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Comparison of Molecular and Microscopic Techniques for Detection of *Treponema pallidum* in Genital Ulcers

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We compared the ability of direct immunofluorescent staining (DFA) and the PCR to detect *Treponema pallidum* in specimens from patients with genital ulcer disease. Touch preparations from 156 patients with genital lesions were fixed in acetone and stained with a fluorescein-labeled monoclonal antibody specific for the 37-kDa antigen of *T. pallidum*. After microscopic examination, the smear was removed from the slide with a swab. DNA was extracted with phenol-chloroform and precipitated with isopropanol. Ten microliters of the extracted DNA was amplified by PCR using primers for the gene encoding the 47-kDa protein of *T. pallidum* and hybridized to an internal probe. Twenty-two of 156 specimens were positive for *T. pallidum* by DFA and PCR, while 127 were negative by both methods, yielding a concordance of 95.5% ($\kappa = 0.84$). Four specimens were positive by PCR and negative by DFA, while three specimens were negative by PCR and positive by DFA. The DFA-negative, PCR-positive specimens may have resulted from the presence of large numbers of leukocytes on the slides, obscuring visualization of treponemes. The DFA-positive, PCR-negative results were not due to inhibition of the PCR since purified *T. pallidum* DNA was amplified when added to aliquots of these specimens. Negative results in these specimens were most likely due to inefficient recovery of their DNA. These data suggest that DFA and PCR are equivalent methods for detection of *T. pallidum* on touch preparations of genital lesions. Further refinements of the PCR assay are necessary for it to significantly improve the detection of *T. pallidum* in genital lesions.

Since *Treponema pallidum* has yet to be cultured in artificial media, the laboratory diagnosis of syphilis depends on the detection of antibodies or the demonstration of organisms by microscopy (10, 21, 23). Serologic tests, however, have poor sensitivity during early disease (4, 21). At initial presentation, up to 30% of patients with primary syphilis may have a negative nontreponemal serologic test (6, 19, 20). Although treponemal tests, such as the fluorescent treponemal antibody-absorption test, become positive before nontreponemal tests, most laboratories perform the latter as a screening test and only confirm positive nontreponemal tests with a fluorescent treponemal antibody-absorption test. Visualization of treponemes by dark-field microscopy is generally considered the gold standard; however, its sensitivity can vary from 79 to 97% (15, 17, 23). In addition, commensal spirochetes can confound the interpretation of smears of oral or anal lesions because they cannot be distinguished morphologically from *T. pallidum* (6, 15, 16, 17). The application of topical agents to treat lesions can also interfere with the interpretation, leading to false-negative results. More recently, detection of *T. pallidum* has been accomplished by direct fluorescent antibody (DFA) staining performed with fluorescein-conjugated polyclonal or monoclonal anti-*T. pallidum* antibodies (6, 7, 17). This test is a suitable alternative to dark-field examination and has a comparable sensitivity to it (6), and since the observation of motility is not a criterion for identification, the DFA can be performed on fixed specimens (6, 7). Rabbit infectivity testing is perhaps the closest one can get to a culture system. However, the technique is impractical in a routine clinical setting and can detect only viable virulent organisms (18, 22).

Molecular biology techniques are being used increasingly in the clinical laboratory. These methodologies, particularly the PCR, have been used in the detection of treponemes. This technique has detected *T. pallidum* in a variety of clinical specimens, including serum (1, 3, 18), cerebrospinal fluid (1, 3, 5, 12, 18), amniotic fluid (1, 3, 18), fixed tissues (1), and lesion exudate (8, 13, 22). However, its performance relative to other tests, particularly in early disease, remains to be validated by extensive clinical studies. In this study, we describe a method that uses PCR to detect *T. pallidum* in smears of human genital lesions after DFA staining.

MATERIALS AND METHODS

Clinical specimens. Glass slides containing touch preparations of genital lesion exudate were obtained from 156 patients with genital ulcer disease from Malawi. Lesions were present for a mean of 14 days. To obtain samples, the lesions were abraded, and a glass slide was pressed against the lesion exudate. The slides were air dried, fixed in acetone for 10 min, and stored at −20°C. Slides were shipped on ice to the United States. Positive control slides were prepared by fixing aliquots of *T. pallidum* (kindly provided by Lola Stamm, School of Public Health, University of North Carolina) onto glass slides with acetone for 10 min. The slides were then rinsed in sterile distilled water, air dried, and stored at −20°C until used.

DFA staining. DFA staining was performed with a fluorescein-conjugated monoclonal antibody to the 37-kDa antigen of *T. pallidum* (Centers for Disease Control, Atlanta, Ga.). Aliquots of the conjugate were diluted 1:100 in sterile phosphate-buffered saline (PBS) containing 2% polyoxyethylene 80 (Tween 80; Sigma Chemical Co., St. Louis, Mo.) and a 1:20,000 dilution of Evan's blue. Fifty microliters of the diluted antibody was spread on patient and positive control slides which were then incubated at 37°C for 45 min in moist chambers. After

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incubation, the slides were rinsed with sterile PBS and then with sterile distilled water. The slides were air dried, a drop of mounting medium (9 parts glycerol plus 1 part PBS [pH 7.2]) was added, and a coverslip was applied. The slides were examined with a model BH-2 fluorescent microscope (Olympus Corporation, Lake Success, N.Y.). Each slide was scanned under 40× dry and 100× oil objectives for 5 min. The presence of at least one intact spirochete showing green fluorescence was the criterion for a positive DFA result.

Sample preparation for PCR. To prevent contamination, all samples were prepared under a laminar-flow hood. After microscopic examination of DFA slides, the coverslips were removed with acetone and the slides were rinsed with sterile distilled water. After air drying, 20 μl of 0.05% (wt/vol) sodium dodecyl sulfate (SDS; Sigma) was placed on the smear, and a sterile dacron-tipped swab (Harwood Products, Guilford, Maine) was used to remove the smear from the slide. The tip of the swab was cut off and placed in a sterile 1.5-ml microcentrifuge vial containing 180 μl of sterile distilled water. The vials were vortexed vigorously, after which the tips were inverted and the vials were centrifuged at 16,000 × *g* to express absorbed liquid.

DNA was obtained as described by Radolf (14). Briefly, 100 μl of the processed specimen was placed in a sterile 1.5-ml microcentrifuge vial, and 77 μl of 5 M NaCl, 15.5 μl of 2 M NaOH, and 7.5 μl of 20% (wt/vol) SDS were added. After mixing, the vials were placed in a boiling water bath for 1 min and then cooled on ice for 5 min. Samples were neutralized by adding 0.8 ml of 0.5 M Tris-HCl (pH 7.0; Sigma) to each vial. Saturated phenol (Amresco, Solon, Ohio) was added, and the vials were shaken for 30 s and then centrifuged at 16,000 × *g* for 3 min. The aqueous phase was transferred to a sterile 1.5-ml microcentrifuge vial containing 0.5 ml of 24:1 chloroform-isoamyl alcohol (Fisher Scientific, Pittsburgh, Pa.), extracted for 30 s, and centrifuged at 16,000 × *g* for 3 min. The aqueous phase was transferred to a sterile 1.5-ml microcentrifuge vial, and the DNA was precipitated overnight at −70°C in 1 ml of isopropanol with 2 μg of salmon sperm DNA (Sigma). After the incubation, the vials were centrifuged at 16,000 × *g* for 30 min at 4°C. The liquid was decanted, and the pellet was air dried for 1 h. The dried pellet was dissolved in 50 μl of sterile distilled water.

PCR. The oligonucleotide primers used amplify a 658-bp fragment of the gene encoding the 47-kDa lipoprotein of *T. pallidum*. The sequence of the primers, concentrations of the PCR mixture, and the cycling conditions used have been described previously by Burstain et al. (1). The reaction mixture contained the following: 10 μl of extracted DNA, 10 μl of 10× PCR buffer (0.5 M KCl, 0.1 M Tris-HCl, nuclease-free bovine serum albumin [50 mg/ml] [pH 8.3]), 10 μl each of forward and reverse primers (70 pmol/μl), 0.8 μl of 25 mM deoxynucleotide triphosphate mix, 3.5 mM $MgCl_2$, 2.5 U of *Taq* polymerase (The Perkin-Elmer Corp., Norwalk, Conn.), and 55.2 μl of sterile distilled water. Amplification was performed in a Gene Amp 9600 Thermalcycler (Perkin-Elmer) for 40 cycles of denaturation at 94°C for 75 s, annealing at 60°C for 75 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min after the 40th cycle. Aliquots of some extracted samples were spiked with purified *T. pallidum* DNA to determine whether inhibition of amplification occurred. The amplified samples were stored at 4°C. To prevent contamination, the amplification process and the subsequent detection methods were performed in a PCR-dedicated amplification laboratory which is physically separated from the sample preparation area.

Gel electrophoresis. After amplification, 25 μl of the PCR product was diluted 1:6 in sample buffer (2.0 g of Ficoll 400, 0.1 g of SDS, 0.025 g of bromphenol blue, 0.37 g of disodium EDTA, 10.0 ml of distilled H_2O) and electrophoresed in a 1% agarose gel at 100 mV for 2 h in TBE buffer (90 mM Tris-HCl, 90 mM boric acid, 2 mM EDTA [pH 8.5]). Molecular weight markers (*Hae*III digest of lambda ϕX174; Promega, Madison, Wis.) were run on each gel. The gels were stained with ethidium bromide (0.25 μg/ml) for 30 min and photographed.

Preparation of *T. pallidum* probe for dot blot hybridization. PCR amplification products were also analyzed by dot blotting onto nylon membranes and hybridization with a PCR-generated, digoxigenin (DIG)-dUTP (Boehringer Mannheim, Indianapolis, Ind.) probe internal to the 658-bp PCR product. The probe was generated in a PCR mix identical to the one described above except that appropriate primers (1) and 8.6 μl of 25 nM DIG-dUTP was added to the reaction mixture. The cycling conditions were also identical to the ones used to amplify the 658-bp fragment. Amplification was confirmed by electrophoresis on a 1% agarose gel and visualization of the predicted 496-bp product. Amplification products were pooled, and the labeled probe was precipitated with 0.1 volume of 3 M sodium acetate and 3 volumes of chilled ethanol at −70°C for 30 min, after which the vials were centrifuged at 16,000 × *g* for 15 min. The supernatant was decanted, and the pellet was washed with 100 μl of 70% chilled ethanol and then centrifuged at 16,000 × *g* for 15 min. The pellet was dried and dissolved in 50 μl of sterile distilled water.

Dot blotting. Aliquots of the patient and control amplification products were denatured by boiling for 1 min. After chilling on ice for 5 min, 1 volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was added. The samples were mixed, and 10 μl of each was spotted onto Hybond positively charged nylon membranes (Amersham, Arlington Heights, Ill.) with a dot blotting manifold (V & P Scientific, San Diego, Calif.). The membranes were denatured in 1.5 M NaCl–0.5 M NaOH for 5 min and then neutralized in 1.5 M NaCl–0.5 M Tris-HCl–1.0 mM EDTA (pH 7.2) for 1 min. The membranes were soaked in 0.4 M NaOH for 20 min and then rinsed in 5× SSC for 1 min and stored in plastic bags at 4°C until hybridization.

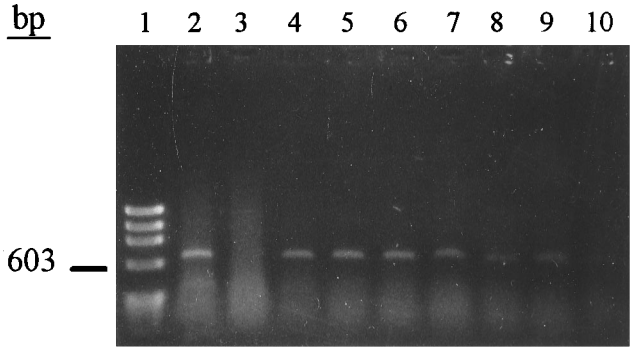

FIG. 1. Agarose gel electrophoresis of PCR amplification products from serially diluted *T. pallidum* removed from glass slides. Lanes: 1, molecular weight markers; 2, positive *T. pallidum* DNA control; 3, negative (no DNA) control; 4 to 10, products from slides containing 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10^1 *T. pallidum* organisms per slide, respectively.

Hybridization. Hybridization and detection were performed by use of the Genius 3 nonradioactive nucleic acid detection kit (Boehringer Mannheim) as described in the manufacturer's instructions. After hybridization, the probe solution was stored at −20°C for later reuse.

Statistical analysis. The kappa (κ) statistic was used to determine agreement between the tests (2).

RESULTS

Sensitivity and specificity of PCR-dot blot. We determined the sensitivity of our PCR assay for the detection of *T. pallidum* from glass slides. Serial 10-fold dilutions (10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 10^0 organisms per slide) of a frozen *T. pallidum* stock were acetone fixed to glass slides. The slides were then processed for PCR in the same fashion as the patient samples described earlier. We found that the lower limit of detection was approximately 10^1 spirochetes (Fig. 1) by gel electrophoresis. When this experiment was repeated, we detected 10^2 organisms. Dot blotting the PCR products of purified *T. pallidum* DNA and detection with the DIG-dUTP-labeled probe resulted in a 10-fold increase in sensitivity over that of visualization of ethidium bromide-stained gels (data not shown).

Specificity was determined by amplification of DNA from several different microorganisms. DNA obtained from *T. pallidum* demonstrated a band on the agarose gel corresponding to the predicted 658-bp amplification product, while DNA from *Borrelia burgdorferi*, *Haemophilus ducreyi*, and *Candida albicans* was not amplified. Specificity of the hybridization reaction was determined by dot blotting amplified DNA from *T. pallidum*, *B. burgdorferi*, *H. ducreyi*, and *C. albicans*. Hybridization with the DIG-dUTP-labeled probe gave a positive signal only with the *T. pallidum* PCR product (Fig. 2).

Concordance of DFA and PCR-dot blot. DFA staining was performed on 156 smears of genital lesion exudate. Twenty-five of the 156 smears were positive for *T. pallidum* by DFA

FIG. 2. Dot blot hybridization of PCR amplification products of DNA isolated from *T. pallidum* (lane 1), *H. ducreyi* (lane 2), *B. burgdorferi* (lane 3), and *C. albicans* with a DIG-dUTP-labeled *T. pallidum* probe (lane 4). Lane 5 contained no DNA in the amplification reaction.

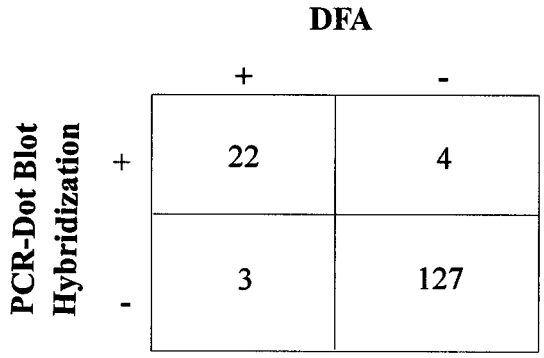

FIG. 3. Results of *T. pallidum* DFA stain versus PCR-dot blot on 156 genital lesion exudate smears.

staining. Twenty-six of the smears were PCR-dot blot hybridization positive. Overall, 22 of the 156 specimens were positive for *T. pallidum* by DFA and PCR-dot blotting, while 127 were negative by both methods, yielding a concordance of 95.5% ($\kappa = 0.84$) (Fig. 3). Without the hybridization step, the concordance of PCR and DFA was 93.6% ($\kappa = 0.83$).

To determine if there had been inhibition of the PCR in the PCR-negative, DFA-positive specimens, aliquots of these samples were spiked with purified *T. pallidum* DNA and then amplified. All four spiked samples demonstrated an amplification product by gel electrophoresis (Fig. 4).

DISCUSSION

Because *T. pallidum* cannot be cultured, serologic and microscopic tests have been the mainstay of the laboratory diagnosis of syphilis (10, 21, 23). While these tests are important in the diagnosis of syphilis, they have drawbacks. In particular, for the diagnosis of early (primary) syphilis, serologic tests suffer from a lack of sensitivity. The commonly used screening tests such as the Venereal Disease Research Laboratory test and the rapid plasma reagin test are approximately 75% sensitive during primary disease (10). Microscopic evaluation (dark field and DFA) of primary lesions has aided in the diagnosis of early syphilis; however, the sensitivities of these techniques are variable (6, 17), and an experienced microscopist is required. Dark-field preparations must be read immediately and cannot be stored or shipped for analysis. The specificities of these methods are good on external genital lesions, but oral or anal lesions may contain indigenous spirochetes which are indistinguishable from *T. pallidum* by dark-field microscopy (6, 15–17, 23) and may cross-react with supposed pathogen-specific monoclonal antibodies (15, 23). For these reasons, alternative methodologies for the diagnosis of syphilis, especially primary syphilis, are desirable.

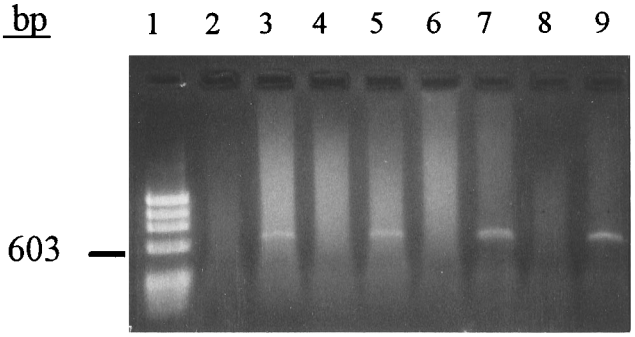

FIG. 4. Agarose gel electrophoresis of PCR products from DFA-positive, PCR-negative samples with or without added *T. pallidum* DNA. Lanes: 1, DNA markers; 2, unspiked sample 14; 3, spiked sample 14; 4, unspiked sample 128; 5, spiked sample 128; 6, unspiked sample 131; 7, spiked sample 131; 8, unspiked sample 132; 9, spiked sample 132.

The PCR has proven to be sensitive and specific in the laboratory diagnosis of a variety of infectious diseases, including syphilis (1, 3, 5, 12, 18, 22, 23). PCR amplification of *T. pallidum* DNA from serum, amniotic fluid, and cerebrospinal fluid has aided in the diagnosis of congenital syphilis (3, 18) and neurosyphilis (5, 11, 12, 18, 23). The application of PCR in the diagnosis of syphilitic skin lesions has not been reported on as extensively. Investigators reported recently, however, success in amplification of *T. pallidum* DNA from skin lesions of experimentally infected rabbits (22) and humans with genital ulcer disease (8, 13). In the present study, we have shown that the DFA test using monoclonal anti-*T. pallidum* antibody and the PCR using *T. pallidum*-specific primers provide highly concordant means of detecting *T. pallidum* in touch preparations of genital lesions.

The specificity of the PCR-dot blot hybridization assay using a DIG-labeled probe was comparable to that demonstrated by other investigators (1, 3). We were not able to amplify or hybridize the probe to DNA from *B. burgdorferi*, another spirochete, or *H. ducreyi*, another cause of genital ulcer disease. We have not attempted to amplify DNA from herpes simplex virus, a third cause of genital ulcer disease (9). Further specificity studies using DNA from commensal treponemes need to be performed to demonstrate the specificity of our assay in samples representative of oral specimens. Previously, however, Burstain et al. (1) were not able to amplify DNA from *Treponema denticola* or *Treponema phagedenis* with the primers used in this study.

Using purified treponemal DNA, several investigators have demonstrated success in amplification of an amount equivalent to one spirochete (1, 3, 5). In the present study, however, we were not able to achieve this level of sensitivity. Our level of sensitivity was approximately 10 to 100 spirochetes fixed to a glass slide. The explanation for the decreased sensitivity relative to that of other studies most likely stems from the additional manipulations to which the samples were subjected. Because this study involved amplification of samples that had been previously DFA stained, washed, and then removed from slides with swabs, spirochetes were likely lost during the process. Because of this, direct comparisons of the sensitivity of the current method with that of other studies is not appropriate.

We included a hybridization step in the detection system, and, as demonstrated by others (1, 3), this resulted in increased sensitivity of our system. We found a 10-fold increase in sensitivity when a DIG-labeled probe to detect purified treponemal DNA was used (data not shown). The use of this dot blot hybridization technique resulted in the detection of *T. pallidum* DNA in three specimens which were not positive by gel electrophoresis alone. Two of these were DFA positive but negative by gel electrophoresis of PCR product. One sample was both DFA and PCR gel electrophoresis negative.

DFA and PCR-dot blot demonstrated good concordance on the 156 slides we examined. Seven samples, however, gave discordant results. Four specimens were PCR-dot blot positive and DFA negative. These results may be due to the presence of large numbers of leukocytes obscuring the visualization of low numbers of treponemes. Three specimens were PCR-dot blot negative and DFA positive. These results did not appear to be due to inhibition of PCR amplification since purified *T.*

pallidum DNA was amplified when added to aliquots of these four specimens. This does not rule out the possibility of partial inhibition in that the amount of spiked DNA added may have been too great to demonstrate inhibition, whereas a lesser amount may still demonstrate inhibition. Negative results in these specimens were most likely due to inefficient recovery of their DNA. Direct amplification of material removed from slides, without DNA purification, might improve sensitivity; however, we had inconsistent results with this approach in previous experiments (data not shown).

Both DFA and PCR have advantages and disadvantages which must be considered. There is an initial investment in expensive capital equipment required for each methodology (fluorescent microscope or thermalcycler). The reagent costs are greater for PCR. However, the availability of the monoclonal antibody could be a limiting factor. Currently, the only source of this reagent is the Centers for Disease Control, which limits the amount provided (although the amount should be sufficient for routine numbers of assays). Both methods allow collection of specimens at remote sites with shipment to a central laboratory for analysis. The ability to use a swab specimen for PCR is a desirable benefit in terms of quality assurance since the shipment of glass slides can lead to breakage. In this study, we received several broken slides which were shipped in plastic slide boxes. The ultimate choice of methodology demands evaluation of these concerns and the sensitivity and specificity of the procedures. Our data, as currently performed, suggest that DFA and PCR are equivalent in sensitivity. The sensitivity coupled with the cost advantage makes DFA appear more desirable. However, further development and refinement of the PCR assay, possibly with multiplex amplification of other genital ulcer disease pathogens such as *H. ducreyi* and herpes simplex virus may ultimately make the molecular approach more practical.

In conclusion, our initial study of PCR and DFA for detection of *T. pallidum* in genital lesions demonstrated good concordance between the two techniques. While we and others have shown that the primers used in this study are sensitive and specific for detection of purified *T. pallidum* DNA, the sensitivity of PCR for detection of *T. pallidum* in genital lesions was not significantly better than that of DFA. In ongoing studies, we are obtaining swabs from genital lesions for PCR amplification which will not be DFA stained prior to extraction of DNA. This may provide a better sample and result in increased sensitivity of PCR. In addition to the theoretical advantages of PCR in terms of sensitivity, this technique should not be susceptible to false positives due to commensal treponemes. This study demonstrates the feasibility of a molecular diagnostic method for the diagnosis of primary syphilis; however, further development and evaluation are required to determine if it provides a significant advantage over DFA staining.

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