JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1991, p. 1976–1984
0095-1137/91/091976-09$02.00/0

Vol. 29, No. 9

Detection by Polymerase Chain Reaction of *Treponema pallidum* DNA in Cerebrospinal Fluid from Neurosyphilis Patients before and after Antibiotic Treatment

GERDA T. NOORDHOEK,[1,2]* ERIK C. WOLTERS,[2] MARJOLIJN E. J. DE JONGE,[1] AND JAN D. A. VAN EMBDEN[1]

Unit Molecular Microbiology, National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven,[1] and Department of Neurology, Academic Hospital, Vrije Universiteit, Amsterdam,[2] The Netherlands

Received 31 January 1991/Accepted 14 June 1991

A polymerase chain reaction with nested primer pairs based on the DNA sequence of the 39-kDa *bmp* gene of *Treponema pallidum* subsp. *pallidum* is described. The method allowed the detection of purified *T. pallidum* DNA equivalent to the amount of DNA in a single bacterium and was specific for *T. pallidum* subspecies. After concentration of DNA, using diatomaceous earth, it was possible to detect about 100 treponemes in 1 ml of cerebrospinal fluid. Cerebrospinal fluid samples from a total of 29 symptomatic and asymptomatic patients with neurosyphilis were tested for the presence of treponemal DNA before and at various intervals after intravenous treatment with penicillin. Prior to the penicillin treatment, we detected *T. pallidum* DNA in 5 of 7 patients with acute symptomatic neurosyphilis, in none of the 4 patients with chronic symptomatic neurosyphilis tested before treatment, and in 2 of 16 patients with asymptomatic neurosyphilis. Unexpectedly, *T. pallidum* DNA was also often detected in cerebrospinal fluid long after intravenous treatment with penicillin, sometimes up to 3 years after therapy.

T. pallidum subsp. *pallidum* (*T. pallidum*), the causative agent of venereal syphilis, belongs to the few pathogenic bacterial species that cannot be cultured in vitro. Therefore, the detection of this microorganism in infected individuals depends either on unsensitive microscopic methods or on inoculation of laboratory animals such as rabbits, guinea pigs, hamsters, or occasionally, monkeys.

In 1924, Chesney and Kemp (3) reviewed the results of rabbit inoculations with cerebrospinal fluid (CSF) from patients with syphilis. Treponemes could be detected in CSF from patients with all stages of the disease. Even when the spinal fluid specimens from patients with early syphilis were normal with regard to cell count, globulin, and the Wasserman reaction, virulent treponemes were found in 15 to 20% of the specimens (3). More recently, Lukehart et al. (17), also using rabbit inoculations, found treponemes in CSF from 12 of 40 patients with untreated primary and secondary syphilis.

The poor sensitivity associated with treponeme detection by microscopic techniques leaves the clinician to depend mainly on anti-*T. pallidum* antibody tests to make the diagnosis. However, in cases of early primary syphilis, late syphilis, and congenital syphilis, serologic tests are not always positive. In some cases, these serologic tests have been reported to be negative, even when *T. pallidum* could be cultivated from CSF, the aqueous humor of the eye, or lymph nodes (28).

Asymptomatic neurosyphilis requires an extensive intravenous penicillin treatment in order to reach a treponemicidal penicillin level in the central nervous system (35, 36). The use of a combination of antibiotics with probenecid to achieve a higher penicillin concentration in the CSF is to be avoided, because probenecid may interfere with the accumulation of penicillin in the parenchyma of the central nervous system (16). An intramuscular treatment regimen with depot penicillin preparations such as benzathine penicillin or procaine penicillin does not consistently yield treponemicidal concentrations in CSF (22, 27, 30, 40). Follow-up serologies of serum and CSF after treatment of patients with syphilis are necessary to verify the effectiveness of the therapy.

The interest in detecting treponemes in the CSF of patients visiting sexually transmitted disease clinics is growing because of an increasing number of patients who are coinfected with human immunodeficiency virus (HIV) and *T. pallidum* (11–13, 23). In cases of latent *T. pallidum* infection, one might expect relapses to occur as a consequence of the immune suppression caused by HIV, comparable to the reactivation of tuberculosis and other infectious diseases associated with HIV infection (5, 7, 20). Furthermore, in HIV-infected individuals, syphilis may be difficult to diagnose because of atypical clinical symptoms and an abnormal humoral immune response which might interfere with a proper serodiagnosis (9, 10).

Inoculation of CSF in rabbits is considered to be the most sensitive test for the detection of *T. pallidum*. Only 10 viable treponemes are needed to cause seroconversion in rabbits (17). The drawbacks of this method are the costs of housing rabbits and the long incubation period. When the inoculum contains only a small number of viable treponemes, more than 2 months may be needed before there is a serological response in the rabbits. For this reason, the method of rabbit inoculation is seldom used for diagnostic purposes in patients.

At present, specific and sensitive detection of microorganisms can be performed quickly after amplification of DNA by the polymerase chain reaction (PCR) (24). This method theoretically allows the detection of a single microorganism, and it has been proven to be of special value for the diagnosis of infectious agents that are difficult to cultivate, such as *Mycobacterium leprae* (38), human papillomavirus (29), and *T. pallidum* (2, 8, 21). With the use of DNA amplification based on sequences of the *tmpA* and *tpf-1* genes of *T.*

* Corresponding author.

pallidum, Hay et al. (9) described a sensitive method for the detection of treponemes in CSF. CSF from 10 of 19 patients with latent or tertiary syphilis and 7 of 28 HIV-positive patients were positive in the *T. pallidum* PCR. Three of the PCR-positive, HIV-infected patients had negative syphilis serology. However, until now, no data were available about the presence of viable treponemes or treponemal DNA in CSF after intravenous penicillin treatment. In this report, we present data on the presence of *T. pallidum* DNA in CSF from patients with symptomatic and asymptomatic neurosyphilis before and up to 8 years after penicillin treatment. We used a method particularly suited to the detection of DNA from treponemes that may have been lysed by freezing and thawing of the clinical samples.

MATERIALS AND METHODS

Clinical specimens. CSF and serum samples were collected from patients attending the departments of neurology at the academic hospitals of the Free University and the University of Amsterdam during the period 1983 to 1990. The patients attended the clinics because they had neurological symptoms in combination with positive syphilis serology or because they had been treated with benzathine penicillin G, orally or intramuscularly, for syphilis of unknown duration or for latent syphilis. Patients with latent syphilis are at risk of developing neurosyphilis, and in The Netherlands, 1 year after treatment, serum and CSF samples from these patients are routinely examined for treponemal antibodies and signs of nonspecific inflammation (34, 36). On the basis of neurologic manifestations, the patients were divided into the following three groups: group A, 7 patients with acute meningovascular or parenchymatous neurosyphilis; group B, 6 patients with chronic (parenchymatous) neurosyphilis; and group C, 16 patients with asymptomatic neurosyphilis. Patients in group C had no neurological symptoms, but CSF samples had abnormal cell counts, immunoglobulin M (IgM) and/or IgG index, and treponemal antibodies. None of the patients entering the study had been treated with intravenous penicillin. CSF and serum samples were collected prior to treatment and one to nine times after antimicrobial therapy at approximately 6-month intervals. All patients received 0.15 million units of aqueous penicillin G per kg of body weight per day intravenously during 15 days.

The detailed results of the various assays done on serum and CSF specimens, such as CSF cell counts, albumin, IgG and IgM indices, and nontreponemal and treponemal antibody tests, such as the Venereal Disease Research Laboratory (VDRL) test, the *T. pallidum* hemagglutination assay, the fluorescent-treponemal-antibody absorption test, and TmpA enzyme-linked immunosorbent assay, will be described elsewhere (37). Only disposable equipment was used to collect and process serum and CSF samples, in order to avoid cross contamination of the clinical specimens. Sera and CSF samples were stored at −20°C. The majority of these specimens were thawed and frozen several times prior to use for PCR.

Negative control CSF specimens were derived from persons attending the hospital for reasons other than a spirochetal infection, for instance, low back pains with or without lumbar intervertebral disc problems. Serum samples from these patients were negative in syphilis tests.

Bacteria. *T. pallidum* Nichols was maintained by serial passage in rabbit testes (31). Treponemes were purified from testicular tissue by urografin gradient centrifugation. After purification, the treponemes were suspended in phosphate-buffered saline (PBS) at a concentration of 10^{10}/ml and were frozen in small aliquots. Cell counts were done by dark-field microscopy.

Extraction of DNA. DNA from *T. pallidum* organisms was purified as described previously (31) by using sodium dodecyl sulfate, proteinase K, and phenol extraction. After ethanol precipitation, the chromosomal DNA was suspended in 10 mM Tris–1 mM EDTA (pH 8; TE), and the concentration was estimated by measurement of the A_{260}. Dilutions of *T. pallidum* DNA from 5 μg/ml to 0.05 pg/ml were made in TE containing 100 μg of herring sperm DNA (Boehringer, Mannheim, Federal Republic of Germany) per ml as a carrier. Aliquots of 5 μl of the DNA dilutions were kept at −20°C in 0.5-ml tubes for use as positive controls in the PCR.

For determination of the specificity of the PCR, DNA was isolated by standard DNA procedures (25) from *T. pallidum* subsp. *pertenue* CDC 2575 and 352 Pariaman (21), *Treponema phagedenis* biotype Reiter, *Borrelia burgdorferi* B31, *Bordetella pertussis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis*, *Salmonella typhi*, *Salmonella typhimurium*, *Salmonella panama*, *Salmonella virchow*, *Salmonella enteritidis*, *Shigella flexneri*, *Shigella sonnei*, *Streptococcus pneumoniae*, *Vibrio cholerae*, human papillomavirus, human placenta, and human intestinal flora. DNA from *Leptospira interrogans*, *Leptospira biflexa*, *Treponema innocens*, and *Treponema hyodysenteriae* was kindly provided by R. P. A. M. Segers, Utrecht, The Netherlands. DNA from *T. pallidum* SS3 and *Treponema denticola* was kindly provided by C. Penn, Birmingham, United Kingdom. DNA from *Haemophilus influenzae* and *Neisseria meningitidis* was kindly provided by L. van Alphen, Amsterdam, The Netherlands; and DNA from *Haemophilus ducreyi* was provided by L. M. Parssons, Albany, N.Y.

Sample preparation for PCR. CSF samples of 250 μl or dilutions of *T. pallidum* cells in CSF were centrifuged for 15 min at 15,000 × *g* in a microcentrifuge. The pellet was suspended in 50 μl of lysis buffer consisting of 50 mM Tris hydrochloride (pH 8.5), 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dithiothreitol, 0.45% Nonidet P-40, proteinase K (100 μg/ml), and herring sperm DNA (100 μg/ml). After 2 to 3 h of incubation at 55°C, the samples were heated for 10 min at 100°C to inactivate the proteinase K and to denature the DNA. The samples were kept on ice for direct processing or were stored at −20°C.

DNA extraction from CSF. To recover free *T. pallidum* DNA from lysed bacteria, we used the method described by Boom et al. (1) in which DNA is concentrated by adsorption to diatoms in the presence of guanidinium thiocyanate (GuSCN). Briefly, the supernatant of 250 μl of CSF was added to 4.5 ml of L6 buffer (120 g of GuSCN, 100 ml of 0.1 M Tris hydrochloride [pH 6.4], 22 ml of 0.2 mM EDTA, 2.6 g of Triton X-100) with 40 μl of diatom suspension (Celite; Janssen Chimica, Beerse, Belgium). Before centrifugation, herring sperm DNA (100 μg/ml) was added to the CSF as a carrier. The mixture was rotated for 30 to 60 min, and after centrifugation, the pellet of diatoms with absorbed DNA was washed twice with L2 buffer (100 g of GuSCN, 100 ml of 0.1 M Tris hydrochloride [pH 6.4]), twice with 70% ethanol, and once with acetone. After drying, the DNA was eluted from the diatoms with 50 μl of TE by overnight incubation at 55°C. Five microliters of the eluate was used in the PCR.

PCR. Amplimers for the PCR were based on the DNA sequence of the 39-kDa basic membrane protein from *T.*

TABLE 1. Sequences of synthetic oligonucleotides in the *bmp* gene of *T. pallidum*[a]

Nucleotide	Sequence (5′–3′)	Location (position no.) within gene
TP 3	CAGGTAACGGATGCTGAAGT	256–275
TP 4	CGTGGCAGTAACCGCAGTCT	762–741
TP 5	GACCTGAGGACTCTCAAATC	500–519
TP 7	CTCAGCACTGCTGAGCGTAG	172–191
TP 8	AACGCCTCCATCGTCAGACC	788–769

[a] DNA sequence published by Dallas et al. (6).

pallidum (6), and these are given in Table 1. Primers TP 7 and TP 8 were used to amplify a 617-bp fragment of the *bmp* gene, and oligonucleotides TP 3 and TP 4 were used to amplify a 500-bp fragment within this 617-bp fragment. The oligonucleotides were synthesized on a DNA synthesizer (type 381A; Applied Biosystems, Inc., Foster City, Calif.). DNA amplification was performed in a total volume of 25 μl (21, 24). The reaction mixture contained RT buffer (14) (50 mM Tris hydrochloride [pH 8.5], 50 mM NaCl, 4 mM $MgCl_2$, 2 mM dithiothreitol) supplemented with 200 μM (each) deoxynucleoside triphosphate, 200 μM primers TP 7 and TP 8, 0.01% bovine serum albumin (DNase and RNase free; Boehringer), 0.6 U of Amplitaq polymerase (Perkin-Elmer Cetus), and 5 μl of sample. After the mixtures were preheated at 94°C for 3 min, 30 or 39 amplification cycles were performed in a DNA thermocycler (Perkin-Elmer Cetus), as follows: 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C. The elongation step at 72°C was extended for 5 s for each subsequent cycle. After the final cycle, the mixture was incubated for 6 min at 72°C to complete the last polymerase reaction. Samples from this first PCR with primer set TP 7-TP 8, designated PCR 1, were diluted 1 to 10 with distilled water, and 5 μl was subjected to a second round of amplification; PCR 2 was done by using primers TP 3 and TP 4 as nested primers. PCR 2 was performed as described for PCR 1, except that the $MgCl_2$ and primer concentrations were different: 5 mM and 40 μM, respectively. Positive and negative controls were included in all experiments and were subjected to the same treatments as the clinical samples were. Positive controls consisted of PBS containing two different concentrations of treponemes, 100/ml and 10,000/ml. Negative controls were placed randomly between the samples and consisted of PBS only or were taken from pooled control CSF samples. We included at least one negative control per six clinical samples. In order to avoid contamination, positive displacement pipettors with disposable pistons (Microman; Gilson Medical Electronics, Villiers-le-Bel, France) were used for pipetting the clinical samples and to dilute the samples after PCR 1. Preamplification buffers were prepared in a building that had not previously been exposed to *T. pallidum* DNA, and clinical samples were handled in a room that was well separated from the room where the amplified DNA was further analyzed (15, 26).

Analysis of PCR products. Eight microliters of the reaction mixture was analyzed by gel electrophoresis in 2% agarose (Seakem) containing ethidium bromide (EB). Southern blotting was done as described by Noordhoek et al. (19) by using the ^{32}P-end-labeled oligonucleotide TP 5 (Table 1) as a probe. After hybridizations, the blots were washed twice at 50°C and autoradiography was performed for 24 h at −70°C by using Kodak X-Omat film (19).

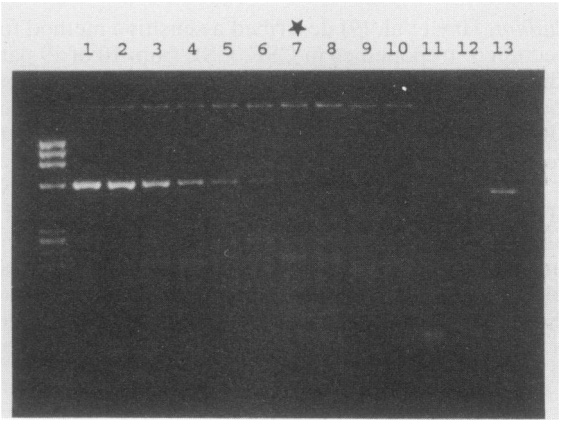

FIG. 1. Sensitivity of the PCR method for detection of *T. pallidum* DNA. Dilutions of target DNA were amplified by 39 cycles of PCR by using the primers TP 7 and TP 8 and were analyzed by EB-containing agarose gel electrophoresis. Lane 1, 25 pg of DNA; lane 2, 5 pg of DNA; lane 3, 1 pg of DNA; lane 4, 200 fg of DNA; lane 5, 40 fg of DNA; lane 6, 8 fg of DNA; lane 7, 1.6 fg of DNA; lane 8, 0.3 fg of DNA; lane 9, 0.1 fg of DNA; lane 10, 0.01 fg of DNA; lanes 11 and 12, H_2O; lane 13, molecular mass marker (500 bp); the unmarked lane contains φX174 DNA digested with *Hae*III-containing fragments of 1,353, 1,078, 872, 603, 310, 281/271, 234, 194, 118, and 72 bp from top to bottom, respectively. Target DNA was diluted in TE plus 100 μg of carrier DNA per ml. The lane marked with a star corresponds to the highest dilution in which a fragment of 617 bp was visible on the EB-stained gel.

RESULTS

Sensitivity of the PCR to detect *T. pallidum* DNA. The primers TP 7 and TP 8 were used to amplify a 617-bp fragment of the *bmp* gene of *T. pallidum*. Fivefold dilutions of purified *T. pallidum* DNA were used to determine the sensitivity of the PCR by protocol PCR 1 described above (Fig. 1). Approximately 1 fg of chromosomal DNA was the minimal amount of DNA needed to detect the 617-bp fragment by hybridization with the ^{32}P-labeled TP 5 probe (data not shown). Assuming a molecular mass of 900 kb for the genome of *T. pallidum* (33), 1 fg of DNA corresponds to about one genome equivalent.

When lysed *T. pallidum* bacteria instead of purified target DNA were used in the PCR, the sensitivity was about 1,000-fold lower and bands differing from the 617-bp fragment were visible on EB-stained agarose gels. This apparent loss of specificity prompted us to develop a PCR protocol with two steps of amplification, using the TP 7-TP 8 primers in the first 30 amplification cycles (PCR 1) and the TP 3-TP 4 primers in a second round of 39 amplification cycles (PCR 2). This protocol, PCR 1 plus PCR 2, allowed us to detect the DNA from an equivalent of about one bacterium. The 500-bp PCR fragment derived from 1.6 fg of DNA was clearly detectable on an EB-stained agarose gel (Fig. 2, lane 6). In addition, this two-step PCR increased the specificity because, except for the 500-bp fragment, no additional fragments were found on EB-stained agarose gels. As expected, an additional band of 617 bp was detected if more than 1 pg of target DNA was present in the sample.

Specificity of the primers. To determine the specificity of the PCR for the amplification of *T. pallidum* DNA, 100 ng of DNA from a variety of different microorganisms, listed in Material and Methods, was subjected to the PCR by using the two sets of primers. Except for DNA from three *T. pallidum* strains, no amplification of the *T. pallidum*-specific

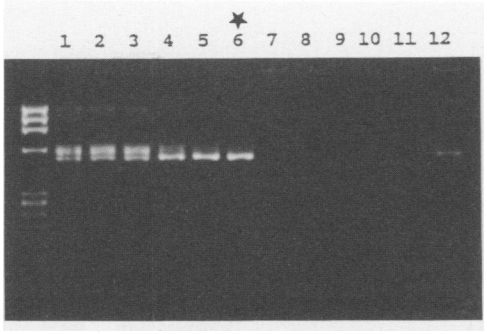

FIG. 2. Sensitivity of the two-step PCR 1 and PCR 2 to detect *T. pallidum* DNA. After 30 amplification cycles with primers TP 7 and TP 8, the PCR product was diluted 1:10 and subjected to 39 cycles in a second PCR with primers TP 3 and TP 4. Lane 1, 25 pg of DNA; lane 2, 5 pg of DNA; lane 3, 1 pg of DNA; lane 4, 40 fg of DNA; lane 5, 8 fg of DNA; lane 6, 1.6 fg of DNA; lane 7, 0.3 fg of DNA; lane 8, 0.1 fg of DNA; lane 9, 0.01 fg of DNA; lanes 10 and 11, H_2O; lane 12, molecular mass marker (500 bp); unmarked lane, φX174 DNA digested with *Hae*III (see legend to Fig. 1). The star indicates the highest dilution in which a fragment of 500 bp was visible on the EB-stained gel.

500-bp fragment was found among DNA from 25 bacterial species belonging to a variety of genera, human papillomavirus, or human placenta or DNA purified from the bacterial mixture derived from the human intestinal flora (Fig. 3). Except for one *E. coli* DNA sample, none of the non-*T. pallidum*-derived target DNAs gave rise to a distinct amplified fragment. In the case of the *E. coli* DNA sample, a 400-bp fragment was seen on the EB-stained agarose gel, but this fragment did not hybridize with the *T. pallidum*-specific oligonucleotide TP 5 (Fig. 3B and C).

Detection of rabbit-derived *T. pallidum* suspended in CSF. To estimate the minimum number of *T. pallidum* cells detectable by the PCR 1 plus PCR 2 protocol, rabbit-derived *T. pallidum* bacteria were diluted in saline and subjected to treatment with Nonidet P-40 and proteinase K prior to amplification of the DNA. The detection limit was found to be two bacteria in 25 μl of the PCR mixture (data not shown). A similar value was obtained when treponemes were diluted in control CSF instead of saline.

Because most of the clinical CSF samples to be analyzed were frozen and thawed several times, the effects of freezing and thawing on the integrity of *T. pallidum* cells were determined. Tenfold dilutions of treponemes in control CSF were made, and samples of 250 μl were subjected to three cycles of freeze-thawing followed by centrifugation, to sediment the intact bacteria. The pellet fraction was treated with 50 μl of lysis buffer. In order to recover the DNA from lysed treponemes, the supernatants were extracted and concentrated to 50 μl by the GuSCN-diatomaceous earth method. Five microliters of the extracts, containing 10% of the original sample of 250 μl, was subjected to amplification by PCR 1 plus PCR 2.

T. pallidum DNA was detectable in the pellet fraction when 250 or more treponemes were present in 5 μl (Fig. 4A, lane 5). The minimum number of treponemes detectable in 5 μl of diatom eluate from the CSF supernatant was 2.5 (Fig. 4B, lane 3). Therefore, about 99% of the *T. pallidum* cells are lysed by three cycles of freezing and thawing. Since 2.5 treponemes were detectable in 10% of the original sample of 250 μl, the detection limit of lysed *T. pallidum* cells amounts to about 100 bacteria per ml of CSF.

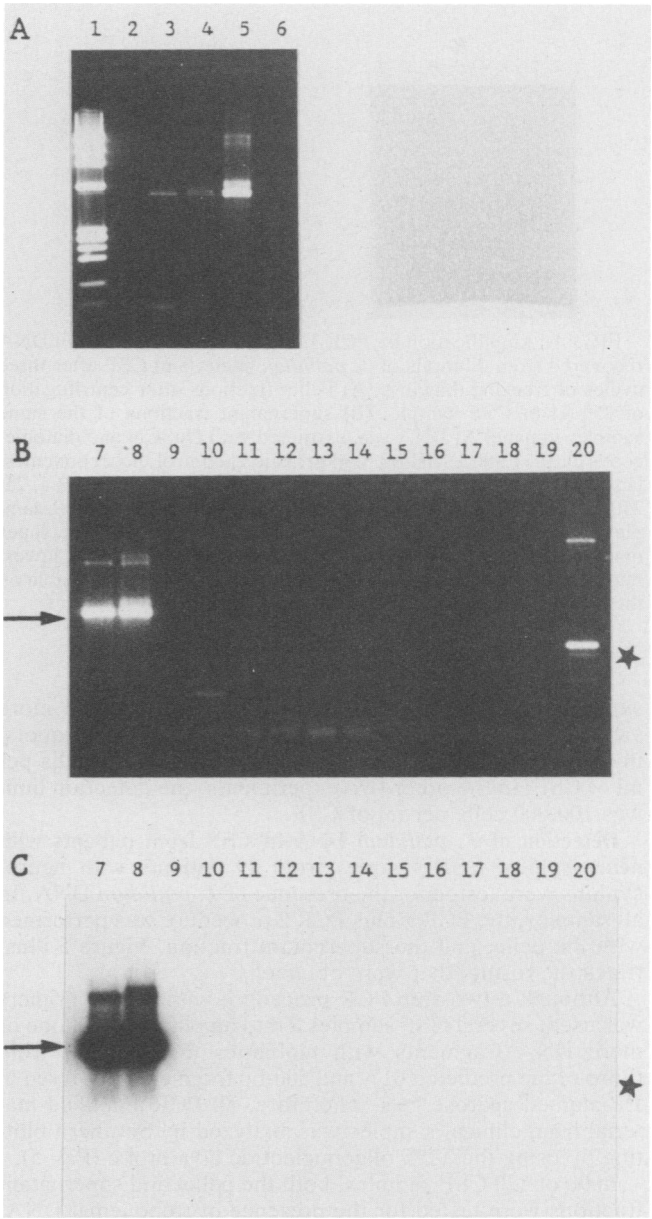

FIG. 3. Specificity of the two-step PCR protocol for detection of *T. pallidum* DNA. DNA from various organisms was tested by PCR 1 plus PCR 2. (A and B) EB-stained agarose gels; (C) Southern blot of the gel in panel B hybridized with the internal probe TP 5. Lane 1, φX174 molecular mass marker (see legend to Fig. 1); lane 2, *T. hyodysenteriae*; lane 3, *T. pertenue* CDC 2575; lane 4, *T. pallidum* SS3; lanes 5, 7, and 8, *T. pallidum* Nichols; lane 6, H_2O; lanes 9 through 20, *T. phagedenis*, *B. pertussis*, *V. cholerae*, *S. panama*, *B. burgdorferi*, *S. enteritidis*, *K. pneumoniae*, *M. tuberculosis*, *S. flexneri*, human papillomavirus, H_2O, and *E. coli*, respectively. Arrows indicate the position of the 500-bp fragment. Stars indicate the position of the nonhybridizing *E. coli* PCR fragment in lane 20.

In order to test the reproducibility of detection of *T. pallidum* DNA in the pellet and in the supernatant fraction of frozen treponeme-containing CSF samples, aliquots of *T. pallidum* dilutions in CSF were subjected to the procedure described above. In all six independent experiments, the detection level was found to be 100 bacteria per ml of

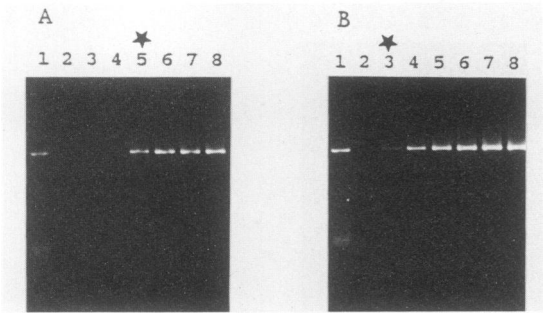

FIG. 4. Amplification by PCR 1 plus PCR 2 of *T. pallidum* DNA recovered from dilutions of *T. pallidum* bacteria in CSF after three cycles of freezing-thawing. (A) Pellet fractions after centrifugation of 250 μl of CSF sample; (B) supernatant fractions of the same samples in panel A; DNA was extracted with GuSCN and diatomeceous earth. Lane 1, 500-bp marker; lane 2, control, no treponemes; lanes 3 through 7, amplified DNA from samples containing 2.5, 25, 250, 2,500, and 25,000 treponemes, respectively. These PCR samples corresponded to 10% of the total CSF sample. The lanes marked with a star correspond to the samples with the lowest number of treponemes in which a fragment of 500 bp was visible on the EB-stained gel.

supernatant. Analysis of the pellet fraction showed more variable results. After freezing-thawing of different aliquots, in four experiments the detection limit was 10,000 cells per ml of CSF; in the other two experiments, the detection limit was 100,000 cells per ml of CSF.

Detection of *T. pallidum* DNA in CSF from patients with neurosyphilis. CSF samples from 29 patients with neurosyphilis were tested for the presence of *T. pallidum* DNA. In all samples, the PCR 1 plus PCR 2 procedure was performed with the pellet and the supernatant fraction. Figure 5 illustrates the results that were obtained.

Although a two-step PCR procedure with nested primers was used, several CSF samples led to amplification of one or more DNA fragments with mobilities that differed from those of the predicted 617- and 500-bp fragments observed in EB-stained agarose gels. Therefore, all PCR-amplified material from clinical samples was analyzed by Southern blotting by using the TP 5 oligonucleotide as a probe (Fig. 5).

In 90 of 120 CSF samples, both the pellet and supernatant fractions were tested for the presence of treponemal DNA. As shown in Table 2, *T. pallidum* DNA was not always detected in both fractions from the same sample. In 30 samples, only one of the fractions could be tested. A sample was considered to be positive when *T. pallidum* DNA was found in one or both fractions.

Figure 6 shows the results of the detection of *T. pallidum* DNA in CSF from seven patients with acute neurosyphilis before and various periods after penicillin treatment. In CSF samples from five of these patients, we detected *T. pallidum* DNA prior to treatment. Unexpectedly, in all patients except one, *T. pallidum* DNA was found in CSF 1 to 21 months after intravenous penicillin treatment. This group of patients included two patients who were PCR negative prior to treatment. None of the 13 CSF samples which were collected from the patients more than 21 months after treatment were positive for *T. pallidum* DNA.

In contrast to the group of patients with acute neurosyphilis, no *T. pallidum* DNA was detected in the four CSF samples available from the group of six patients with chronic syphilis prior to treatment (Fig. 7). Again, unexpectedly, *T.*

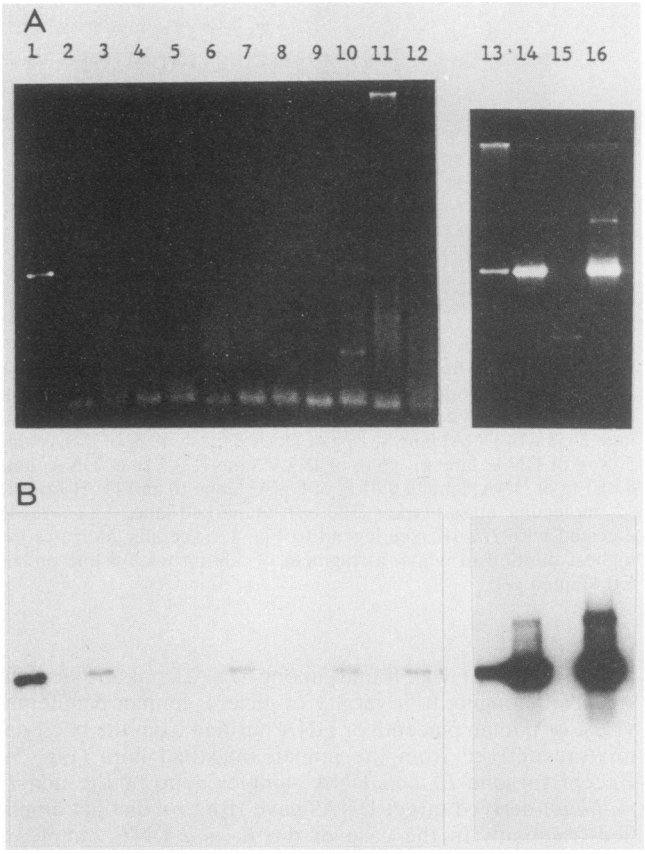

FIG. 5. Analysis of CSF samples from patients with neurosyphilis for the presence of *T. pallidum* DNA after amplification following the PCR 1 plus PCR 2 protocol. Lane 1, molecular mass marker (500 bp); lanes 2 to 16, CSF from individual patients. (A) EB-stained gels; (B) Southern blots after hybridization with the TP 5 probe.

pallidum DNA was detected in CSF from four patients 7 to 48 months after penicillin treatment.

The CSF samples from 16 patients with asymptomatic neurosyphilis were analyzed. Only 2 of the 16 pretreatment CSF samples were positive by the DNA test (Fig. 8). In CSF samples from four patients, *T. pallidum* DNA was detected 3 to 12 months after treatment. CSF from one patient showed a positive PCR 37 months after treatment, but reinfection could not be excluded in this case.

All CSF and serum samples were subjected to serological investigations. As a rule, VDRL, *T. pallidum* hemagglutination assay, and fluorescent-treponemal-antibody absorption test results for serum and CSF decreased slowly after treatment. There was no correlation whatsoever between the VDRL or specific antibody titers and the presence of

TABLE 2. Detection of *T. pallidum* DNA by PCR in the pellet and supernatant fractions of CSF samples[a]

No. of samples	Pellet	Supernatant
6	+	+
8	+	−
5	−	+
71	−	−

[a] +, positive in PCR; −, negative in PCR.

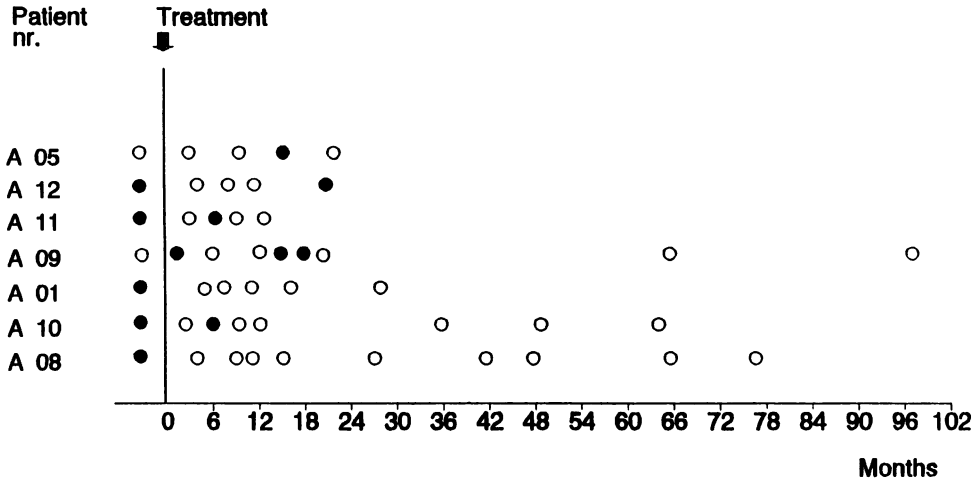

FIG. 6. Detectability by PCR 1 plus PCR 2 of *T. pallidum* DNA in CSF from patients with acute symptomatic neurosyphilis before and after treatment. ●, positive in PCR; ○, negative in PCR.

treponemes in CSF. For instance, serum and CSF from patient A 9 had negative VDRL test results, and CSF from patients A 11 and A 12 had negative VDRL test results. For most patients with chronic and asymptomatic neurosyphilis, serum VDRL test results were positive, but CSF VDRL test results were negative before treatment.

DISCUSSION

In the last 2 years, the PCR technology has proven its value for the direct demonstration of fastidious pathogenic microorganisms in clinical material. Because the PCR consumes little time and because it is very sensitive and specific, this test has many advantages over conventional methods, even in light of the knowledge that PCR tests with the present-day technology are not inexpensive and that successful operation needs special physical containment conditions to avoid contamination of samples with amplicons.

Detection of *T. pallidum* by PCR can be of value for the diagnosis of a *T. pallidum* infection when other methods fail. Promiscuous individuals are at high risk for coinfections with HIV and *T. pallidum* (11). In such cases, the currently used serologic tests for detection of syphilis might fail, and examination of CSF by PCR may be the only method to prove an infection with *T. pallidum*.

Hay and colleagues (8) were able to detect by PCR approximately 65 treponemes in 500 μl of CSF. This was done by concentrating the bacteria by centrifugation; this was followed by lysis in a nonionic detergent. The same method was followed by others for the detection of spirochetes like *B. burgdorferi* (18) and *L. interrogans* (32). We were not able to use such methods to detect *T. pallidum* DNA in CSF, because our clinical samples were frozen and thawed several times during the period of storage, probably resulting in the lysis of more than 99% of the treponemal cells. Although an equivalent of a single *T. pallidum* bacterium in PBS was detectable by PCR, the sensitivity of the method was significantly less when treponemes were suspended in CSF. Centrifugation of clinical CSF samples also caused concentration of inhibitors of the PCR in the pellet fraction. This was also mentioned by Burstain et al. (2). For this reason, we used the method of extraction of DNA from CSF with diatomaceous earth. The recovery of DNA after diatom extraction is higher than after phenol extraction followed by ethanol precipitation (1). In combination with a PCR with nested primers, we were able to detect treponemal DNA with a sensitivity of about 100 genome equivalents per ml of CSF. This indicates that the sensitivity of our method is approximately the same as those of the methods described by Burstain et al. (2) and Hay et al. (8), in which 1 to 10

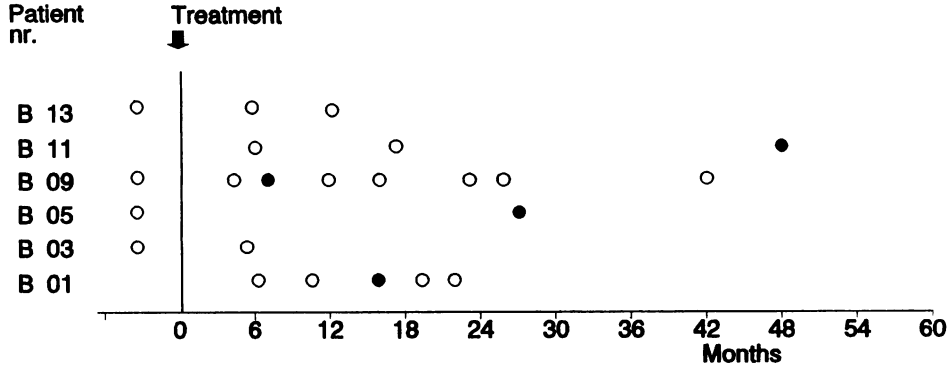

FIG. 7. Detectability by PCR 1 plus PCR 2 of *T. pallidum* DNA in CSF from patients with chronic neurosyphilis before and after treatment. ●, positive in PCR; ○, negative in PCR.

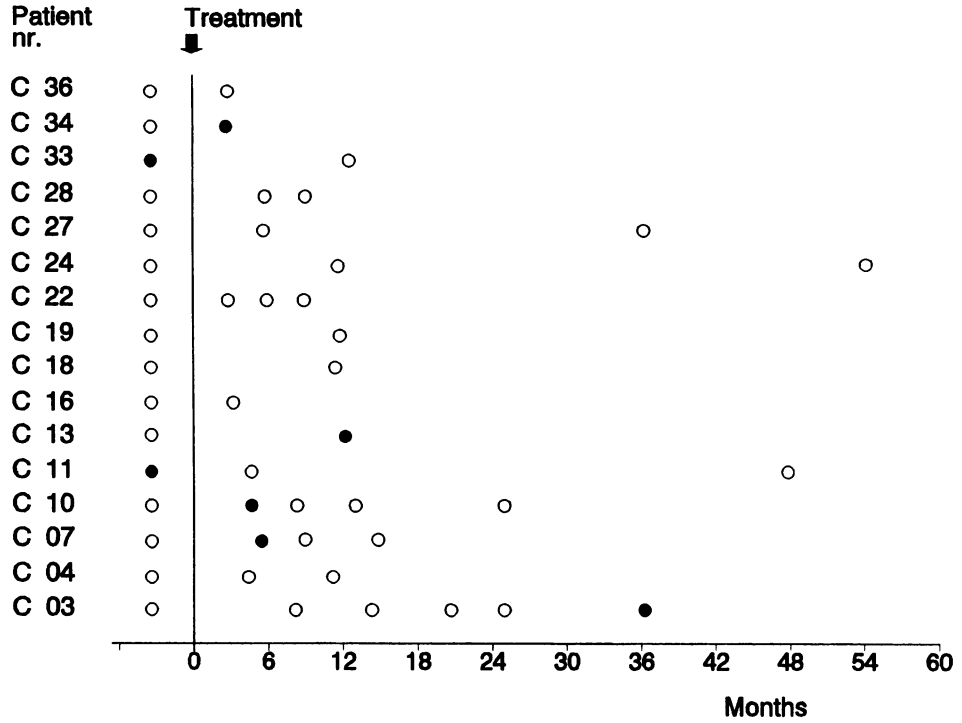

FIG. 8. Detectability by PCR 1 plus PCR 2 of *T. pallidum* DNA in CSF from patients with asymptomatic neurosyphilis before and after treatment. ●, positive in PCR; ○, negative in PCR.

organisms per 50 μl of sample are needed for a positive PCR result. Because we occasionally observed the amplification of nontreponemal DNA, we confirmed all PCR amplicons from patient samples by hybridization with the *T. pallidum*-specific oligonucleotide probe.

Most of the CSF samples were collected in the pre-PCR period, and no special care was taken to avoid degradation of DNA during storage. Therefore, part of the treponemal DNA originally present in the clinical samples may have been degraded. Consequently, the number of PCR-positive samples is likely to be an underestimation of the number of samples which contained *T. pallidum* DNA at the moment of collection. In order to prevent the action of Mg^{2+}-dependent DNases, we now add EDTA to the tubes after we collect fresh clinical CSF samples.

The potential to amplify and thus detect single DNA molecules in clinical material can easily result in false-positive signals caused by the amplification of stray DNA, as discussed by Schochetman et al. (26): "PCR, its power is its own weakness." For this reason, we chose a DNA sequence from a gene which has never been manipulated in our laboratory, the sequence from the *bmp* gene of *T. pallidum* published by Dallas et al. (6).

Even if all precautions to avoid contamination are taken, it is very difficult to exclude occasional false-positive results. However, *T. pallidum* is not a free-living microorganism, and so contamination in the laboratory by *T. pallidum* is unlikely. In addition, the CSF specimens used in this study were not kept in rooms in which cloned *T. pallidum* DNA or *T. pallidum* cultures were handled. In each experiment in which CSF samples were investigated, we included negative controls, which were subjected to the complete extraction and PCR procedures. None of these controls were positive. On the basis of our results, we estimate the chance to get a false-positive reaction caused by contamination during the procedures to be less than 4%. Because the percentage of positive tests on clinical material was about 14%, we presume that the majority of these results were truly positive. During the 2 years we have performed DNA amplifications with the same set of primers, we have had no major problems caused by contamination.

Comparison of the sensitivity of the PCR method with the "gold standard" of inoculation in rabbits was not possible in this study, because the stored CSF samples were not tested in rabbits. The detectability of *T. pallidum* DNA by PCR is expected to differ from this gold standard because by PCR, DNA from viable as well as from nonviable *T. pallidum* is detected.

The results of our PCR study are not directly comparable to results of other investigations on the persistence of *T. pallidum* after penicillin treatment. In previous studies on penicillin-treated patients with syphilis (4, 17, 39), rabbit inoculations were used to monitor the presence of treponemes in CSF or lymph nodes, and furthermore, the administration of penicillin was different from the intravenous route, which was used in our study.

This is the first study in which series of CSF samples from patients before and at various times after antibiotic treatment have been investigated for the presence of *T. pallidum* DNA. We detected *T. pallidum* DNA in CSF from five of seven patients with acute neurosyphilis before treatment. The treatment with intravenous penicillin is presumed to have been adequate (35), because the symptoms of acute neurosyphilis disappeared and the levels of treponemal antibodies and signs of infection in the spinal fluid decreased. However, in five of seven patients, treponemal DNA was found to be present in CSF; in one sample it was even present 21 months after treatment.

From the results of this study, it appears that the presence of *T. pallidum* DNA in CSF does not necessarily mean that these patients were treated inadequately, because none of the patients had a clinical relapse 2 to 9 years after treatment. More research is needed to determine whether the treponemal DNA detected by PCR originates from treponemes that are viable at the time of sampling or from killed or lysed bacteria. DNA is known to be a very stable biopolymer, and our data suggest that it might remain present in CSF for long periods of time after the effective killing of *T. pallidum* by intravenous penicillin treatment. Therefore, the detection of *T. pallidum* DNA by PCR seems to be of limited use to check the efficacy of antibiotic treatment of patients with neurosyphilis. Studies on the stability of DNA from killed *T. pallidum* in CSF are needed to draw a conclusion from the results of this study.

ACKNOWLEDGMENTS

We are grateful to R. P. A. M. Segers, C. Penn, L. van Alphen, and L. M. Parssons for the supply of DNA from various species; M. C. M. Dirks for collecting samples; and C. A. M. Hartgerink, M. P. T. M. Mommers, and F. Abbruscato for technical assistance.

REFERENCES

1. **Boom, R., C. J. A. Sol, M. M. M. Salimans, C. L. Jansen, P. M. E. Wertheim-van Dillen, and J. van der Noorda.** 1990. Rapid and simple method for purification of nucleic acids. J. Clin. Microbiol. **28**:495–503.
2. **Burstain, J. M., E. Grimprel, S. A. Lukehart, M. V. Norgard, and J. D. Radolf.** 1991. Sensitive detection of *Treponema pallidum* by using the polymerase chain reaction. J. Clin. Microbiol. **29**:62–69.
3. **Chesney, A. M., and J. E. Kemp.** 1924. Incidence of Spirochaeta pallida in cerebrospinal fluid during early stage of syphilis. J. Am. Med. Assoc. **22**:1725–1728.
4. **Collart, P., L.-J. Borel, and P. Durel.** 1964. Significance of spiral organisms found, after treatment, in late human and experimental syphilis. Br. J. Vener. Dis. **40**:81–89.
5. **Collins, F. M.** 1989. Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. Clin. Microbiol. Rev. **2**:360–377.
6. **Dallas, W. S., P. H. Ray, J. Leong, C. D. Benedict, L. V. Stamm, and P. J. Bassford, Jr.** 1987. Identification and purification of a recombinant *Treponema pallidum* basic membrane protein antigen expressed in *Escherichia coli*. Infect. Immun. **55**:1106–1115.
7. **Harries, A. D.** 1990. Tuberculosis and human immunodeficiency virus infection in developing countries. Lancet **335**:387–390.
8. **Hay, P. E., J. R. Clarke, R. A. Strugnell, D. Taylor-Robinson, and D. Goldmeier.** 1990. Use of the polymerase chain reaction to detect DNA sequences specific to pathogenic treponemes in cerebrospinal fluid. FEMS Microbiol. Lett. **68**:233–238.
9. **Hay, P. E., J. R. Clarke, D. Taylor-Robinson, and D. Goldmeier.** 1990. Detection of treponemal DNA in the CSF of patients with syphilis and HIV infection using the polymerase chain reaction. Genitourin. Med. **66**:428–432.
10. **Hicks, C. B., P. M. Benson, G. P. Lupton, and E. C. Tramont.** 1987. Seronegative secondary syphilis in a patient infected with the human immunodeficiency virus (HIV) with Kaposi sarcoma. Ann. Intern. Med. **107**:492–495.
11. **Hook, E. W., III.** 1989. Syphilis and HIV infection. J. Infect. Dis. **160**:530–534.
12. **Johns, D. R., M. Tierney, and D. Felsenstein.** 1987. Alteration in the natural history of neurosyphilis by concurrent infection with the human immunodeficiency virus. N. Engl. J. Med. **316**:1569–1572.
13. **Katz, D. A., and J. R. Berger.** 1989. Neurosyphilis in acquired immunodeficiency syndrome. Arch. Neurol. **46**:895–898.
14. **Krawetz, S. A., R. T. Pon, and G. H. Dixon.** 1989. Increased efficiency of the Taq polymerase catalyzed polymerase chain reaction. Nucleic Acids Res. **17**:819.
15. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
16. **Lancet.** 1984. Plugging the penicillin leak with probenicid. Lancet **ii**:499.
17. **Lukehart, S. A., E. W. Hook III, S. A. Baker-Zander, A. C. Collier, C. W. Critchlow, and H. H. Handsfield.** 1988. Invasion of the central nervous system by *Treponema pallidum*: implications for diagnosis and treatment. Ann. Intern. Med. **109**:855–862.
18. **Malloy, D. C., R. K. Nauman, and H. Paxton.** 1990. Detection of *Borrelia burgdorferi* using the polymerase chain reaction. J. Clin. Microbiol. **28**:1089–1093.
19. **Noordhoek, G. T., P. W. M. Hermans, A. N. Paul, L. M. Schouls, J. J. van der Sluis, and J. D. A. van Embden.** 1989. *Treponema pallidum* subspecies *pallidum* (Nichols) and *Treponema pallidum* subspecies *pertenue* (CDC 2575) differ in at least one nucleotide: comparison of two homologous antigens. Microb. Pathog. **6**:29–42.
20. **Noordhoek, G. T., and J. D. A. van Embden.** 1991. Yaws, an endemic treponematosis reconsidered in the HIV era. Eur. J. Clin. Microbiol. Infect. Dis. **10**:4–5.
21. **Noordhoek, G. T., B. Wieles, J. J. van der Sluis, and J. D. A. van Embden.** 1990. Polymerase chain reaction and synthetic DNA probes: a means of distinguishing the causative agents of syphilis and yaws? Infect. Immun. **58**:2011–2013.
22. **Polnikorn, N., R. Witoonpanich, M. Vorachit, S. Vejjajiva, and A. Vejjajiva.** 1980. Penicillin concentrations in cerebrospinal fluid after different treatment regimens for syphilis. Br. J. Vener. Dis. **56**:363–367.
23. **Rufli, T.** 1989. Syphilis and HIV infection. Dermatologica **179**:113–117.
24. **Saiki, R. K., D. H. Gelfand, S. Stoffer, S. J. Scharf, R. Higuchi, G. R. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Schochetman, G., C.-H. Ou, and W. K. Jones.** 1988. Polymerase chain reaction. J. Infect. Dis. **158**:1154–1157.
27. **Schoth, P. E. M., and E. C. Wolters.** 1987. Penicillin concentrations in serum and CSF during high dose intravenous treatment for neurosyphilis. Neurology **37**:1214–1216.
28. **Smith, J. L., and C. W. Israel.** 1968. Treponemes in aqueous humor in late seronegative syphilis. Trans. Am. Acad. Ophthalmol. Otolaryngol. **72**:63–75.
29. **Snijders, P. J., A. J. C. van den Brule, H. F. J. Schrijnemakers, G. Snow, C. J. L. M. Meijer, and J. M. M. Walboomers.** 1990. The use of general primers in the polymerase chain reaction permits the detection of a broad spectrum of human papillomavirus genotypes. J. Gen. Virol. **71**:173–181.
30. **Van der Valk, P. G. M., E. J. Kraai, P. C. van Voorst Vader, H. Haaxma-Reiche, and J. A. M. Snijder.** 1988. Penicillin concentrations in cerebrospinal fluid (CSF) during repository treatment regimen for syphilis. Genitourin. Med. **64**:223–225.
31. **Van Embden, J. D. A., H. J. van der Donk, R. V. van Eijk, H. G. van der Heide, J. A. de Jong, M. F. van Olderen, A. D. Osterhaus, and L. M. Schouls.** 1983. Molecular cloning and expression of *Treponema pallidum* DNA in *Escherichia coli* K-12. Infect. Immun. **42**:187–196.
32. **Van Eys, G. J. J. M., C. Gravekamp, M. J. Gerritsen, W. Quint, M. T. E. Cornelissen, J. Ter Schegget, and W. J. Terpstra.** 1989. Detection of leptospires in urine by polymerase chain reaction. J. Clin. Microbiol. **27**:2258–2262.
33. **Walker, E. M., J. K. Arnett, and S. J. Norris.** 1991. Genome size and chromosome conformation of *Treponema pallidum* subsp. *pallidum*, abstr. H-202, p. 188. Abstr. 91st Gen. Meet. Am. Soc. Microbiol. 1991. American Society for Microbiology, Washington, D.C.
34. **Wolters, E. C.** 1987. Neurosyphilis: a changing diagnostic problem? Eur. Neurol. **26**:23–28.

35. **Wolters, E. C.** 1987. Treatment of neurosyphilis. Clin. Neuropharm. **10**:143–154.
36. **Wolters, E. C., E. A. H. Hische, J. A. Tutuarima, L. van Trotsenburg, R. V. W. van Eijk, J. D. Bos, T. M. Starink, L. J. Emsbroek, and H. J. van der Helm.** 1988. Central nervous system involvement in early and late syphilis: the problem of asymptomatic neurosyphilis. J. Neurol. Sci. **88**:229–239.
37. **Wolters, E. C., G. T. Noordhoek, and E. A. H. Hische.** Unpublished data.
38. **Woods, S. A., and S. T. Cole.** 1989. A rapid method for the detection of potentially viable *Mycobacterium leprae* in human biopsies: a novel application of PCR. FEMS Microbiol. Lett. **65**:305–310.
39. **Yobs, A. R., J. W. Clark, S. E. Mothershed, J. C. Bullard, and C. W. Artley.** 1968. Further observations on the persistence of *Treponema pallidum* after treatment in rabbits and humans. Br. J. Vener. Dis. **44**:116–130.
40. **Zenker, P. N., and R. T. Rolfs.** 1990. Treatment of syphilis, 1989. Rev. Infect. Dis. **12**(Suppl. 6):S590–S609.