Yonsei Medical Journal
Vol. 35. No. 2, 1994

Detection of *Treponema pallidum* by Polymerase Chain Reaction in the Cerebrospinal Fluid of Syphilis Patients

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To find out if polymerase chain reaction could be used as a diagnostic tool for detecting neurosyphilis, we have applied the PCR for the detection of Treponema pallidum DNA in the cerebrospinal fluid (CSF) of syphilis patients. The results of the PCR of the CSF in 26 patients with at various stages of illness were compared with the results of other conventional tests used in the WHO criteria. T. pallidum was detected in the CSF of patients at all stages of syphilis, which indicates that they invade the central nervous system from the early stages of infection. However, the presence of T. pallidum in the CSF was not correlated with the results of other tests used in the WHO criteria, and its significance in the diagnosis of neurosyphilis should further be evaluated.

Key Words: PCR, *Treponema pallidum*, CSF

Syphilis is a chronic, sexually transmitted disease involving multiple organs including skin, and one of the most serious complications that can result from the invasion of the *Treponema pallidum* is neurosyphilis. Neurosyphilis is usually a late sequela but the actual invasion of the central nervous system (CNS) takes place in the early stages and may result in asymptomatic neurosyphilis. If left untreated, 7~9% of the asymptomatic neurosyphilis progresses to symptomatic neurosyphilis (Gjestland, 1955; Kofman, 1956). Asymptomatic neurosyphilis can not be diagnosed clinically, and several reports have suggested that the character of the symptomatic neurosyphilis has been modified from the classic parenchymatous or meningovascular form to manifest subtle and nonspecific symptoms (Hooshmand *et al.* 1972; Jordan, 1988; Simon, 1985). Therefore, in any stage of the disease, cerebrospinal fluid (CSF) must be tested for the evidence of syphilitic involvement. Due to inability to culture the *T. pallidum* in vitro, serological methods are widely used to diagnose syphilis. However, in case of neurosyphilis, it is impossible to make a diagnosis by the serological tests for syphilis alone. Protein content, mononuclear cell count, albumin quotient or IgG index of cerebrospinal fluid (CSF) are also tested, but no definite diagnostic criteria are yet established (Hooshmand *et al.* 1972; Kolar and Burkhart, 1977). Recent advan-ces in the molecular biology have opened new horizons in the research of various diseases. The polymerase chain reaction (PCR) has been developed to amplify a certain segment of DNA (Saiki *et al.* 1985). Using the PCR, few attempts have been made to amplify *T. pallidum*-specific genomic sequences in order to evaluate the usefulness of the PCR as a diagnostic tool for syphilis (Hay *et al.* 1990; Burstain *et al.* 1991; Grimprel *et al.* 1991; Noordhoek *et al.* 1991).

In our study, CSF was collected from pa-

Received March 8, 1994

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*This work was supported by the Yonsei University Faculty Research Grant for 1992.

tients with early and late stages of syphilis and 658 bp DNA fragment, which is a part of *T. pallidum*-specific gene encoding the 47 kDa surface membrane antigen, was amplified. The results were compared with those of the conventional tests to evaluate the usefulness of the PCR as a diagnostic tool to detect the CNS involvement of *T. pallidum*.

MATERIALS AND METHODS

Clinical samples

CSF was collected from 26 patients who visited the Department of Dermatology, Severance Hospital for 1 year beginning in May, 1991. The samples were taken from 2 patients with primary syphilis, 14 with secondary syphilis, and 7 with early latent syphilis and 3 with late latent syphilis. For negative controls, CSF from 5 patients with other neurologic diseases, who showed negative results in serum VDRL and TPHA, test were used.

Comparative bacterial strains

T. pallidum, Nichols strain, obtained from Centers for Desease Control (CDC; Atlanta, GA, U.S.A.) was inoculated into the rabbit testicles for serial passage and used to determine the sensitivity of the PCR and also as positive controls. In order to determine the specificity of the reaction, DNA of *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrhoeae, Neisseria meningitidis, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae* (all of the above are generous gifts from Professor Yun-Sop Chong), *Treponema phagedenis* (CDC; Atlanta, GA, U.S.A.), *T. denticola* (ATCC 33520), and *T. hyodysenteriae* (ATCC 31212, ISU Research Foundation) were extracted and amplified using the same set of primers used for the amplification of the *T. pallidum DNA*.

DNA separation from samples

Modified proteinase K method (Kawasaki, 1990) was used to extract DNA from *T. pallida* in the CSF. CSF was vortexed and 100 μl was taken to be mixed with 50 μl of lysis buffer (proteinase K 60 μg/ml, 0.05% Tween 20 in 100 mM Tris-HCl, pH 8.5). The mixture was incubated for 3 hours at 55℃, and 150 μl of 25:24:1 mixture of phenol:chloroform: isoamyl alcohol (P/C/IAA) was added. It was mixed gently and centrifuged at 12,000 g for 20 seconds. The supernatant was carefully moved into a new microtube using a large-bore micro pipette tip. P/C/IAA extraction was repeated, and the supernatant was gently mixed with equal amount of chloroform and centrifuged. The supernatant was moved to a 0.5 ml microtube and 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol chilled to −20℃ were added and mixed gently. The mixture was kept for more than one hour at −20℃ or for more the 30 minutes at −70℃. It was then centrifuged at 13,000 g for 10 minutes and the supernatant was discarded. DNA pellet was washed with cold 70% ethanol and dried. The pellet was dissolved in 20 μl of distilled water and used as the template for the PCR.

DNA extraction from other bacterial strains

One ml each of *T. phagedenis, T. vincentii, T. denticola and T. hyodysenteriae* were taken from their culture brothes and centrifuged briefly at 14,000 g. To the pellet, 100 μl of distilled water and 50 μl of lysis buffer were added and incubated for 3 hours at 55℃. DNA was extracted by the same procedure as was used for the extraction from clinical specimens. Colonies were collected from each culture media for other strains of bacteria, and the identical procedure was done to extract their DNA. Dried pellets of DNA were dissolved in distilled water.

Detection of *T. pallidum* DNA by PCR

For oligonucleotide primers, 648-669 (47-1) and 1284-1305 (47-2) nucleotides of the sense and antisense strands of the tpp47 were synthesized by a DNA synthesizer (Applied Biosystems, Foster city, CA, U.S.A.). In a 0.5 ml microcentrifuge tube, 20 μl of template DNA, 25 pmol of each primers, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 50 mM KCl, 50 μM

of each dNTPs (Pharmacia P-L Biochemials, Milwaukee, Wisconsin, U.S.A.), and 1.5 unit of *Taq* DNA polymerase (Perkin-Elmer Cetus Corporation, Eme-ryville, CA, U.S.A.) were mixed to a total volume of 50 μl and covered with mineral oil. The samples were first denatured for 5 minutes at 94℃, annealed for 1 minute at 58℃, and extended for 1 minute at 72℃ with a DNA thermal cycler (Pharmacia, Uppsala, Sweden). Subsequently, each steps were repeated for 1 minute for 39 cycles. The last extension at 72℃ was done for 5 minutes and the reaction was terminated at 4℃. To the 10 μl of the reaction product, 2 μl of the gel loading buffer was added and electrophoresed in a 1.5% agarose gel (Boehringer Mannheim, Mannheim, Germany) at 50 volts for about 2 hours. The gel was stained with ethidium bromide for 30 minutes and observed under UVB illuminator.

Determination of the PCR sensitivity

T. pallida extracted from the rabbit testicles were diluted to 10^3, 10^2, 10^1 and 10^0 organisms per 100 μl of normal human serum. Each diluted sera was treated by proteinase K lysis buffer, and processed and amplified according to the protocols for the clinical samples. DNA extracted from the *T. pallida* were also diluted to 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg. They were amplified according to the same protocol.

Determination of the PCR specificity

T. pallidum, Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes, S. pneumoniae, Neisse-ria gonorrhoeae, N. meningitidis, Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae, T. phagedenis, T. denticola and T. hyodysenteriae were extracted of their DNA and 100 pg of each DNA was used as the templates for the above protocol.

DNA restriction enzyme analysis

To verify if the synthesized DNA fragments are specific for *T. pallidum, Kpn I* (Boehringer Mannheim, Mannheim, Germany) and *Pst* I (Boehringer Mannheim, Mannheim, Germany) were used to digest the fragments. The digested products were electrophoresed on a 2% agarose gel.

Dot blot hybridization

Every reaction products of the clinical samples was verified by dot blot hybridization utilizing digoxigenin in a nonradioactive DNA labeling and detection kit (Boehringer Mannheim, Mannheim, Germany). From the *T. pallidum*, Nichols strain, 10 ng of DNA was extracted and amplified. A specific band was identified by an agarose gel electrophoresis and the DNA was eluted using a Geneclean II kit (Bio 101, La Jolla, CA, U.S.A.) to use as the probes in the hybridization. Digoxigenin-11-dUTP was incorporated into the eluted probes by random primed labeling. Reaction products of the clinical samples were applied to a nylon membrane (TM-NYS, Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) and treated in a denaturation buffer, neutralization buffer, and 20× SSC for 10, 10, and 5 minutes, respectively. UV cross linker (Hoefer Scientific Instu-ments, San Francisco, U.S.A.) was used to link the DNA to the membrane with 120 mJ/cm² of UVB. Prehybridization and hybridization were done for 1 and 6 hours, respectively, at 69℃ and washed with 2× SSC-0.1% SDS and 0.1× SSC-0.1% SDS. After washing, the membrane was reacted with antidigoxigenin-alkaline phosphatase and then with nitroblue tetrazolium and 5-bromo-4-chloro-3-inolyl phosphate for 16 hours.

Statistical evaluation

Significance of the test results was evaluated by a Fishers' exact test using the SPSS/PC + program for the IBM-PC compatible computers. Except for small sized samples, 95% confidence interval was calculated.

RESULTS

Sensitivity of PCR

T. pallidum diluted to 10 organisms/100 μl of normal human serum was positive by ethidium bromide stain after PCR, and 1 or-

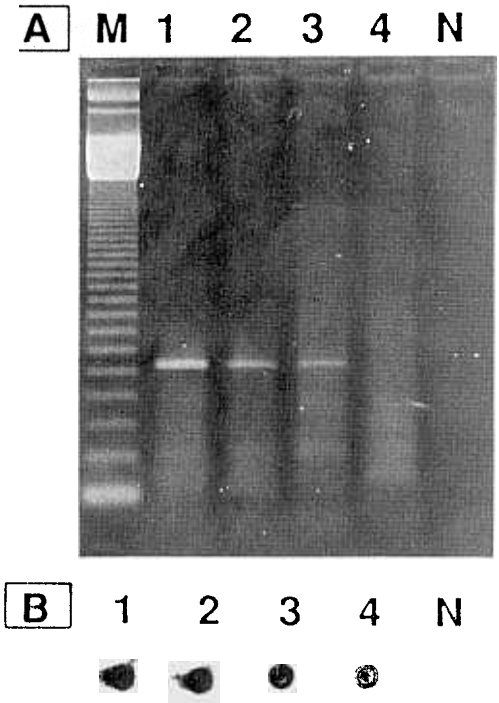

Fig. 1. Sensitivity of the PCR in relation to the number of organisms. Treponema pallida were diluted in 100 μl of normal human serum and amplified using 47-1 and 47-2 primers. (A) Ethidium bromide stain, (B) dot blot hybridization, Lane 1, 1×10^3 organisms: lane 2, 1×10^2; lane 3, 1×10^1; lane 4, 1×10^0; N, negative control; M, marker DNA (123 bp DNA ladder; 123, 246, 369, 492, 615, 738, 861, and 984 bp from bottom to top).

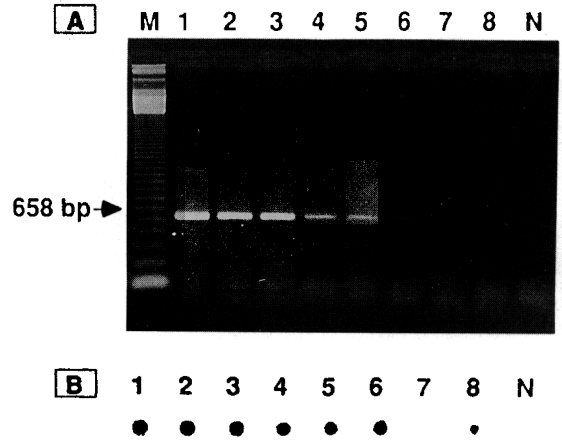

Fig. 2. Sensitivity of the PCR in relation to the concentration of treponemal DNA. (A) Ethidium bromide stain, (B) dot blot hybridization. Lane 1, 10 ng; lane 2, 1 ng; lane 3, 100 pg; lane 4, 10 pg; lane 5, 1 pg; lane 6, 100 fg. lane 7, 10 fg; lane 8, 1 fg; N, negative control; M, marker DNA (123 bp DNA ladder).

ganism/100 μl of normal human serum showed positive result by dot blot hybridization (Fig. 1). With purified *T. pallidum* DNA, 100 fg and 1 fg of DNA were detectable by ethidium bromide staining and dot blot hybridization, respectively (Fig. 2).

Specificity of PCR

All other comparative bacterial DNAs did not be amplified, but only the *T. pallidum* DNA was amplified by the PCR using the 47-1 and 47-2 primers (Fig. 3).

DNA restriction enzyme analysis

To verify whether synthesized DNA fragment is specific for *T. pallidum*, DNA restriction enzymes, *Kpn* I and *Pst* I, were used to digest the synthesized DNA fragments. By *Kpn* I digestion, 87 and 571 bp fragments and by Pst I digestion, 141 and 517 bp DNA fragments were produced (Fig. 4). Accordingly, 658 bp DNA fragment synthesized by the PCR using 47-1 and 47-2 primers is thought to be identical to the 658 bp DNA intercalated between the nucleotides 648 and 1305 of the *tpp47* gene.

Detection of *T. pallidum* DNA from the CSF

Of the 26 patients, 10 (38%) showed positive results in more than one category of Dattner's criteria (Table 1). PCR of the CSF from syphilis patients showed positive reaction in 4 of 16 (25%) primary and secondary syphilis patients, in 2 of 7 (29%) early latent syphilis patients, and in 2 of 3 (67%) late latent syphilis patients (Table 2). No significant differences could be found in the PCR and other test results between the early symptomatic and early latent stages (Table 3). Conventional

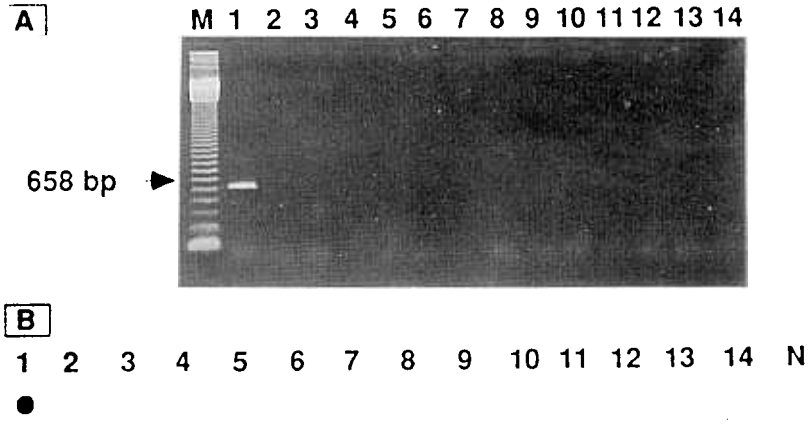

Fig. 3. *Specificity of the PCR. DNA from Treponema pallidum and various organisms were amplified using 47-1 and 47-2 primers. (A) Ethidium bromide stain, (B) dot blot hybridization. N, negative control; M, marker DNA (123 bp DNA ladder).*

1: Treponema pallidum
2: Treponema phagedenis
3: Treponema vincentii
4: Treponema denticola
5: Treponema hyodysenteriae
6: Escherichia coli
7: Enterobacter cloacae
8: Streptococcus pneumoniae
9: Streptococcus pyogenes
10: Staphylococcus aureus
11: Staphylococcus epidermidis
12: Neisseria gonorrhoeae
13: Neisseria meningitidis
14: Klebsiella pneumoniae

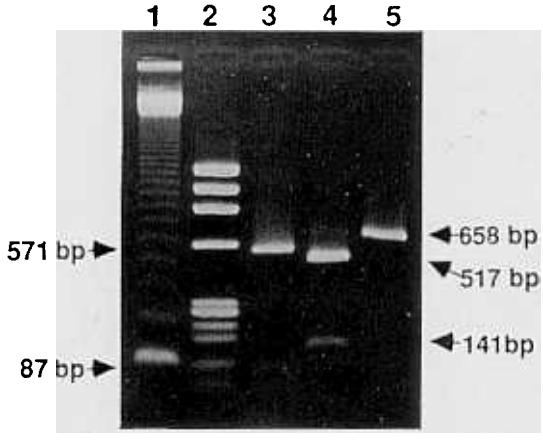

Fig. 4. *Digestion of the PCR. product by DNA restriction enzymes. Amplified 658 bp DNA fragment was digested into 87 and 571 bp fragments by Kpn I (lane 3), and into 141 and 517 bp fragments by Pst I (lane 4). Lane 1, marker DNA (123 bp DNA ladder); Lane 5, undigested 658 bp DNA; Lane 2, ϕX 147 DNA digested by Hae III (1353, 1078, 872, 603, 310,281/271, 234, 194, 118 and 72 bp DNA fragments from top to bottom).*

Table 1. Syphilis patients with more than one positive CSF test according to WHO criteria

Clinical stage	No. of cases	No. positive	% positive
Syphilis patients	26	10	38[19~57]
Primary & secondary	16	5	31[13~49]
Early latent	7	3	43[24~62]
Late latent	3	2	67[14~100]

]: 95% confidence interval (%)

Table 2. Rate of the syphilis patients with PCR positive results in their CSF

Clinical stage	No. of cases	No. positive	% positive
Syphilis patients	26	8	31[13~49]
Primary & secondary	16	4	25[4~46]
Early latent	7	2	29[0~62]
Late latent	3	2	67[14~100]
Negative control	5	0	0

Table 3. Incidences of abnormal CSF findings in patients with syphilis at various stages

Test results	Primary & secondary (n=16)	Early latent (n=7)	Late latent (n=3)	p value*
PCR positive	4(25) [4~46]	2(29) [0~62]	2(67) [14~100]	1.0000
VDRL positive	0	2(29) [0~62]	0	0.0830
WBC>5/mm^3	4(25) [4~46]	1(14) [0~39]	0	1.0000
Protein>40mg%	4(25) [4~46]	2(29) [0~62]	2(67) [14~100]	1.0000

*Between Primary & secondary and Early latent
( ): %, [ ]: 95%confidence interval(%)

Table 4. Comparison of the incidences of abnormal CSF findings between PCR positive and negative gruops

Test results	PCR positive (n=8)	PCR negative (n=18)	p value
VDRL positive	1(13) [0~36]	1(6) [0~17]	0.5290
WBC>5/mm^3	1(13) [0~36]	4(22) [3~41]	1.0000
Protein>40mg%	4(50) [15~85]	4(22) [3~41]	0.1970

( ): %, [ ]: 95% confidence interval (%)

test results were compared to the results of the PCR but the presence of *T. pallidum* in CSF had no significant influence on the other test results (Table 4).

DISCUSSION

T. pallidum is known to invade the CNS from the early stages of infection. Pleocytosis or increased protein concentration of the CSF are observed in 9% of primary syphilis patients and in 30~70% of secondary syphilis patients (Wolters *et al.* 1988). Abnormal CSF test results without neurologic signs are called asymptomatic neurosyphilis and it is known that, if untreated, 7~9% of the cases progress to symptomatic neurosyphilis (Gjestland, 1955; Kofman, 1956). However, even the diagnosis of symptomatic neurosyphilis may be problematic as the symptoms of neurosyphilis tend to be rather atypical these days, presenting as seizure, stroke, dizziness, personality change, or cervical spondylosis. Therefore, the diagnosis of neurosyphilis must be confirmed by the examination of the CSF but no definite diagnostic criteria is yet established. WHO (1982) has adopted the criteria of Dattner *et al.* (1951), i. e., positive CSF VDRL test, pleocytosis, and increased protein concentration, for the diagnosis of neurosyphilis but the test results can be normal even when the neurosyphilis is symptomatic (Hooshmand *et al.* 1972; Kolar and Burkhart, 1977; Lee *et al.* 1983). PCR has recently been applied for the detection of *T. pallidum* in syphilis patients due to its remarkable sensitivity and specificity. Hay *et al.* (1990) have identified the organism in CSF of late latent and tertiary syphilis patients and Grimprel *et al.* (1991) have had positive results from the amniotic fluid, fetal blood and CSF. Noordhoek *et al.* (1991) have also compared the PCR results in CSF before and after treatment. To establish a more effective method of detecting neurosyphilis, we have applied the PCR for the detection of *T. pallidum* DNA in the CSF of syphilis patients in various stages of illness. The results of the PCR was compared with the results of other conventional tests used in WHO criteria and

the significance of the presence of *T. pallidum* DNA in CSF was evaluated.

The sensitivity of the PCR using 47-1 and 47-2 primers have been evaluated by diluting the *T. pallidum* itself and its purified DNA. *T. pallida* extracted from the rabbit testicles and their purified DNA were serially diluted. After amplification and dot blot hybridization, *T. pallida* and their DNA dilutions both showed positive reactions in dilutions equivalent to 1 organism. DNA from four organisms belonging to genus *Treponema* and nine other bacterial species were extracted and amplified using the 47-1 and 47-2 primers which resulted in a positive reaction only with the *T. pallidum* DNA. Thus, the PCR using the above primers was considered sensitive and specific enough to be used for further study.

Overall 38% of the patients showed positive results in CSF in at least one of the WHO criteria. PCR was positive in 25% in early symptomatic syphilis, 29% in early latent syphilis, and 67% in late latent syphilis, showing an overall positivity of 31% in CSF. The above findings indicate that *T. pallidum* does invade the CNS from the early stages of infection. The results of the PCR and other tests were compared to see if they show any significant difference according to the stage of the disease. Number of patients in the late stage of the disease was too small but the results did not show any statistical significance between the early symptomatic and early latent stages. There was one patient in the early latent stage who showed positive results in the PCR as well as all other tests. Only one patient each in the three stages showed positive results in PCR and one of the other tests. The number of the samples was too small to draw any statistical significance but the presence of *T. pallidum* in CSF does not seem to have any correlation with the results of Dattner's criteria. Four patients who were positive in PCR but negative in all other tests should be further followed to discriminate between mere invasion without actual neurosyphilis and the initial stage of involvement later progressing to actual neurosyphilis. The overall positivity of the PCR, 25 %, in the primary and secondary syphilis patients in our study coincides with the result of Lukehart *et al.* (1988) obtained by the rabbit infectivity test. To find out whether the presence of *T. pallidum* results in any laboratory abnormalities, the results of the tests in Dattner's criteria were compared according to the result of the PCR. It turned out that the presence of *T. pallidum* does not induce any consistent laboratory abnormalities in the CSF.

We have utilized a PCR using primers that amplify 658 bp DNA fragments corresponding to the *T. pallidum*-specific 47 kDa surface antigen *T. pallidum* was detected in the CSF of patients with all stages of syphilis which indicate that they invade the CNS from the early stages of infection but they did not show any significant relevance to the WHO diagnostic criteria. Further study is necessary to evaluate the significance of its presence in the diagnosis of neurosyphilis.

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