our experimental conditions, we found no cross-reactivity between these DNAs and the 2.3-kb *T. vaginalis* probe (Fig. 2). We evaluated the reactivity of the probe with other trichomonads. The 2.3-kb *T. vaginalis* probe reacted with *T. foetus* and *P. hominis* DNAs (Fig. 3).

By using vaginal discharges with known amounts of *Trichomonas* isolates, we found that the limiting signal recognized by the probe corresponds to the DNA of about 200 cells per dot (Fig. 2, dot B4). We extended the use of the *T. vaginalis* probe to the analysis of 25 clinical samples of women with suspected trichomoniasis and 73 women with no specific signs of vaginitis. Figure 4 shows the strong positivity of 14 samples from women with clinical signs of acute trichomoniasis (Fig. 4, dots A12, B3, B7, C12, C13, F3, G4, G5, G6, G7, G8, G9, G10, and G11). The results were confirmed by the wet mount diagnostic technique, which showed a high number of motile flagellated organisms. Although six samples were not positive by wet mount analysis, they showed reactivity with the probe (Fig. 4, dots A3, B1, B2, B10, B12, and C14), and protozoans grew in cell cultures with Vagicult. The remaining clinical samples were negative by all methods.

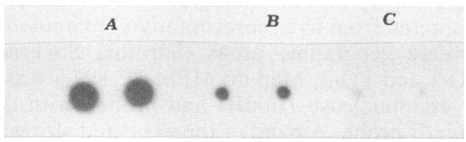

FIG. 3. Autoradiograph of a dot blot hybridization of a 2.3-kb DNA probe with DNA from some trichomonads. (A) *T. vaginalis*; (B) *T. foetus*; (C) *P. hominis*.

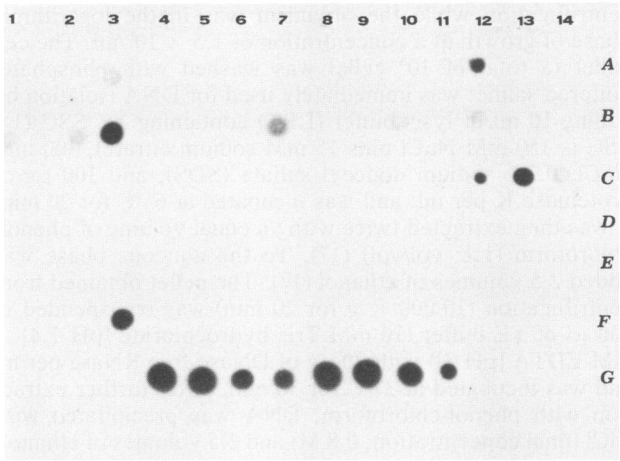

FIG. 4. Autoradiograph of a dot blot hybridization of DNA obtained from 98 clinical samples of vaginal discharges probed with the 2.3-kb *T. vaginalis* probe. Dots A12, B3, B7, C12, C13, F3, G4, G5, G6, G7, G8, G9, G10, and G11 were also positive by wet mount examination and the cell culture method. Dots A3, C1, C2, C10, C12, and C14 were positive only by the cell culture method.

DISCUSSION

In the past few years, several genes from pathogenic microorganisms have been cloned and used as probes for the detection of agents of infectious diseases (8, 13, 22). Such probes, derived from basic molecular biology studies, have been extended to applied biotechnology.

Isolation of DNA from *T. vaginalis* and other protozoans is relatively difficult because of the presence of active DNases. Therefore, we found that it was extremely important to use inhibitors of DNase activity in order to obtain intact DNA. Among these, we used proteinase K, which has successfully been used to prevent DNA degradation during the isolation of DNA from other protozoans such as *G. lamblia*, *Trypanosoma brucei*, *P. falciparum*, and *Leishmania* spp. (23).

The method described by Wang and Wang (31) for *T. vaginalis* DNA isolation is difficult to perform, time-consuming, and expensive. Using this method, they reported a yield of 150 μg of purified DNA per 10^9 cells. In this report, we describe a simple and rapid method that gave a good yield of DNA that could be used in polymorphism studies, gene cloning, and genomic library preparation.

Our probe was highly specific for *T. vaginalis*. The specificity of the DNA probe was particularly important for diagnostic use, since women with trichomoniasis are known to be at risk for coinfections by other urogenital tract pathogens. These include bacteria responsible for vaginosis, vulvovaginal candidiasis, and sexually transmitted diseases caused by *C. trachomatis*, herpes simplex virus type 2, and *N. gonorrhoeae* (7). No cross-reactivities among several urogenital pathogens (herpes simplex virus type 2, *C. albicans*, group B streptococci, *N. gonorrhoeae*, *Enterococcus* spp., *C. trachomatis*, *G. vaginalis*) or protozoans such as *G. lamblia*, *P. falciparum*, *E. histolytica*, *L. major*, and *L. infantum* were demonstrated with our 2.3-kb fragment. It was not surprising that the 2.3-kb DNA probe recognized DNA from other trichomonads. This should not limit the use of this probe for the identification of *T. vaginalis* in clinical vaginal samples, however. The possibility of a false-positive result because of the presence in the human vagina of *P. hominis*, an inhabitant of the human large intestine, or *T. foetus*, a bovine parasite, is unlikely. These trichomonads cannot be readily transplanted into the vagina, the natural habitat of *T. vaginalis*.

Our DNA probe recognized all the *T. vaginalis* isolates from different geographic areas tested. Furthermore, the sensitivity of the probe in the dot blot hybridization technique allowed the identification of an amount of *T. vaginalis* DNA corresponding to about 200 protozoans present in a sample containing human and bacterial DNAs. According to Philip et al. (21), this low concentration can be found only in fewer than 5% of women with vaginal trichomoniasis. We stress, however, that recovery of DNA is never 100%, so the real number may be even lower. Sensitivity could be increased further by using probes of antisense oligonucleotides against rRNA, as has been suggested for *Plasmodium* spp. (32). However, basic knowledge of *T. vaginalis* rRNA is still lacking.

Our probe may overcome some common diagnostic problems. First, although the wet mount method has the advantages of being inexpensive and simple to perform, it lacks acceptable sensitivity, because a minimal concentration of 10^4 organisms per ml of vaginal fluid appears to be necessary for detection of the protozoan (5). Second, culture techniques require that the protozoans are viable, and a minimal inoculum of 10 to 10^4 is necessary (5). Finally, a single monoclonal antibody is unable to recognize *T. vaginalis* surface antigens from organisms obtained from diverse geographical areas because of their high heterogeneities (2, 3, 5, 16).

Although the use of the DNA probe reported here gave encouraging results and its sensitivity in examining clinical samples is equal to that of the culture method, its use as a routine diagnostic tool awaits further studies. First, a simple and reliable method for the isolation of DNA from all clinical samples, including vaginal swabs, must be developed. Second, nonradioactive methods should be used for the detection of nucleic acid hybridizations. This could be achieved by using the biotin-avidin complex, fluorochromes, or chemiluminescent molecules.

ACKNOWLEDGMENTS

We thank P. Melis and R. Negri for criticism in reading the manuscript and Mauro Colombo for helpful discussion.

This work was supported by grant 89.05352.MZ77 from the Consiglio Nazionale delle Richerche and, in part, by a grant from MPI.

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