Nested polymerase chain reaction for assessing the clinical course of tuberculous meningitis

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Neurology 2005;64;1789-1793
DOI 10.1212/01.WNL.0000162052.13838.B2

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Abstract—The authors examined the usefulness of nested PCR (N-PCR) to detect *Mycobacterium tuberculosis* (MTB) DNA in CSF for assessing the clinical course of tuberculous meningitis (TBM). N-PCR successfully detected MTB DNA in all nine CSF samples from patients with suspected TBM. During anti-tuberculosis treatments, N-PCR results converted from positive to negative, correlating with the improvement of the patient's clinical condition.

NEUROLOGY 2005;64:1789–1793

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In the diagnosis of tuberculous meningitis (TBM), PCR has been widely used to detect *Mycobacterium tuberculosis* (MTB) DNA in CSF samples as a more rapid, sensitive, and specific diagnostic method as compared to the conventional bacteriologic detection such as direct smear for acid-fast bacilli (AFB) and MTB cultures.[1-5] Recently, nested (N) PCR technique has added drastically increasing sensitivity and specificity of DNA amplification to the conventional single step (S) PCR.[6-9] However, N-PCR analysis of CSF samples has yet to be widely used in TBM diagnosis owing to its laborious and time-consuming procedures.[6-9] In addition, there are few reports that have determined its accurate minimum detection sensitivity and application with regard to the assessment of the clinical course of TBM. In this study, we examined the usefulness of N-PCR for the diagnosis of TBM and for assessing the clinical course of TBM.

Methods. Nine serial patients with suspected TBM and 20 non-TBM control patients were selected among patients who were admitted to our hospital between July 2000 and April 2004. All nine suspected TBM patients met the established clinical criteria and supporting evidence for TBM[1,6,9,10] (table 1), and were classified as two confirmed cases (positive CSF culture or AFB smear) and seven highly probable cases (meeting all the clinical criteria and with all three supporting evidences positive, but having no bacterial isolation).[6,9] A total of 36 CSF samples from these nine patients were collected. Of 36 samples, 27 were available from the seven highly probable patients who had follow-ups of more than at least 2 weeks. The non-TBM control group consisted of four cases of bacterial meningitis, three cryptococcal meningitis, three viral meningitis, six multiple sclerosis, and one each CNS lupus, CNS malignant lymphoma, hepatic insufficiency, and neuro Behçet disease. The diagnoses for the non-TBM control cases were based on their specific clinical and laboratory findings.

For DNA extraction and purification, the phenol-chloroform method and ethanol precipitation were used. For the two subsequent amplification steps of N-PCR, two pairs of oligonucleotide primers capable of specifically amplifying the MPB64 gene[5,6] of MTB were prepared. The outer primers used in the first step PCR (S-PCR) were F-1: 5'-ATCCGCTGCCAGTCGTCTTCC-3' and R-1: 5'-CTCGCGAGTCTAGGCCAGCAT-3'. The inner primers used in the second step PCR (N-PCR) were F-2: 5'-CATTGTGCAAGGTGAACTGAGC-3' and R-2: 5'-AGCATCGAGTCGATCGCGGAA-3'. As the template, 2 μL of the extracted DNA specimen at the first step or 2 μL of the S-PCR product at the second step was added to the PCR reaction-solution mixture (total reaction volume 20 μL). These preparations were subjected to the previous reported protocol.[6] The S- and N-PCR products were analyzed by an automatic electrophoresis system. The presence of a 239 base-pair (bp) band for S-PCR and a 194 bp band for N-PCR indicated successful amplification.

For PCR amplification markers, we prepared original plasmids that were inserted a DNA fragment (239 bp) of the MPB64 gene. The nucleotide sequence of the inserted DNA fragment was confirmed by direct sequencing in both strands. Concentrations of the plasmid DNA were measured by a UV spectrophotometer at least three times, and then eight serial preparations of 10-fold dilutions were prepared (1 to 10^7 copies/2 μL). To examine the minimum detection sensitivity of N-PCR, we constructed imitative CSF samples containing each of these plasmid preparations (1 to 10^7 copies). Using the same procedure carried out for the actual CSF analysis, we then performed extraction and purification of the DNA from these imitative samples.

Results. Table 1 summarizes the clinical features upon admission (before the anti-tuberculosis treatment [ATT]) and the outcomes in the nine suspected TBM patients. Conventional S-PCR only revealed positive results in 2 (22.2%) out of the nine patients and these cases were the confirmed (culture positive) cases (Cases 1 and 2) (figure, A). N-PCR results were positive in all 9 patients (100%) (figure, B). The 20 patients in the non-TBM control group had all negative N-PCR results (data not shown).

The results of the minimum detection sensitivity study for the S- and N-PCR are shown in the figure. When using the same experimental conditions as that for the actual CSF samples, the detection limit for the conventional S-PCR was only to 10^4 copies/2 μL extracted plasmid from imitative CSF sample (figure, C). In contrast, the limit for the N-PCR was 1 to 10 copies/2 μL extracted plasmid (figure, D).

Table 2 summarizes the results of the diachronic study, which includes cultures for MTB, the S- and N-PCRs, and

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Supported by a grant from the Ministry of Education, Science and Culture of Japan (High-Tech Research Center, Nihon University).

Received November 4, 2004. Accepted in final form February 2, 2005.

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Table 1 *The summary of clinical features, CSF findings, PCR results, and outcome in nine patients with suspected TBM*

	Confirmed cases		Highly probable cases	
	Case 1	Case 2	Case 3	Case 4
Age/sex	73/M	76/M	35/F	65/F
Medical history (background disease)	Diabetes mellitus, renal cancer, hypertension	CML, atrial fibrillation, hypertension	SLE	Diabetes mellitus
Administration of corticosteroid, mg/d	—	—	PSL:20	—
Clinical stage upon admission*	III	III	II	I
CSF findings upon admission (before treatment)				
Cells/μL (M:P)	288 (75:141)	165 (462:34)	208 (170:455)	107 (161:0)
Protein, mg/dL	299	569	300	70
Glucose, mg/dL	13	46	13	48
Cl, mEq/L	96	97	94	122
ADA, IU/L	23.4	12.3	16.3	7.8
AFB smear	−	−	−	−
Tb culture	+	+	−	−
Single PCR (figure 1A)	+	+	−	−
Nested PCR (figure 1B)	+	+	+	+
Cranial MRI findings				
Meningeal enhancement	+	+	+	−
Hydrocephalus	+	−	+	−
Cerebrovascular disorder	+	+	+	−
Intracranial focal mass	+	−	+	−
MTB outside CNS				
Pulmonary (sputum or Ball)	+	+	−	−
Gastric aspirate	+	−	−	−
Urine	−	−	−	−
Peripheral blood	−	−	−	−
Treatment				
INH, mg/d	500	600	800	450
Intrathecal administration, mg	−	−	100 (3 times/wk)	−
RFP, mg/d	600	600	600	450
PZA, g/d	1.5	1.5	1.5	1.5
EB, mg/d	750	−	750	−
SM, g/d	−	1	1	1
Anticonvulsant	Phenytoin	−	−	−
V-P shunt	+	−	+	−
Complication	SIADH/hyponatremia, acute renal failure, DIC/ARDS	Drug-induced hepatopathy, drug-induced paracusis	SIADH/hyponatremia, left facial palsy	−
Outcome†	Death	Recovery	Recovery	Recovery
Clinical criteria (A) and supporting evidence (B)	All A and three B (1,2,3)	All A and all B	All A and three B (1,2,4)	All A and three B (1,2,4)

A = The clinical criteria suggestive for TBM are fever, headache, and neck stiffness of more than 1 week duration.

B = Supporting evidence for TBM include 1) compatible abnormal CSF findings that included increased white cell counts with lymphocyte predominating, hypoglycorrhachia, protein concentration > 100 mg/dL, adenosine deaminase (ADA ≧ 10 IU/L and negative results for routine bacterial and fungal cultures; 2) MRI findings suggesting tuberculous involvement of the CNS (basal exudates, hydrocephalus, and intracranial focal mass, etc.); 3) presence of tuberculosis in the body outside of the CNS or a history of tuberculosis; and 4) clinical response to anti-tuberculosis therapy.

The suspected TBM cases were classified as "confirmed" cases (having the bacterial isolation for MTB such as CSF culture or AFB smear positive) or "highly probable" cases (meeting all the above clinical criteria and with all three supporting evidence positive).

* According to the clinical stages defined by the British Medical Research Council stage I, no definite neurologic symptoms; II, signs of meningeal irritation with slight clouding of consciousness and neurologic defects; III, severe clouding of consciousness and neurologic defects.

† Outcome classified as recovery with minor or no neurologic impairment, severe neurologic impairment, and death.

TBM = tuberculous meningitis; CML = chronic myelocytic leukemia; SLE = systemic lupus erythematosus; APS = antiphospholipid syndrome; RPGN = rapidly progressive glomerulonephritis; CRF = chronic renal failure; MS = multiple sclerosis; ATL = adult T-cell leukemia; M = mononuclear cell; P = polymorphonuclear cell; ADA = adenosine deaminase; AFB = acid-fast bacilli; Tb = tuberculosis; MTB = *Mycobacterium tuberculosis*; INH = isoniazid; RFP = rifampicin; PZA = pyrazinamide; EB = ethambutol; SM = streptomycin sulfate; DX = dexamethasone; PSL = prednisolone; V-P shunt = ventricle-peritoneal shunt; SIADH = syndrome of inappropriate secretion of antidiuretic hormone; DIC = disseminated intravascular coagulation; ARDS = adult respiratory distress syndrome.

***Table 1** (Continued)*

Highly probable cases				
Case 5	Case 6	Case 7	Case 8	Case 9
52/M	24/F	44/F	59/F	44/M
Alcoholism, alcoholic cirrhosis, diabetes mellitus	SLE, lupus nephritis APS	RPGN, CRF (hemodialysis)	MS, hyperlipidemia	ATL
—	PSL:20	PSL:20	PSL:30	—
II	I	III	II	III
18 (27:26)	30 (69:21)	60 (41:138)	40 (121:0)	117 (345:7)
135	25	70	359	87
54	30	52	78	48
96	118	116	125	130
8.6	4.4	N.D.	3.7	3.9
−	−	−	−	−
−	−	−	−	−
−	−	−	−	−
+	+	+	+	+
+	−	−	−	+
+	−	−	+	−
+	−	+	−	−
+	+	−	−	−
+	−	−	−	+
+	−	−	−	+
−	−	−	−	−
−	−	−	−	−
500	500	200	600	500
−	−	−	−	−
450	450	225	450	450
1	2	1.5	2	2
−	−	−	−	−
1	1	−	1	1
Phenytoin	−	Valproate sodium	−	Phenytoin, phenobarbital
−	−	−	+	−
Symptomatic epilepsy	−	Symptomatic epilepsy, acute pancreatitis	−	Leukemic meningitis (intrathecal administration of carcinostatics)
Recovery	Recovery	Recovery	Recovery	Death
All A and all B	All A and three B (1,2,4)	All A and three B (1,2,4)	All A and three B (1,2,4)	All A and three B (1,2,3)

other CSF findings that were collected from the seven highly probable patients (Cases 3 through 9) during the clinical treatment course. There were all negative results for the conventional cultures and S-PCRs in the 27 serial CSF samples. In contrast, N-PCR revealed significant alterations during the clinical course for all seven highly probable patients, with positive results observed in 11 out of the 27 serial CSF samples (40.7%). Among these, the 194 bp bands of the N-PCR converted from positive to negative during various periods throughout ATT in the six patients (Cases 3 through 8) who demonstrated improvement in both their clinical conditions and routine CSF findings. In particular, the intensity of the 194 bp bands gradually decreased in Cases 7 and 8, whereas in Case 9, in which the patient died due to aggravation of the malignant background disease (adult T-cell leukemia), the 194 bp band remained positive throughout the clinical course. (An autopsy was not done in this patient, since permission could not be obtained from the family.)

Discussion. Although several investigators have discussed the minimum detection sensitivity of N-PCR for MTB DNA,[6-9] there have been no studies that have reported actual copy numbers for the purified MTB DNA. Using the original plasmid preparations, we established the minimum detection sensitivity of N-PCR to be between 1 and 10 copies/2 μL, which was 1,000 to 10,000 times higher than that for S-PCR.

At present, although the gold standard in diagnosis of TBM is bacterial isolation, that still has sev-

Table 2 *The results of single PCR, nested PCR, culture, and other CSF findings during the clinical course in the seven highly probable cases (cases 3 to 9)*

	Weeks									Months			
	0 (upon admission)	1	2	3	4	5	6	7	8	3	4	5	
Patient Serial CSF findings	Before treatment	During treatment											Outcome
Case 3													
Cells/μL	208	348	130	342	330	474	272	130	59	20	9	19	Recovery
Protein, mg/dL	300	660	890	830	570	670	390	249	144	105	125	460	
Glucose, mg/dL	13	20	70	135	140	91	76	86	104	99	78	57	
Tuberculosis culture	–	–	–	–	–	–	–	–	–	–	–	–	
Single PCR	○	○			○		○		○	○	○	○	
Nested PCR	●	●			●		●		○	○	○	○	
Intrathecal administration of INH						←			→				
Case 4													
Cells/μL	107	263	41	14	7								Recovery
Protein, mg/dL	70	148	31	30	36								
Glucose, mg/dL	48	45	40	42	51								
Tuberculosis culture	–		–		–								
Single PCR	○		○										
Nested PCR	●		○										
Case 5													
Cells/μL	18	23	25		19			7		6	10	6	Recovery
Protein, mg/dL	135	62	46		69			42		33	41	42	
Glucose, mg/dL	54	71	78		75			77		71	80	101	
Tuberculosis culture	–	–	–		–			–		–	–	–	
Single PCR	○	○	○		○					○	○	○	
Nested PCR	●	●	○		○					○	*	○	
Case 6													
Cells/μL	30	7	9		11		2				4		Recovery
Protein, mg/dL	25	16	23		29		30				28		
Glucose, mg/dL	30	38	40		32		34				40		
Tuberculosis culture	–		–		–		–				–		
Single PCR	○		○		○		○				○		
Nested PCR	●		○		○		●				○		
Case 7													
Cells/μL	60	3	4		3		19		9				Recovery
Protein, mg/dL	70	68	46		52		62		74				
Glucose, mg/dL	52	76	44		42		63		43				
Tuberculosis culture	–		–				–		–				
Single PCR	○		○				○		○				
Nested PCR	●		*				*		○				
Case 8													
Cells/μL	40	31	24	28	8	15	22	17	7	8	2	1	Recovery
Protein, mg/dL	359	304	293	434	341	297	288	580	673	303	348	472	
Glucose, mg/dL	78	63	67	61	60	57	55	50	55	76	84	77	
Tuberculosis culture	–		–		–			–	–		–		
Single PCR	○		○		○			○			○		
Nested PCR	●		*		○			○			○		
Case 9													
Cells/μL	117	95	34	2	1	1	17						Death
Protein, mg/dL	87	87	104	78	55	47	106						
Glucose, mg/dL	48	49	65	55	47	59	51						
Tuberculosis culture	–		–			–							
Single PCR	○		○			○							
Nested PCR	●		●			●							

● = Clearly positive; * = slightly positive; ○ = negative; INH = isoniazid.

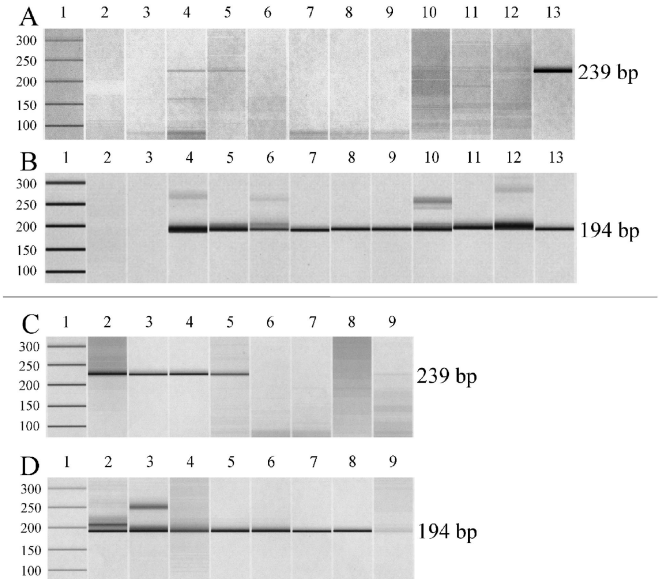

Figure. The results of single (S) and nested (N) PCR. Analysis performed by the Agilent 2100 bioanalyzer system (Agilent Technologies, Waldbronn, Germany). (A) The results of S-PCR in Cases 1 through 9 before treatment (i.e., upon admission). Lane 1: DNA molecular weight marker; lanes 2, 3: negative controls; lane 4: Case 1; Lane 5: Case 2; lane 6: Case 3; lane 7: Case 4; lane 8: Case 5; lane 9: Case 6; Lane 10: Case 7; lane 11: Case 8; lane 12: Case 9; lane 13: positive control. Positive results for the S-PCR analysis were only found in Cases 1 and 2, who were also the confirmed cases with positive cultures for Mycobacterium tuberculosis*. The 239 base-pair (bp) bands are slightly observed in lanes 4 and 5. (B) The results of N-PCR in Cases 1 through 9 before treatment (i.e., upon admission). Lanes 1 through 13 are the same as described in (A). The N-PCR exhibited positive results in all cases (Cases 1 through 9). The 194 bp bands are clearly observed in lanes 4 through 12. (C, D) The results of the minimum detection sensitivity study using extracted plasmid preparations for S-PCR (C) and for N-PCR (D). Lane 1: DNA molecular weight marker; lane 2: 10^7 copies/2 μL plasmid; lane 3: 10^6 copies/2 μL plasmid; lane 4: 10^5 copies/2 μL plasmid; lane 5: 10^4 copies/2 μL plasmid; lane 6: 10^3 copies/2 μL plasmid; lane 7: 10^2 copies/2 μL plasmid; lane 8: 10 copies/2 μL plasmid; lane 9: 1 copy/2 μL plasmid. The detection limit for conventional S-PCR was to 10^4 copies/2 μL extracted plasmid (C). The detection limit for N-PCR was 1 to 10 copies/2 μL extracted plasmid (D).*

eral complex issues. In the present study, both conventional cultures and S-PCRs were positive in only 2 out of 9 patients (22.2%). These results suggest that when the amount of MTB in the CSF from suspected TBM patients is low, it may be difficult to demonstrate MTB presence by using conventional methods. In the present diachronic study, only N-PCR results revealed significant alterations during the clinical course for all seven highly probable patients. To our knowledge, there has only been one other previous diachronic study in which serial N-PCR was performed to examine CSF samples collected during the clinical treatment course from 12 patients with AIDS with suspected TBM.[9] We speculated that the diachronic alterations of the N-PCR results might reflect the clinical ATT response more sensitively than conventional methods. However, the assessment of the clinical course of TBM is difficult, since TBM has many complications, such as hydrocephalus and vasculopathy, which are really not dependent upon the presence of bacterial protein or DNA in the CNS but rather on the immunologic reaction of the host. Consequently, N-PCR is a much more sensitive, specific, and useful method as compared to other conventional methods such as MTB cultures and S-PCR. To establish the usefulness and superiority of N-PCR, both for TBM diagnosis and in the assessment of the clinical course of TBM against other conventional methods, it will be necessary to accumulate and evaluate a larger number of patients with suspected TBM.

Acknowledgment

The authors thank N. Sato, M. Shimamura, and K. Sugama for technical assistance. They also thank their colleagues within the Division of Neurology, Nihon University School of Medicine, for collecting samples and for providing data.

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