JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1991, p. 62–69
0095-1137/91/010062-08$02.00/0

Vol. 29, No. 1

Sensitive Detection of *Treponema pallidum* by Using the Polymerase Chain Reaction

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Received 7 August 1990/Accepted 16 October 1990

We have developed a sensitive assay for *Treponema pallidum* subsp. *pallidum* (*T. pallidum*), the agent of venereal syphilis, based upon the polymerase chain reaction (PCR). A 658-bp portion of the gene encoding the 47-kDa membrane immunogen was amplified, and the PCR products were probed by DNA-DNA hybridization with a 496-bp fragment internal to the amplified DNA. The assay detected approximately 0.01 pg of purified *T. pallidum* DNA, and positive results were obtained routinely from suspensions of treponemes calculated to contain 10 or more organisms and from some suspensions calculated to contain a single organism. Specific PCR products were obtained for the closely related agent of yaws, *Treponema pallidum* subsp. *pertenue*, but not with human DNA or DNAs from other spirochetes (including *Borrelia burgdorferi*), skin microorganisms, sexually transmitted disease pathogens, and central nervous system pathogens. *T. pallidum* DNA was detected in serum, cerebrospinal fluids, and amniotic fluids from syphilis patients but not in nonsyphilitic controls. *T. pallidum* DNA was also amplified from paraffin-embedded tissue. The diagnosis of syphilis by using PCR may become a significant addition to the diagnostic armamentarium and a valuable technique for the investigation of syphilis pathogenesis.

Syphilis is a sexually transmitted disease caused by the spirochete *Treponema pallidum* subsp. *pallidum* (*T. pallidum*). Although effective therapy has been available for more than four decades, the disease continues to be a global public health problem (33). Two recent developments underscore the need for more effective means of diagnosing this infection: (i) substantial annual increases in new cases have been reported in the United States since 1986 (5) and (ii) a large proportion of patients with human immunodeficiency virus infection are coinfected by *T. pallidum* (15), and they may be predisposed to more severe syphilitic manifestations, such as neurosyphilis or retinitis (3, 20, 35).

The diagnosis of syphilis is complicated because *T. pallidum* is one of the few major bacterial pathogens of humans that cannot be cultivated on artificial medium. Current methods for detection of *T. pallidum* in clinical specimens are either insensitive, as in the case of dark-field microscopy (46), or impractical, as with rabbit intratesticular inoculation (rabbit infectivity testing or RIT) (21, 47). Serologic tests for syphilis are the mainstays of laboratory diagnosis; however, these lack sensitivity, specificity, or both for certain forms of the disease, particularly primary syphilis, congenital syphilis, and neurosyphilis (12, 21, 43, 46).

The polymerase chain reaction (PCR) can selectively amplify the copy number of a target gene more than 10^6-fold (39). PCR, therefore, has great potential for improving the ability to diagnose infectious diseases caused by fastidious or slowly growing microorganisms (8), and it has already been employed for the detection of many such bacterial pathogens, including *Mycoplasma pneumoniae* (2), *Mycobacterium tuberculosis* (10), *Legionella pneumophila* (25, 44), and *Borrelia burgdorferi* (31, 37). In this study, we report the development of an exquisitely sensitive assay for *T. pallidum* that is based upon amplification of the gene encoding the pathogen-specific and highly conserved 47-kDa membrane immunogen (*tpp47*) (9, 16, 26, 27, 32, 36, 45). Detection of *T. pallidum* by amplification of a portion of the tmpA gene was recently reported by Hay et al. (13).

(This work was presented in part at the 90th Annual Meeting of the American Society for Microbiology, Anaheim, Calif., 13 to 17 May 1990.)

MATERIALS AND METHODS

Bacterial strains and culture media. *T. pallidum* subsp. *pallidum* Nichols (*T. pallidum*) and the Haiti B strain of *T. pallidum* subsp. *pertenue* were passaged by intratesticular inoculation of New Zealand White rabbits and extracted in phosphate-buffered saline, pH 7.4, approximately 10 and 14 days later, respectively (34). For isolation of chromosomal DNA, treponemes were purified by Percoll density gradient centrifugation (11). The Dal-1 strain of *T. pallidum* was isolated from the amniotic fluid of a pregnant woman with untreated syphilis. Eight additional clinical isolates of *T. pallidum*, three from genital ulcers and five from cerebrospinal fluids (CSFs), were isolated in Seattle, Wash. Thioglycolate stock cultures of the nonpathogenic *Treponema phagedenis* biotype Reiter and *Treponema denticola* were subcultured in Spirolate broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 10% heat-inactivated rabbit serum and incubated at 34°C for 3 days. *B. burgdorferi* was grown in BSKII medium at 35°C. The concentrations of spirochetes were determined at ×400 by dark-field microscopy (24).

Clinical isolates of *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, viridans group streptococci, *Candida albicans*, *Neisseria meningitidis*, and *Corynebacterium* sp. were the generous gifts of Sue Tyrone (Department of Microbiology,

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University of Texas Southwestern Medical Center at Dallas [UTSWMCD]). A clinical isolate of *Neisseria gonorrhoeae* was provided by Alice Erwin (Department of Microbiology, UTSWMCD). *Cryptococcus neoformans* and *Streptococcus agalactiae* (group B beta-hemolytic streptococcus) were the respective gifts of Mary Lipscomb and Rita Gander (Department of Pathology, UTSWMCD). *Listeria monocytogenes* was provided by Christopher Lu (Department of Internal Medicine, UTSWMCD). Cells from the human T-cell line H9 containing the BAG strain of human immunodeficiency virus type 1 were a gift of Mark Till (Department of Microbiology, UTSWMCD). *Haemophilus ducreyi* was obtained from the Dallas County Public Health Department. A mixture of herpes simplex viruses types I and II was provided by James P. Luby (Department of Internal Medicine, UTSWMCD).

Plasmids and DNAs. Plasmid pPH47.2, a pUC19 derivative containing *tpp47* on a 2.4-kbp DNA insert (17), was provided by Pei-Ling Hsu (University of Texas Health Sciences Center at Houston) and purified by CsCl density gradient centrifugation (41).

Chromosomal DNAs from *T. pallidum*, *T. phagedenis* biotype Reiter, *T. denticola*, *B. burgdorferi*, *N. meningitidis*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Cryptococcus neoformans*, human T-cell line H9 containing human immunodeficiency virus type 1, *Streptococcus pyogenes*, viridans group streptococci, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Corynebacterium* species, *Candida albicans*, *H. ducreyi*, *N. gonorrhoeae*, and herpes simplex virus types 1 and 2 were prepared and purified by using modifications of established procedures (41). Final concentrations of some of the DNAs were estimated by measurement of A_{260}. Chromosomal DNA of *Chlamydia trachomatis* serovar L2 was a gift from Richard S. Stephens (University of California, San Francisco). *Haemophilus influenzae* type b and pUC19 DNA were provided by Eric J. Hansen (Department of Microbiology, UTSWMCD). *Leptospira interrogans* serovar pomona DNA was generously provided by Niles Charon (University of West Virginia, Morgantown). *Borrelia hermsii* DNA was donated by Alan G. Barbour (University of Texas Health Sciences Center at San Antonio). *Escherichia coli* DNA was purchased from Sigma Chemical Co. (St. Louis, Mo.).

Clinical specimens. Amniotic fluids from untreated syphilis patients and from a normal patient, obtained following informed consent for evaluation of fetal maturity, were generously provided by George D. Wendel (Department of Obstetrics and Gynecology, UTSWMCD). Normal sera and CSFs and sera and CSFs from adults with untreated syphilis were obtained by one of the co-authors (J.D.R.) from the Clinical Immunology Laboratory of Parkland Memorial Hospital; neurosyphilis was diagnosed if a patient had a compatible clinical syndrome, CSF pleocytosis (>5 leukocytes per mm^3), and a reactive CSF-Venereal Disease Research Laboratory (VDRL) test. Infectivity testing of amniotic fluids was performed by the method of Turner et al. (47). Use of the clinical specimens for these investigations was approved by the UTSWMC Institutional Review Board.

Preparation of samples for PCR. Two different methods were used to prepare samples for PCR. In the first method, whole *T. pallidum* was serially diluted in sterile isotonic saline; 10- to 20-μl portions containing the desired numbers of organisms were placed in 1.5-ml microcentrifuge tubes, boiled for 10 min, and then amplified as described below. Alternatively, 50-μl samples of saline containing serially diluted treponemes or 100 μl of a clinical specimen were subjected to alkaline lysis and DNA extraction by using a modification of the technique described by Kogan et al. (22). First, portions from separate 5 M NaCl, 5 N NaOH, and 10% sodium dodecyl sulfate (SDS) stock solutions were added to the sample to give final concentrations of 1 M NaCl, 1 M NaOH, and 2% SDS. The mixture then was boiled for 1 min and neutralized with 4 volumes of 0.5 M Tris hydrochloride (pH 7.0). DNA was extracted by using phenol–chloroform (50:50, vol/vol) and precipitated at −70°C with 2 μg of salmon sperm DNA in 2 volumes of 100% ethanol or in 0.6 to 1 volume of 100% isopropanol. The DNA pellets were then dissolved in 50 μl of water for PCR.

TABLE 1. Sequences of oligonucleotide primers

Primer	Sequence	Coding strand	Location (nt)[a]
47-1	GACAATGCTCACTGAGGATAGT	+	648–669
47-2	ACGCACAGAACCGAATTCCTTG	−	1284–1305
47-3	TTGTGGTAGACACGGTGGGTAC	+	713–734
47-4	TGATCGCTGACAAGCTTAGGCT	−	1187–1208

[a] nt, Nucleotides.

PCR of paraffin-embedded tissues. Testes from normal and syphilitic rabbits were cut into 2- to 3-mm pieces with a no. 11 scalpel and fixed in 10% phosphate-buffered Formalin. Specimens were embedded in paraffin and either stained by Warthin-Starry silver in the University of Texas Southwestern Histopathology Laboratory or placed into microcentrifuge tubes for DNA extraction by the method of Shibata et al. (42).

PCR. Oligonucleotide primers 47-1 and 47-2, derived from nucleotides 648 to 669 and 1284 to 1305 of the respective sense and antisense strands of *tpp47* (17) (Table 1), were synthesized by standard phosphoramidite chemistry (1) in the core facility of the Howard Hughes Medical Institute, UTSWMCD. Concentrations of *Taq* DNA polymerase (Perkin Elmer/Cetus, Norwalk, Ct.), oligonucleotide primers, deoxyribonucleotides (Pharmacia, Piscataway, N.J.), and Mg^{2+}, as well as PCR cycle lengths, numbers, and temperatures, were optimized as previously described (19). All PCRs were performed in final volumes of 100 μl. A typical reaction contained 50 mM KCl, 10 mM Tris hydrochloride, 3 mM $MgCl_2$ (pH 8.3), 100 μg of gelatin per ml, 70 pmol of primers, 300 μM deoxyribonucleotides, 2.5 U of *Taq* polymerase, and various amounts of purified DNA or boiled whole treponemes. Reactions were performed for 40 cycles in a Thermocycler (Perkin Elmer/Cetus) by using the following parameters: (i) denaturation for 1 min, 15 s at 94°C, (ii) annealing for 1 min, 15 s at 60°C, and (iii) extension for 1 min at 72°C. After the 40th cycle, specimens were incubated at 72°C for an additional 10 min and then stored at 4°C until analyzed. To prevent false-positives due to contamination, all specimens were prepared under a laminar flow hood in a PCR-dedicated facility remote from the principal laboratory.

Agarose gel electrophoresis and DNA-DNA hybridization analyses. One-tenth of each reaction volume (10 μl) was analyzed by electrophoresis through a 1% agarose gel containing TBE buffer (0.089 M Tris hydrochloride, 0.089 M Borate, and 0.002 M EDTA) and 0.25 μg of ethidium bromide per ml. Gels were photographed under UV illumination and in some cases transferred to 0.2-μm-pore-size nitrocellulose (Schleicher & Schuell, Keene, N.H.) for Southern blot analysis. Other specimens were applied directly to nitrocellulose by using a Minifold I apparatus (Schleicher & Schuell) for dot blot DNA-DNA hybridization.

The DNA probe for hybridizations consisted of an internal 496-bp fragment encompassing nucleotides 713 to 1208 of *tpp47* (17). Although originally isolated as a *Kpn*I-*Hin*dIII fragment from the insert of pPH47.2 (17), in later experiments the probe was synthesized by PCR by using plasmid pPH47.2 as the DNA template and oligonucleotides 47-3 and 47-4 as the primers (Table 1). The probe was radiolabeled with [α-^{32}P]dCTP (New England Nuclear, Wilmington, Del.) by using random hexanucleotide labeling (Boehringer Mannheim, Indianapolis, Ind.). Southern and dot blot hybridizations were performed overnight at 65°C in 1 M NaCl–10% dextran sulfate (Pharmacia)–1% SDS–100 μg of sheared salmon sperm DNA per ml and with 10^6 cpm of the labeled probe per ml. After hybridization, blots were washed three times at 22°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% SDS followed by two similar washes at 65°C. Autoradiography was performed at 22°C on Fuji X-ray film for 1 to 24 h, depending on the specific activity of the probe.

Determination of the sensitivity of the assay on the basis of statistical analysis of treponemal suspensions. *T. pallidum* was freshly isolated from a single infected rabbit testis by extraction into medium composed of 50% sterile isotonic saline and 50% heat-inactivated normal rabbit serum (vol/vol). The treponemal suspension was then diluted in the same medium to obtain a concentration of organisms (approximately 10 per high-power dry field) which permitted accurate enumeration under dark-field illumination by using a microscope that had previously been precisely calibrated with a hemacytometer. The average concentration of treponemes was carefully determined from this suspension by three investigators, each counting a minimum of 20 fields. Sequential 10-fold serial dilutions were then prepared such that 50-μl portions were calculated to contain 1,000, 100, 10, and 1 organisms. DNA was extracted by alkaline lysis (as described above) from duplicate suspensions calculated to contain 10 or more organisms and from six suspensions calculated to contain a single organism. The results obtained following amplification of the extracted DNAs were compared with the numbers of treponemes in each suspension predicted (0.97 confidence level) by the binomial probability law for random particles in free solution (48).

RESULTS

Development of the PCR assay. The positions of primers 47-1 and 47-2 (Table 1) within the nucleotide sequence of *tpp47* (17) predicted that a 658-bp fragment would be generated following PCR of either *T. pallidum* chromosomal DNA or chimeric plasmids containing the cloned gene. A fragment of the appropriate size was visualized following PCR of *T. pallidum* DNA and pPH47.2 but not following amplification of pUC19 or reaction mixtures without DNA (Fig. 1A). Southern analysis confirmed that the products obtained from *T. pallidum* and pPH47.2 were specific for *tpp47* and that specific PCR products were absent from the negative controls (Fig. 1B).

Sensitivity of the PCR assay. The sensitivity of the assay was initially determined by using serially diluted, purified *T. pallidum* chromosomal DNA. On the basis of an average *T. pallidum* chromosome size of 9×10^9 Da (28), the DNA equivalent of about 10^7 treponemes (0.10 μg) was detected by dot blot hybridization of unamplified DNA (Fig. 2A); with prolonged exposure times, approximately 10 ng of unamplified DNA was detected (data not shown). Approximately 0.01 pg, or the DNA equivalent of a single treponeme, was

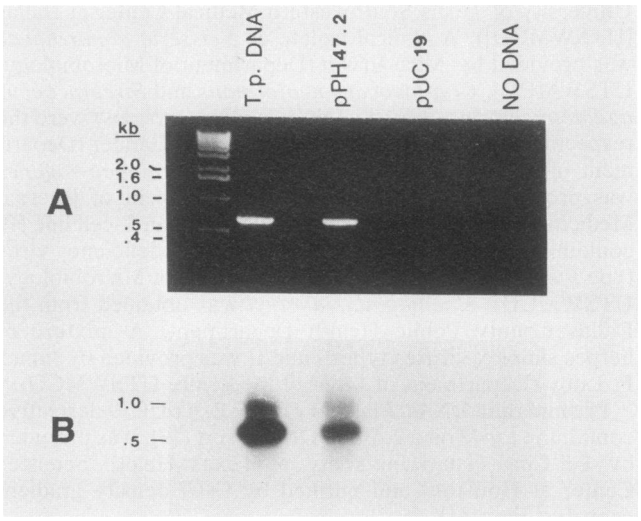

FIG. 1. Amplification of the 47-kDa immunogen gene of *T. pallidum* (*tpp47*). DNA templates consisting of *T. pallidum* chromosomal DNA (T.p. DNA), *tpp47* cloned into pUC19 (pPH47.2), pUC19, and no DNA are shown above the lanes of a 1% agarose gel stained with ethidium bromide (A) and the corresponding Southern blot probed with the 496-bp internal fragment (B). Kilobase pair standards are shown on the left of each panel.

detected following amplification (Fig. 2A). Positive PCR results were also obtained from a suspension of boiled, whole *T. pallidum* calculated to contain a single organism (Fig. 2B).

Interestingly, PCR failures consistently occurred in samples containing 10^4 or greater numbers of boiled whole treponemes (Fig. 2B). Because PCR can be inhibited by serum and tissue components (14), it was surmised that the testicular extracts present in samples containing the higher concentrations of treponemes were responsible for the failures. This was confirmed by the findings that 10^4 and greater numbers of treponemes purified on Percoll density gradients amplified appropriately and that the PCR was inhibited by the addition of either normal rabbit testicular extract or

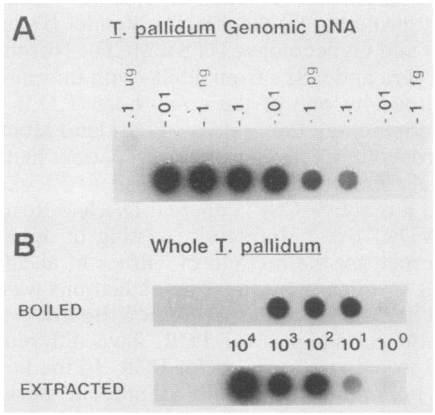

FIG. 2. Sensitivity of the PCR assay for purified DNA and whole organisms. (A) Dot blot hybridization of *T. pallidum* Nichols DNA before (row 1) and after (row 2) amplification. The amounts of DNA used in each reaction are shown above the lanes. (B) PCR of serially diluted *T. pallidum* Nichols after boiling or after alkaline lysis and DNA extraction.

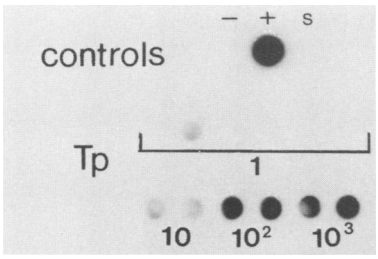

FIG. 3. Determination of assay sensitivity by statistical analysis. PCR results are shown for the DNAs extracted from six *T. pallidum* (Tp) suspensions calculated to each contain a single organism and from duplicate suspensions calculated to contain 10, 100, or 1,000 organisms. Controls consisted of PCR mixtures without (−) and with (+) pPH47.2 plasmid DNA and a sham-extracted aliquot (S) of the medium used to harvest and dilute the treponemes.

greater than 10^4 human leukocytes to reaction mixtures containing purified *T. pallidum* DNA (data not shown). Extraction of DNA following alkaline lysis eliminated the PCR failures with a sensitivity comparable to that obtained with boiled treponemes (Fig. 2B).

To confirm as rigorously as possible that the assay's sensitivity approached the detection of DNA from a single spirochete, DNA for PCR was extracted from serially diluted treponemal suspensions and the PCR results were compared with the numbers of organisms predicted to be in the suspensions by the binomial probability law (48). Positive PCR results were obtained for all of the suspensions calculated to contain 10 or more treponemes and for one of the six suspensions calculated to contain a single organism (Fig. 3). Probability analysis predicted (0.97 confidence level) that two of the latter six suspensions contained at least one organism.

Assay specificity. PCR was performed by using DNAs purified from a number of pathogenic and nonpathogenic spirochetes. Specific products were obtained only following amplification of DNAs from *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* (Fig. 4A). Equally important, each of eight *T. pallidum* clinical isolates amplified successfully from a small number of organisms (Fig. 4B).

Specimens from patients with genital ulcers (chancres) will inevitably contain skin flora and/or other sexually transmitted disease pathogens. For this reason, the specificity of the assay was investigated further by using a panel of potential skin and sexually transmitted disease contaminants. Specific PCR products were not obtained from skin flora (Fig. 5A) or from other sexually transmitted disease pathogens (Fig. 5B). In the case of *N. gonorrhoeae*, heterogeneous PCR products were observed on the agarose gel, but these did not hybridize with the *tpp47*-specific probe (Fig. 5B). Such nonspecific priming occurred only when microgram quantities of *N. gonorrhoeae* DNA were subjected to PCR (data not shown). No PCR products were obtained from a variety of central nervous system pathogens including *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Streptococcus agalactiae*, *H. influenzae* type b, *N. meningitidis*, *E. coli*, *Cryptococcus neoformans*, and human immunodeficiency virus (data not shown). Finally, amplification of purified *T. pallidum* DNA or whole *T. pallidum* was unaffected by amounts of human DNA ranging from 3.3 pg to 3.3 μg, the DNA equivalents of 10^0 to 10^6 human cells, respectively (data not shown).

Detection of *T. pallidum* in clinical specimens. Positive PCR results were obtained from serum, CSF, and amniotic fluids of untreated syphilis patients but not from control specimens. Representative results are shown in Fig. 6. Both the serum and the CSF from the patient with early latent syphilis were PCR positive (Fig. 6, dots 1 and 3); interestingly, the CSF of this patient had normal clinical laboratory parameters. The other PCR-positive CSF (Fig. 6, dot 5) was from a patient with syphilitic meningitis, while the two PCR-negative CSFs (Fig. 6, dots 6 and 7) were from patients with general paresis. Two amniotic fluids from untreated pregnant women were PCR positive (Fig. 6, dots 9 and 10). The more weakly reactive of the two (Fig. 6, dot 10) was dark-field negative according to two different observers. Treponemes were isolated from both amniotic fluids by RIT.

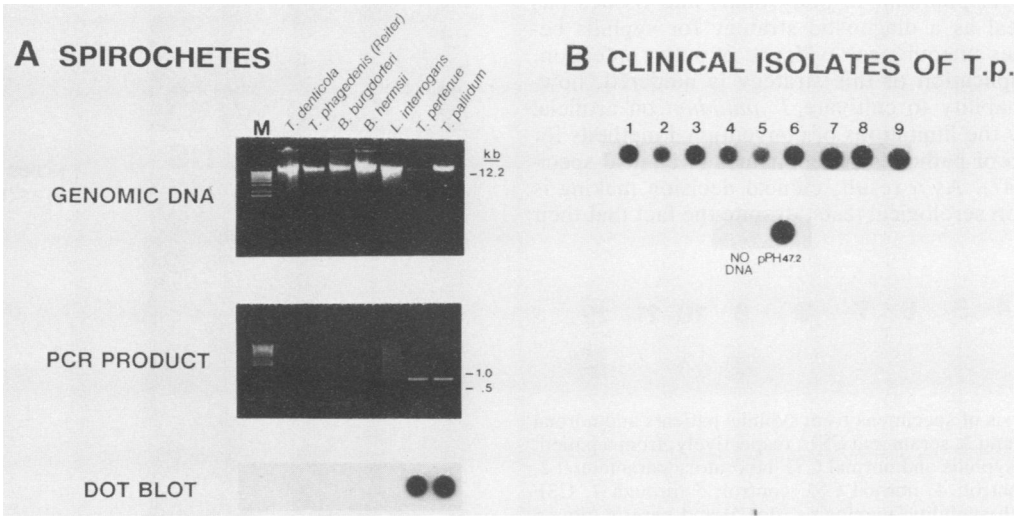

FIG. 4. Analysis of spirochete DNAs. (A) Agarose gel electrophoresis and dot blot analyses following amplification of genomic DNAs purified from species of *Treponema*, *Borrelia*, and *Leptospira*. Kilobase pair standards corresponding to marker lanes (M) are shown at the right. (B) Dot blot hybridization following PCR of approximately 10 *T. pallidum* Nichols organisms (dot 9) and clinical isolates from Seattle, Wash. (dots 1 to 5, 7, and 8), and from Dallas, Tex. (dot 6).

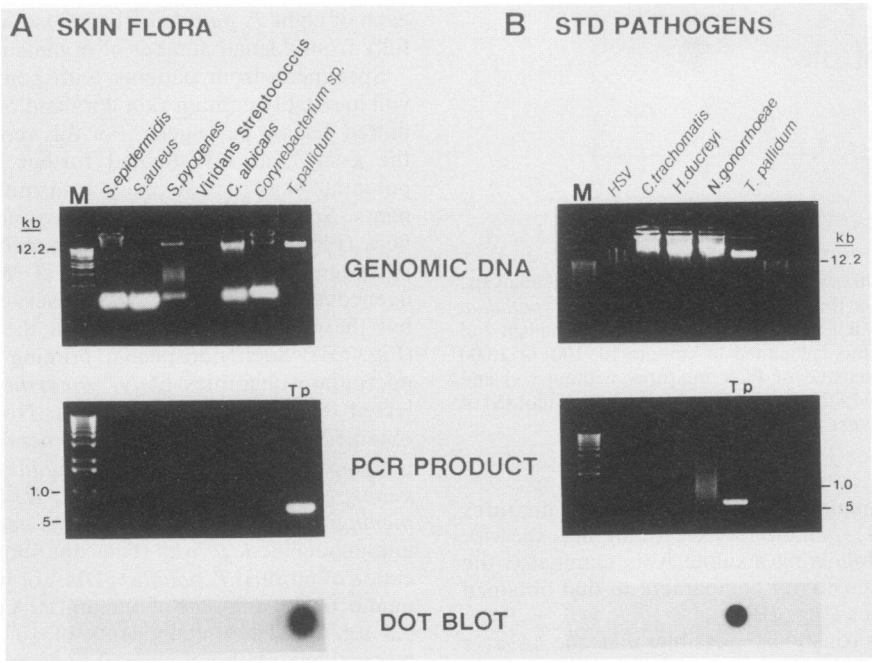

FIG. 5. Analysis of DNAs from skin commensals (A) and sexually transmitted disease (STD) pathogens (B). Agarose gels of unamplified DNAs are shown in upper panels, while agarose gels and dot blot hybridizations of the resulting PCR products are presented in the middle and lower panels, respectively. Kilobase pair standards corresponding to marker lanes (M) are shown next to each panel.

PCR of DNA from paraffin-embedded rabbit testes. Shibata and co-workers (42) have demonstrated that DNA can be amplified from chemically fixed, paraffin-embedded tissues. PCR analysis of archival specimens could therefore help to resolve a number of questions concerning syphilis in the preantibiotic and perhaps even the prehistoric eras. To demonstrate the feasibility of such investigations, *T. pallidum* DNA was amplified successfully from a single 5-μm section of a syphilitic rabbit testis (Fig. 7).

DISCUSSION

Detection of *T. pallidum* in body fluids and tissues has enormous appeal as a diagnostic strategy for syphilis because it provides unequivocal evidence of active infection. Widespread application of this strategy is hindered, however, by the inability to cultivate *T. pallidum* on artificial medium and by the limitations of conventional methods for direct detection of pathogenic treponemes in clinical specimens (21, 46, 47). As a result, clinical decision making is often based upon serological tests, despite the fact that their

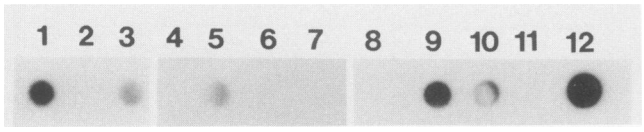

FIG. 6. Analysis of specimens from syphilis patients and normal controls. Dots: 1 and 3, serum and CSF, respectively, from a patient with early latent syphilis and normal CSF laboratory parameters: 2, normal serum control; 4, normal CSF control; 5 through 7, CSF from patients with syphilitic meningitis (dot 5) and paresis (dots 6 and 7); 8, normal amniotic fluid; 9 and 10, dark-field-positive and dark-field-negative amniotic fluids, respectively, from pregnant women with untreated syphilis; 11, sham preparation (PCR mixture without DNA); 12, positive control (10 ng of pPH47.2).

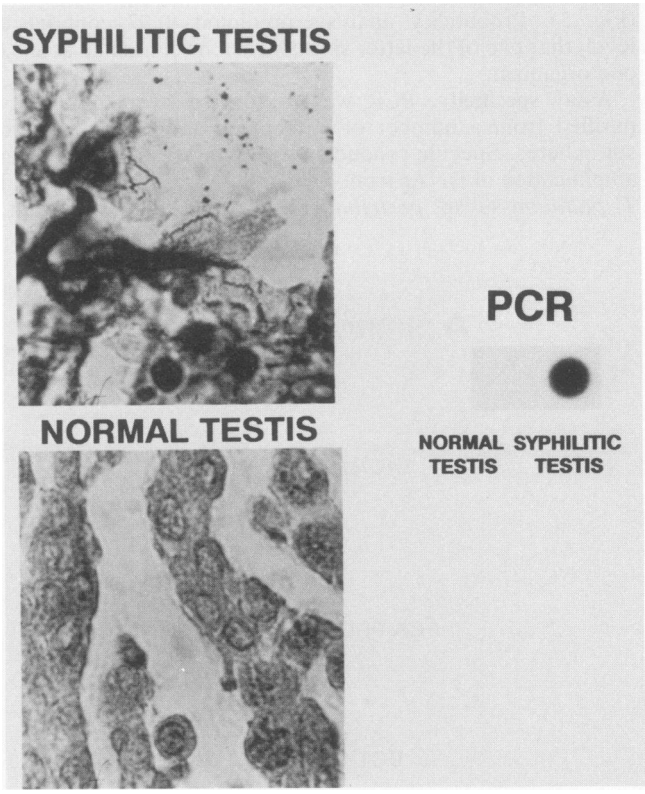

FIG. 7. Detection of *T. pallidum* Nichols in paraffin-embedded tissue. Single thick sections from syphilitic and normal rabbit testes were stained by Warthin-Starry silver or processed for PCR and dot blot analysis (magnification, ×400).

results may not correlate with disease activity (12, 21, 43, 46). We used PCR to develop a highly sensitive and specific assay for *T. pallidum* in the belief that such an assay might help to circumvent some of these problems.

Syphilis specimens, such as blood and CSF, may contain extremely low concentrations of treponemes (6, 23, 47). It was therefore essential that the assay's sensitivity approach the theoretical ability of PCR to detect a single organism (8, 39). To maximize the efficiency of amplification, reaction parameters were carefully optimized (19). Detection of PCR products was maximized by using a large duplex DNA probe, rather than a synthetic oligonucleotide, since this could be radiolabeled to a much greater specific activity. In addition, a large probe would presumably minimize false-negatives resulting either from sequence variation of *tpp47* among wild-type strains or from base pairs substituted by potential *Taq* polymerase "infidelity." Several experiments, including one based upon probability analysis, confirmed that the assay's sensitivity did in fact approach the detection of a single copy of *tpp47*.

Inasmuch as clinical specimens are likely to contain large numbers of cells of human and/or microbial origin, it was equally important that the assay be highly specific for *T. pallidum*. Although considerable data already existed to support the *T. pallidum* specificity of the 47-kDa immunogen and its corresponding gene (16, 26, 27, 32, 36), the specificity of the assay was investigated extensively. The occasional visualization of nonspecific PCR products on ethidium bromide-stained agarose gels (as in the case of *N. gonorrhoeae*) emphasized the need for routinely employing DNA-DNA hybridization with the *tpp47*-specific probe as a means of enhancing specificity as well as sensitivity. In addition to *T. pallidum* subsp. *pallidum*, specific PCR products were obtained only from genomic DNA of *T. pallidum* subsp. *pertenue*. This finding was not surprising given the extraordinarily close genetic relatedness of the pathogenic treponemes (29) and the highly conserved antigenicity of their 47-kDa immunogens (9, 26, 45). Amplification of *tpp47* as performed in the present study will therefore not be able to distinguish syphilis and yaws any better than conventional diagnostic modalities. On the other hand, it is noteworthy that the PCR assays developed by us and by others (31, 37) can readily distinguish between *T. pallidum* and *B. burgdorferi*; cross-reactivity between the two spirochetes is a major problem in serodiagnostic tests for syphilis and Lyme disease (7, 18). While the PCR assay for *T. pallidum* has yet to be validated by extensive clinical evaluation, it seems reasonable to proceed to such evaluations under the presumption that it specifically amplifies only DNA from pathogenic treponemes.

Inhibition of PCR by rabbit testicular tissue and human leukocytes was a significant and unexpected problem. Other investigators apparently have circumvented similar problems by using proteolysis, DNA extraction, or both to eliminate the inhibitory substance(s) prior to PCR (2, 4, 10, 14, 22, 30, 40). In our hands, alkaline lysis followed by phenol-chloroform extraction proved to be a sensitive and reliable method for sample preparation. More recently, we have obtained generally satisfactory results by using differential centrifugation to separate the majority of human cells from spirochetes in spinal and amniotic fluids (unpublished data). Nevertheless, we find that occasional specimens known to contain *T. pallidum* are PCR positive only following DNA extraction. Investigators therefore need to consider the possibility that PCR inhibition may be a cause of false-negative results.

RIT can detect a single treponeme (21, 24), a sensitivity which has established it as the "gold standard" for identification of *T. pallidum* in clinical specimens. Accordingly, investigators in both pre- and the postantibiotic eras used RIT to provide important insights into syphilis pathogenesis (6, 21, 23, 47). These studies demonstrated that *T. pallidum* invades the central nervous system early in the course of syphilitic infection and that isolation of treponemes from the CSF does not necessarily correlate with CSF laboratory abnormalities or a patient's neurological status. To illustrate the latter point, *T. pallidum* can be isolated from the CSFs of approximately 10% of early syphilis patients with normal CSF clinical laboratory parameters (6, 23), while RIT of CSF from neurosyphilis patients, particularly those with parenchymatous disease, is frequently negative (21, 47). The preliminary PCR results reported here are consistent with these earlier studies. Recently, Hay et al. (13) reported that their PCR assay detected *T. pallidum* in over half of the spinal fluids from patients with latent and tertiary syphilis. While potentially important, these results are inconsistent with published RIT analyses of spinal fluids from late syphilis patients (21, 23) and they were not corroborated by companion RIT data. It is clear, therefore, that the PCR assay for *T. pallidum* will require extensive clinical comparison with RIT before its precise role in syphilis diagnosis is ascertained. Such evaluations will also be necessary to determine whether the detection of treponemal DNA by PCR, rather than of virulent organisms by RIT, will engender errors in clinical decision making.

In addition to its obvious diagnostic applications, detection of *T. pallidum* by PCR may prove to be a valuable tool for clinical and laboratory investigation of syphilis. For example, enhanced detection of *T. pallidum* in clinical specimens will provide further insights into the kinetics and routes of dissemination by the organism. Detection of minute numbers of treponemes in tissue culture studies may help elucidate some of the poorly understood aspects of *T. pallidum*-mammalian cell interactions. Finally, PCR-based detection of *T. pallidum* in paraffin-embedded tissues and archaeological specimens (38) will facilitate analyses of samples that predate the antibiotic era. Such investigations may help to resolve many of the controversies surrounding syphilis in the modern era, not the least of which is the mysterious origin of the disease.

ACKNOWLEDGMENTS

We gratefully acknowledge Esther Robinson and Martin Goldberg for their excellent technical assistance, George Wendel and Pablo Sanchez for dark-field examination and RIT of amniotic fluids, Donald McIntire for assistance with statistical analysis, Robin Isaacs and Yankel Kupersztoch for helpful suggestions, and Pei-Ling Hsu for subcloning *tpp47* into pUC19. We also thank George H. McCracken, Edmund C. Tramont, E. Russell Alexander, and Robert T. Rolfs for their support and encouragement.

This study was partially supported by Public Health Service grants AI-26756 to J.D.R., AI-16692 and AI-17366 to M.V.N., and AI-18988 and NS23677 to S.A.L. from the National Institutes of Health and by contract 927056 from the Centers for Disease Control to J.D.R. J.D.R. is a scholar of the American Foundation for AIDS Research. J.M.B. is a research fellow of the Howard Hughes Medical Institute.

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