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Novel Wide-Range Quantitative Nested Real-Time PCR Assay for *Mycobacterium tuberculosis* DNA: Clinical Application for Diagnosis of Tuberculous Meningitis▿

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Although the "gold standard" for diagnosis of tuberculous meningitis (TBM) is bacterial isolation of *Mycobacterium tuberculosis*, there are still several complex issues. Recently, we developed an internally controlled novel wide-range quantitative nested real-time PCR (WR-QNRT-PCR) assay for *M. tuberculosis* DNA in order to rapidly diagnose TBM. For use as an internal control calibrator to measure the copy number of *M. tuberculosis* DNA, an original new-mutation plasmid (NM-plasmid) was developed. Due to the development of the NM-plasmid, the WR-QNRT-PCR assay demonstrated statistically significant accuracy over a wide detection range (1 to 10^5 copies). In clinical applications, the WR-QNRT-PCR assay revealed sufficiently high sensitivity (95.8%) and specificity (100%) for 24 clinically suspected TBM patients. In conditional logistic regression analysis, a copy number of *M. tuberculosis* DNA (per 1 ml of cerebrospinal fluid) of >8,000 was an independent risk factor for poor prognosis for TBM (i.e., death) (odds ratio, 16.142; 95% confidence interval, 1.191 to 218.79; *P* value, 0.0365). In addition, the copy numbers demonstrated by analysis of variance statistically significant alterations ($P < 0.01$) during the clinical treatment course for 10 suspected TBM patients. In simple regression analysis, the significant correlation ($R^2 = 0.597$; $P < 0.0001$) was demonstrated between copy number and clinical stage of TBM. We consider the WR-QNRT-PCR assay to be a useful and advanced assay technique for assessing the clinical treatment course of TBM.

Tuberculous meningitis (TBM) is the severest form of infection of *Mycobacterium tuberculosis*, causing death or severe neurological defects in more than half of those affected in spite of antituberculosis treatment (ATT) (1, 2, 8, 18). The diagnosis of TBM remains a complex issue, because the most widely used conventional bacteriological detection methods, such as direct smear for acid-fast bacilli (AFB) and culture for *M. tuberculosis*, are unable to rapidly detect *M. tuberculosis* with sufficient sensitivity in the acute phase of TBM (3–13, 18, 19). In 2006, we designed a novel internally controlled quantitative nested real-time PCR (QNRT-PCR) assay based on TaqMan PCR (Applied Biosystems) (15). Moreover, based on this original QNRT-PCR (OR-QNRT-PCR) assay, an improved wide-range QNRT-PCR (WR-QNRT-PCR) assay was developed (17). For use as a "calibrator" in WR-QNRT-PCR assay, a new internal control was constructed (17). In the preliminary experiments, the WR-QNRT-PCR assay demonstrated significantly improved quantitative accuracy and had a wide detection range (1 to 10^5 copies) compared to what was seen for the OR-QNRT-PCR assay (17).

In this study, we tried to quantitatively detect *M. tuberculosis* DNA in actual cerebrospinal fluid (CSF) samples by using the WR-QNRT-PCR assay. In addition, the clinical usefulness of this novel assay technique for the rapid and accurate diagnosis of TBM and for assessing the clinical course of TBM was examined.

MATERIALS AND METHODS

This study was approved by the Nihon University Institutional Review Board.

Clinical specimens and control. Clinical specimens from 24 patients with clinically suspected TBM and 29 non-TBM control patients were collected between 1998 and 2005. A total of 67 CSF samples were collected from these 24 patients. Of 67 CSF samples, 43 were available serially from the 10 patients (cases 3 and 8 to 16) who had follow-ups of more than at least 2 weeks. In addition, the extracted DNA specimen from *M. tuberculosis* standard strain H37Rv (ATCC 25618) was used as the positive control in this study.

The 29 non-TBM control patients consisted of 4 cases of bacterial meningitis, 3 of cryptococcal meningitis, 12 of viral meningitis, 6 of multiple sclerosis, and 1 each of central nervous system (CNS) lupus, CNS malignant lymphoma, hepatic insufficiency, and neuro-Behçet's disease. The diagnoses for the non-TBM control cases were based on their specific clinical and laboratory findings. Moreover, to determine the analytical specificity and cross-reactivity of our assays, extracted DNA specimens from six additional reference strains of non-*M. tuberculosis* species—*M. bovis* BCG (ATCC 19274), *M. avium* (ATCC 15769), *M. intracellulare* (ATCC 15985), and clin-

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TABLE 1. Sequences of primers and TaqMan probes for PCR assays

Objective	PCR product size (bp)	Target	Type	Sequence[a]
WR-QNRT-PCR assay				
First-step PCR	239	Wild *M. tuberculosis* DNA (MPT64) or W-plasmid	WF1: outer wild forward primer	5′-ATCCGCTGCCAGTCGTCTTCC-3′; total of 21 nucleotides, A:2, T:6, G:4, C:9 (G+C, 62%)
			WR1: outer wild reverse primer	5′-CTCGCGAGTCTAGGCCAGCAT-3′; total of 21 nucleotides, A:4, T:4, G:6, C:7 (G+C, 62%)
		New internal control (NM-plasmid)	MF1: outer mutation forward primer	5′-TCGATTCTGTCCCACCGCCGT-3′; total of 21 nucleotides, A:2, T:6, G:4, C:9 (G+C, 62%)
			MR1: outer mutation reverse primer	5′-AGACTCGACGCGTAGTCCTCG-3′; total of 21 nucleotides, A:4, T:4, G:6, C:7 (G+C, 62%)
Second-step PCR	77	Wild *M. tuberculosis* DNA (MPT64) or W-plasmid	TqMn-WF2: TaqMan inner wild forward primer	5′-GTGAACTGAGCAAGCAGACCG-3′; total of 21 nucleotides, A:7, T:2, G:7, C:5 (G+C, 57%)
			TqMn-WR2: TaqMan inner wild reverse primer	5′-GTTCTGATAATTCACCGGGTCC-3′; total of 22 nucleotides, A:4, T:7, G:5, C:6 (G+C, 50%)
		New internal control (NM-plasmid)	TqMn-MF2: TaqMan inner mutation forward primer	5′-AGATCGGATAGCCAGCACGGA-3′; total of 21 nucleotides, A:7, T:2, G:7, C:5 (G+C, 57%)
			TqMn-MR2: TaqMan inner mutation reverse primer	5′-TGCGCTGCGTCGACATATTCTA-3′; total of 22 nucleotides, A:4, T:7, G:5, C:6 (G+C, 50%)
		Wild *M. tuberculosis* DNA (MPT64) or W-plasmid	TqMn-W-VIC: TaqMan probe-wild-VIC	5′-VIC-TATCGATAGCGCCGAATGCC GG-TAMRA-3′; total of 22 nucleotides, A:5, T:4, G:7, C:6 (G+C, 59%)
		New internal control (NM-plasmid)	TqMn-M-FAM: TaqMan probe-mutation-FAM	5′-FAM-ATGGGACGGCTAGCAATCC GTC-TAMRA-3′; total of 22 nucleotides, A:5, T:4, G:7, C:6 (G+C, 59%)
OR-QNRT-PCR assay				
First-step PCR	239	Wild *M. tuberculosis* DNA (MPT64) and old internal control (M-plasmid)	WF1	5′-ATCCGCTGCCAGTCGTCTTCC-3′
			WR1	5′-CTCGCGAGTCTAGGCCAGCAT-3′
Second-step PCR	77	Wild *M. Tb* DNA (MPT64) and old internal control (M-plasmid)	TqMn-WF2	5′-GTGAACTGAGCAAGCAGACCG-3′
			TqMn-WR2	5′-GTTCTGATAATTCACCGGGTCC-3′
		Wild *M. tuberculosis* DNA (MPT64)	TqMn-W-VIC	5′-VIC-TATCGATAGCGCCGAATGCC GG-TAMRA-3′
		Old internal control (M-plasmid)	TqMn-M-FAM	5′-FAM-ATGGGACGGCTAGCAATCC GTC-TAMRA-3′
Conventional single and nested PCR assays				
First-step PCR	239	Wild *M. tuberculosis* DNA (MPT64)	WF1	5′-ATCCGCTGCCAGTCGTCTTCC-3′
			WR1	5′-CTCGCGAGTCTAGGCCAGCAT-3′
Second-step PCR	194	Wild *M. tuberculosis* DNA (MPT64)	WF2: inner forward primer	5′-CATTGTGCAAGGTGAACTGAGC-3′
			WR2: inner reverse primer	5′-AGCATCGAGTCGATCGCGGAA-3′
Internal control	196	Human β-globin gene	HBB-F: human β-globin forward primer	5′-GGCAGACTTCTCCTCAGGAGTC-3′
			HBB-R: human β-globin reverse primer	5′-CTTAGACCTCACCCTGTGGAGC-3′

[a] Underlining indicates artificial sequence; double underlining indicates restriction site. FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

ically isolated *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* strains—were tested.

Smear and culture. All CSF samples from patients with suspected TBM were examined microscopically for AFB and by culture for *M. tuberculosis*. First, CSF aliquots of 1 to 2 ml were concentrated by centrifugation (3,000 × *g* for 10 min) for conventional bacteriology. The sediments were used to prepare smears for direct examination of AFB by auramine-rhodamine and Ziehl-Neelsen stain and cultured by inoculation on a Bactec MGIT 960 system (Becton Dickinson Diag-

TABLE 2. Summary of basal clinical features of 24 patients with suspected TBM[a]

Case type[b] and patient no.	Age (yr)/sex[c]	Clinical stage[d]	Basal CSF findings (before treatment)						Single-PCR assay	Nested-PCR assay	OR-QNRT-PCR assay (copies/ml CSF)	WR-QNRT-PCR assay (copies/ml CSF)	Cranial MRI findings[f]	*M. tuberculosis* outside CNS[g]	Period up to initial sample collection	ATT response	Outcome after ATT
			Cells (per μl)	Protein (mg/dl)	Glucose (mg/dl)	ADA[e] (IU/l)	AFB smear	*M. tuberculosis* culture									
Confirmed cases																	
1	73/M	III	288	299	13	23.4	−	+	+	+	1.0×10^5	28721	ME, HC, CVD, IFM	Sp, GA	About 3 wk	Noneffective	Death
2	76/M	III	165	569	46	12.3	−	+	+	+	6.4×10^4	10028	ME, CVD	Sp	2 Days	Effective	Recovery
3	28/M	III	605	434	25	16.3	−	+	−	+	8.8×10^4	22571	ME, HC, CVD, IFM	Sp	About 1 mo	Noneffective	Death
4	38/M	II	76	637	18	6.5	−	+	−	+	3.0×10^4	7161	HC, IFM	Sp, GA	1 Day	Effective	Recovery
5	53/F	III	344	354	38	10.3	−	+	+	+	6.0×10^4	4547	IFM	−	1 Day	Effective	Recovery
6	72/F	III	247	329	57	18.4	−	+	−	+	3.3×10^3	6340	HC, IFM	−	7 Days	Noneffective	Death
7	34/M	II	612	320	18	20.2	−	+	+	+	3.8×10^3	1243	−	−	Not available	Effective	Recovery
8	42/M	II	418	456	36	22.6	−	+	+	+	1.8×10^4	10532	ME, IFM	Sp	About 2 wk	Effective	Recovery
Highly probable cases																	
9	35/F	II	208	300	13	16.3	−	−	−	+	3.6×10^3	7892	ME, HC, CVD, IFM	−	7 Days	Effective	Recovery
10	65/F	I	107	70	48	7.8	−	−	−	+	5.6×10^3	1904	−	−	3 Days	Effective	Recovery
11	52/M	II	18	135	54	8.6	−	−	−	+	2.7×10^3	5858	ME, HC, CVD, IFM	Sp, GA	1 Day	Effective	Recovery
12	24/F	I	30	25	30	4.4	−	−	−	+	1.9×10^4	5436	IFM	−	1 Day	Effective	Recovery
13	44/F	III	60	70	52	N.D.	−	−	−	+	1.5×10^4	9600	CVD	−	1 Day	Effective	Recovery
14	59/F	II	40	359	78	3.7	−	−	−	+	5.6×10^3	5112	HC	−	About 1 mo	Effective	Recovery
15	44/M	III	117	87	48	3.9	−	−	−	+	2.2×10^4	8400	ME	Sp, GA	About 3 wk	Noneffective	Death
16	40/M	III	800	188	66	12	−	−	−	+	1.1×10^4	7050	CVD	−	1 Day	Effective	Recovery
17	30/F	III	720	211	50	9.7	−	−	−	+	3.1×10^3	5596	IFM	−	5 Days	Effective	Recovery
18	20/F	II	442	164	46	17.6	−	−	−	−	Not detected	Not detected	IFM	GA	Not available	Effective	Recovery
19	63/M	III	75	84	47	15.9	−	−	−	−	4.8×10	76	ME	Sp	Not available	Effective	Recovery
20	63/F	II	34	294	30	12.7	−	−	−	+	3.2×10^2	188	HC	−	Not available	Effective	Recovery
21	53/M	III	76	81	82	16.9	−	−	−	+	8.4×10^3	2592	ME, IFM	Sp	1 Day	Effective	Recovery
22	51/M	III	227	155	34	12.7	−	−	−	+	5.2×10^3	636	ME, CVD, IFM	−	1 Day	Effective	Recovery
23	66/M	III	129	120	58	4.7	−	−	−	+	2.2×10^3	1600	ME	−	4 Days	Effective	Recovery
24	2/F	II	193	119	30	8.3	−	−	−	+	1.1×10^3	1444	ME, HC	−	Not available	Effective	Recovery

[a] The clinical criteria suggestive for TBM are fever, headache, and neck stiffness of more than 1 week in duration. Supporting evidence for TBM includes (i) compatible abnormal CSF findings that included increased white cell counts with lymphocytes predominating, hypoglycorrhachia, a protein concentration of >100 mg/dl, adenosine deaminase at ≥10 IU/liter, and negative results for routine bacterial and fungal cultures; (ii) magnetic resonance imaging findings suggesting tuberculous involvement of the CNS (basal exudates, hydrocephalus, and intracranial focal mass, etc.); (iii) presence of tuberculosis in the body outside of the CNS or a history of tuberculosis; and (iv) clinical response to ATT.

[b] The suspected TBM cases were classified as "confirmed" cases (having bacterial isolation for *M. tuberculosis*, e.g., being CSF culture positive) or "highly probable" cases (meeting all the above clinical criteria and with all three supporting evidences positive). The 2 "confirmed" cases (cases 1 and 2) and 7 "highly probable" cases (cases 9 to 15) correspond to cases 1 to 9 in our previous papers (see references 14 and 15.

[c] M, male; F, female.

[d] Clinical stages are defined according to the British Medical Research Council as follows: stage 0, no definite neurologic symptoms; stage I, slight signs of meningeal irritation with slight clouding of consciousness; stage II, moderate signs of meningeal irritation with moderate disturbance of consciousness and neurologic defects; stage III, severe disturbance of consciousness and neurologic defects.

[e] ADA, adenosine deaminase.

[f] MRI, magnetic resonance imaging; ME, meningeal enhancement; HC, hydrocephalus; CVD, cerebrovascular disorder; IFM, intracranial focal mass; −, no finding.

[g] Sp, sputum; GA, gastric aspirate; −, no finding.

TABLE 3. Assay results and clinical parameters

Assay	Result	No. with indicated result among: Clinically suspected TBM cases (24 cases)	No. with indicated result among: Negative control group (35 samples)	Sensitivity (%)	Specificity (%)	Predictive values (%) Positive	Predictive values (%) Negative
M. tuberculosis culture	+	8	0	33.3	100	100	68.6
	−	16	35				
Single-PCR assay	+	5	0	20.8	100	100	64.8
	−	19	35				
Nested-PCR assay	+	22	0	91.7	100	100	94.6
	−	2	35				
OR-QNRT-PCR assay	+	23	0	95.8	100	100	97.2
	−	1	35				
WR-QNRT-PCR assay	+	23	0	95.8	100	100	97.2
	−	1	35				

nostic Instrument Systems, Sparks, MD). The cultures were incubated under an atmosphere containing 5% CO_2 at 37°C and observed for 12 weeks before they were discarded.

Conventional single- and nested-PCR assays. The DNA specimens including *M. tuberculosis* DNA were extracted and purified from the 250 μl of CSF samples by previously reported conventional phenol-chloroform method and ethanol precipitation (15, 17).

Two sets of primer pairs, outer primers WF1 and WR1 and inner primers WF2 and WR2, which were specific for the MPB64 protein of *M. tuberculosis* (MPT64; GenBank accession no. NC_000962) were prepared (Table 1). In the single (i.e., first-step) PCR assay, 2 μl of the extracted DNA specimen including *M. tuberculosis* DNA as a template was added to 18 μl of the previously reported PCR solution mixture (15, 17) containing 20 pM each of outer primers WF1 and WR1 and then subjected to the following protocol using GeneAmp PCR system 9700 (Perkin Elmer, Norwalk, CT): an initial denaturation at 96°C for 3 min followed by 35 cycles of amplification with denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min.

In nested (i.e., second-step) PCR, 2 μl of the single PCR product as a template was added to 18 μl of the PCR mixture containing 20 pM each of outer primers WF2 and WR2 and then subjected to an initial denaturation at 96°C for 3 min followed by 25 cycles of amplification with denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min and a final extension at 72°C for 10 min. The PCR products were analyzed by Agilent 2100 bioanalyzer system (Agilent Technologies, Waldbronn, Germany). The presence of a 239-bp band for single PCR and a 194-bp band for nested PCR indicated successful amplification.

In addition, for use as an internal control, a pair of primers that were specific for the human β-globin gene (HBB; GenBank accession no. L48217) was prepared (Table 1). The 196-bp HBB fragment as an internal control was amplified in another tube under the same assay conditions.

OR-QNRT-PCR assay. For the OR-QNRT-PCR assay, two types of original plasmid, wild plasmid (W-plasmid) and mutation plasmid (M-plasmid), were constructed (15). The original W-plasmid was prepared for use as the standard template to construct the standard curve in the second step of this assay (15). The original M-plasmid was constructed based on the W-plasmid for use as the old internal control (15). The DNA specimens were extracted and purified from 250 μl of CSF samples added by the 1,000 copies of M-plasmid in advance by previously reported conventional phenol-chloroform method and ethanol precipitation (15).

The OR-QNRT-PCR assay consists of two consecutive PCR amplification steps, which were conventional PCR at the first step and RT-PCR (TaqMan) at the second step, using an ABI Prism 7700 sequence detector (Applied Biosystems, Foster City, CA). Two sets of primer pairs, outer primers WF1 and WR1 at the first step and inner primers TqMn-WF2 and TqMn-WR2 at the second step, which were specific for wild MPT64, were prepared (Table 1). In addition, for use in second-step PCR, two types of TaqMan probes, TqMn-W-VIC for detecting wild MPT64 and TqMn-M-FAM for detecting the old internal control (i.e., M-plasmid), were prepared (Table 1). As the template, 2 μl of the extracted DNA specimen at the first step and 2 μl of the first-step PCR product at the second step were used. All procedures of this assay were subjected to the previously reported protocol (15). (The first-step PCR was set at 35 amplification cycles.)

WR-QNRT-PCR assay. For the WR-QNRT-PCR assay, the original new-mutation plasmid (NM-plasmid) was constructed based on M-plasmid for use as a new internal control (17). The DNA specimens were extracted and purified from the 250 μl of CSF samples added by the 1,000 copies of NM-plasmid in advance by use of the previously reported method (17).

The WR-QNRT-PCR assay consists of two consecutive PCR amplification steps, as does the OR-QNRT-PCR assay (15, 17). In first-step PCR, 2 μl of the extracted DNA specimen including the new internal control as a template was amplified by using the outer primers WF1 and WR1 or MF1 and MR1 (Table 1) at 25 amplification cycles (17). In second-step PCR, 2 μl of the first-step PCR product was used as a template. The wild MPT64 fragment was amplified by the inner primers TqMn-WF2 and TqMn-WR2 and detected by specific TaqMan probe TqMn-W-VIC (Table 1). The new internal control, i.e., NM-plasmid, was amplified by the inner primers TqMn-MF2 and TqMn-MR2 and detected by the specific TaqMan probe TqMn-M-FAM (Table 1). All procedures of this assay were subjected to the previously reported protocol (17).

Quantitative detection for *M. tuberculosis* DNA. The initial copy number of *M. tuberculosis* DNA in CSF samples was calculated based on the amplification ratio against the 1,000 copies of the old internal control (M-plasmid) in the OR-QNRT-PCR assay or the new internal control (NM-plasmid) in the WR-QNRT-PCR assay (15, 17). For *M. tuberculosis*, it is universally acceptable that a single copy of the MPT64 gene represented one bacterial cell (3, 4). Therefore, we considered that the copy numbers calculated by both the OR- and WR-QNRT-PCR assays corresponded to the *M. tuberculosis* bacterial cell numbers in CSF samples.

Blinded (randomized) assay. For comparative evaluation of respective diagnostic assays, conventional single- and nested-PCR assays and OR- and WR-QNRT-PCR assays were performed against the blinded (randomized) 10 CSF samples labeled A to J. These blinded samples were selected in the clinically obtained CSF samples stored at −80°C. The 10 blinded CSF samples, samples A to J, correspond to a cryptococcal meningitis sample, a case 1 sample, a case 2 sample, a viral meningitis sample, a CNS lupus sample, a case 3 sample, a case 8 sample, another viral meningitis sample, a multiple sclerosis sample, and a case 9 sample, respectively.

Statistical analysis. The statistical analysis was calculated using data analysis software program SPSS 13.0 for Windows. A P value of <0.05 was considered statistically significant.

RESULTS

Clinical features of the patients. Table 2 summarizes the clinical features of the 24 suspected TBM patients upon admission (before ATT). All 24 patients (i) met the clinical criteria and (ii) demonstrated supporting evidence for TBM (shown in Table 2) (4, 6, 11, 12, 14, 15) and were classified as 8 "confirmed" cases (cases 1 to 8) (CSF culture positive for *M. tuberculosis*) and 16 "highly probable" cases (cases 9 to 24) (meeting all the clinical criteria and with 3 positive for supporting evidences but having no bacterial isolation). Of these

TABLE 4. Blind (randomized) assay results

Blinded (randomized) sample	Result for:				Sample source
	Single-PCR assay[a]	Nested-PCR assay[b]	OR-QNRT-PCR assay (copies/ml CSF)[c]	WR-QNRT-PCR assay (copies/ml CSF)[d]	
A	−	−	−	−	Cryptococcal meningitis (negative control)
B	+	+	+ (2.5×10^5)	+ (25,647)	Case 1
C	−	+	+ (8.6×10^4)	+ (12,214)	Case 2
D	−	−	−	−	Viral meningitis (negative control)
E	−	−	−	−	CNS lupus (negative control)
F	−	+	+ (1.2×10^5)	+ (18,236)	Case 3
G	+	+	+ (9.2×10^3)	+ (9,542)	Case 8
H	−	−	−	−	Viral meningitis (negative control)
I	−	−	−	−	Multiple sclerosis (negative control)
J	−	+	+ (2.7×10^3)	+ (8,705)	Case 9

[a] Rate of consistency to previous assay results, 90%.
[b] Rate of consistency to previous assay results, 100%.
[c] Rate of consistency to previous assay results, 100%. Wilcoxon signed-rank test *P* value, 0.0052.
[d] Rate of consistency to previous assay results, 100%. Wilcoxon signed-rank test *P* value, 0.147 (no significant difference).

24 patients, the 2 "confirmed" cases (cases 1 and 2) and 7 "highly probable" cases (cases 9 to 15) correspond to cases 1 to 9 reported in our previous papers (14, 15). In addition, the periods up to initial sample collection available from these 24 patients are shown in Table 2. This period was approximately consistent with the period from the development of symptoms to the beginning of ATT for suspected TBM patients and was therefore considered to be clinically important. The results of respective diagnostic assays, including *M. tuberculosis* culture, conventional single- and nested-PCR assays, and OR- and WR-QNRT-PCR assays, are summarized and evaluated clinically in Tables 3 and 4.

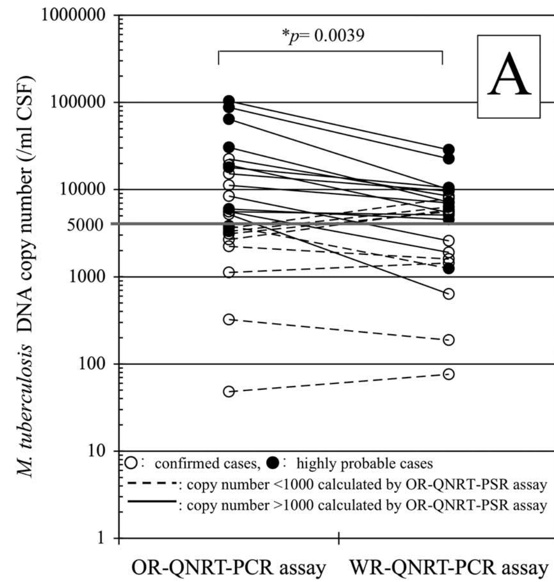
WR-QNRT-PCR assay results for CSF samples. (i) Upon admission. For WR-QNRT-PCR assay, the quantitative detection of *M. tuberculosis* DNA was possible in 23 cases out of the 24 suspected TBM patients (95.8%) upon admission (Tables 2 and 3). The measured copy numbers of *M. tuberculosis* DNA (per 1 ml of CSF) are shown in Table 2. However, the WR-QNRT-PCR assay revealed no amplification for all 35 CSF samples collected from the 29 patients in the non-TBM control group and the six reference strains of non-*M. tuberculosis* species (Table 3). OR-QNRT-PCR assay results are also shown in Table 2. The sensitivities and specificities of both the OR- and WR-QNRT-PCR assays were equivalent (Table 3). However, there was the significant difference ($P = 0.0039$) between the OR- and WR-QNRT-PCR results by Wilcoxon signed-rank testing (Fig. 1A). In particular, copy numbers of more than 1,000 calculated by the OR-QNRT-PCR assay tend to reveal more values than do WR-QNRT-PCR assay results (Fig. 1A). This trend indicated the unfavorable influence of overamplification and the instability of the M-plasmid as the old internal control in the OR-QNRT-PCR assay.

In the blinded (randomized) assay (Table 4), both OR- and WR-QNRT-PCR results were completely consistent with the assay results shown in Table 2. However, there was a significant difference ($P = 0.0052$) in copy numbers (per ml of CSF) between the blinded assay and the previous assay results of OR-QNRT-PCR by Wilcoxon signed-rank test (Table 4). In WR-QNRT-PCR, the copy numbers were not statistically significantly different between blinded assay and previous assay results (Table 4). These results indicated that the WR-QNRT-PCR assay had sufficient reproducibility and was more stable and reliable as a diagnostic method than conventional assay techniques, including OR-QNRT-PCR.

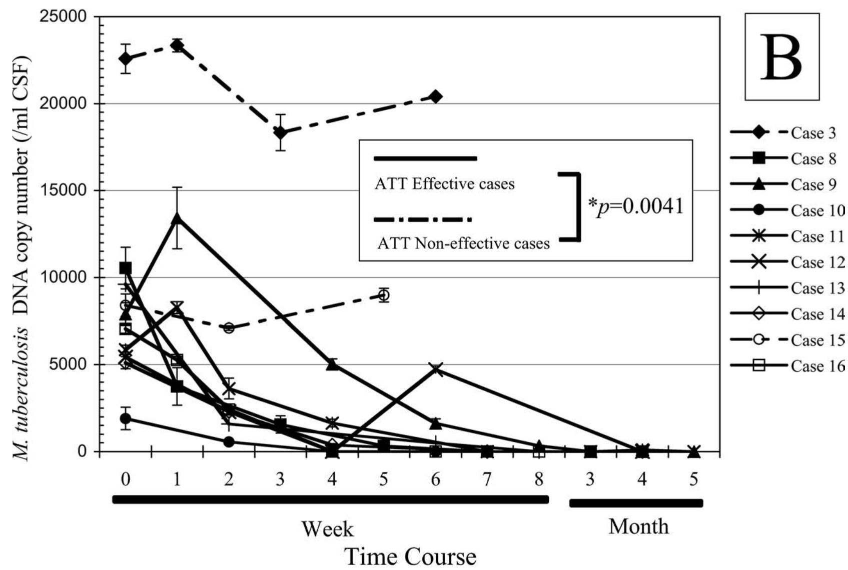
In conditional logistic regression analysis, *M. tuberculosis* culture positivity, *M. tuberculosis* DNA copy numbers of >8,000 (per ml of CSF) calculated by WR-QNRT-PCR assay, and more than 2 weeks up to initial sample collection, were independent risk factors for a poor prognosis for TBM (i.e., death) (Table 5) (for these three risk factors, statistics were, respectively, as follows: odds ratio [OR] = 37.368, 95% confidence interval [95% CI] = 1.233 to 1132.781, $P = 0.0375$; OR = 16.142, 95% CI = 1.191 to 218.79, $P = 0.0365$; OR = 32.501, 95% CI = 1.709 to 618.21, $P = 0.0205$). The copy number of >8,000 (per ml of CSF) as the threshold value was set based on the 75th percentile (8,146 copies) of WR-QNRT-PCR assay results in all 24 cases (Table 2). However, the conventional single- and nested-PCR assays, which were qualitative examinations, were not statistically significant risk factors for a poor prognosis for TBM (Table 5).

(ii) Diachronic study. Table 6 summarizes the diachronic study results, which include *M. tuberculosis* cultures, conventional single- and nested-PCR assays, the WR-QNRT-PCR assay, and other routine CSF findings, for the 10 patients: 2 "confirmed" cases (cases 3 and 8) and 8 "highly probable" cases (cases 8 to 16). Among these 10 patients, cases 8 to 15 correspond to cases 1 to 8 in our previous paper (16). The cultures for *M. tuberculosis* revealed positive results for only 3

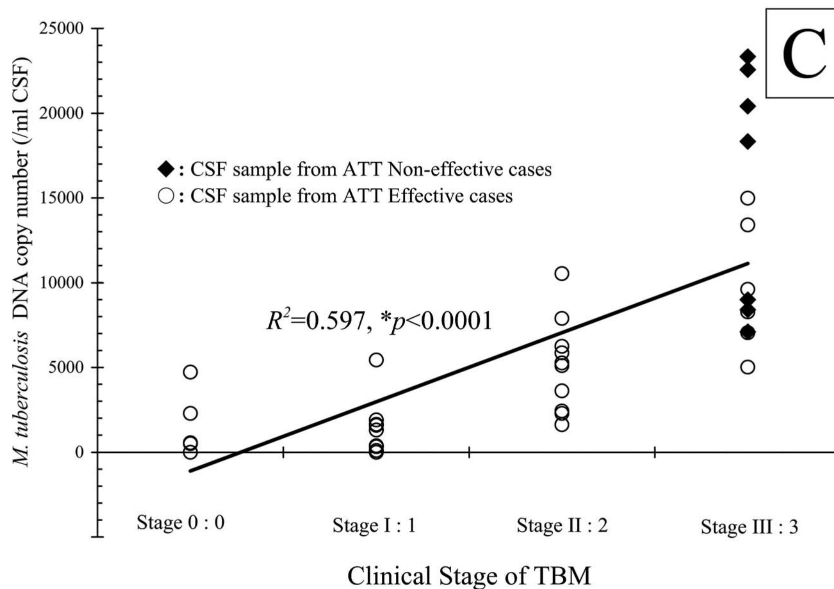
FIG. 1. Statistical analysis for WR-QNRT-PCR assay in clinical application. (A) Statistical comparison between OR- and WR-QNRT-PCR results by Wilcoxon signed-rank test. (B) The progress of *M. tuberculosis* DNA copy numbers calculated by the WR-QNRT-PCR assay during a clinical time course for 10 suspected TBM patients (cases 3 and 8 to 16). A statistical comparison between the ATT-effective cases (cases 8 to 14 and 16) and the ATT-noneffective cases (cases 3 and 8) was calculated by repeated-measures ANOVA. (C) Result of simple regression analysis between *M. tuberculosis* DNA copy number (*y* axis) and clinical stage of TBM (*x* axis).



*p= 0.0039
A
M. tuberculosis DNA copy number (/ml CSF)
1000000
100000
10000
5000
1000
100
10
1
○: confirmed cases, ●: highly probable cases
: copy number <1000 calculated by OR-QNRT-PSR assay
: copy number >1000 calculated by OR-QNRT-PSR assay
OR-QNRT-PCR assay
WR-QNRT-PCR assay



B
M. tuberculosis DNA copy number (/ml CSF)
25000
20000
15000
10000
5000
0
ATT Effective cases
ATT Non-effective cases
*p=0.0041
Case 3
Case 8
Case 9
Case 10
Case 11
Case 12
Case 13
Case 14
Case 15
Case 16
0 1 2 3 4 5 6 7 8 3 4 5
Week
Month
Time Course



C
M. tuberculosis DNA copy number (/ml CSF)
25000
20000
15000
10000
5000
0
◆: CSF sample from ATT Non-effective cases
○: CSF sample from ATT Effective cases
R^2=0.597, *p<0.0001
Stage 0 : 0
Stage I : 1
Stage II : 2
Stage III : 3
Clinical Stage of TBM

TABLE 5. Independent predictors for poor prognosis of TBM (conditional logistic regression model)[a]

Independent predictor	OR	95% CI	*P* value[b]
Age (yr)	1.040	0.953–1.134	0.3803 (NSD)
Culture for *M. tuberculosis*	37.368	1.233–1132.781	0.0375
Single-PCR assay	0.069	0.001–3.236	0.1731 (NSD)
Nested-PCR assay	6.125	0.489–76.770	0.1600 (NSD)
M. tuberculosis DNA copy no. of >8,000 (WR-QNRT-PCR assay)	16.142	1.191–218.79	0.0365
More than 2 wk to initial sample collection	32.501	1.709–618.21	0.0205

[a] The association of the specifically diagnostic parameters with risk for the poor prognosis for TBM (death) was tested using conditional logistic regression analysis. A backwards elimination procedure was used for multivariable analysis. For multivariate risk predictors, the adjusted OR values are given with the 95% CI values.

[b] NSD, no significant difference.

(from cases 3 and 8) out of 43 serial CSF samples collected during the clinical treatment course. In contrast, the quantitative detection of *M. tuberculosis* DNA was possible for 25 CSF samples (58.1%) in the WR-QNRT-PCR assay. In analysis of variance (ANOVA), the WR-QNRT-PCR assay results revealed significant alterations ($P < 0.01$) during the clinical treatment course in 10 patients (Table 6 and Fig. 1B). In addition, the copy numbers of *M. tuberculosis* DNA (per 1 ml of CSF) gradually decreased to below the detection limit of the WR-QNRT-PCR assay for the eight patients (cases 8 to 14 and 16) for whom ATT was effective and who demonstrated improvement in both their clinical stages and routine CSF findings during the clinical treatment course (Table 6 and Fig. 1B). However, for cases 3 and 15, for whom ATT was noneffective and who died due to aggravation of TBM, the copy numbers were continually at a high level throughout the clinical course (Table 6 and Fig. 1B). The pattern (trend) in the alterations of *M. tuberculosis* DNA copy numbers during the clinical treatment course demonstrated significant difference ($P = 0.0041$) between the ATT-effective cases (cases 8 to 14 and 16) and the ATT-noneffective cases (cases 3 and 8) by repeated-measures ANOVA (Fig. 1B). Moreover, in simple regression analysis, significant correlation ($R^2 = 0.597$, $P < 0.0001$) was demonstrated between the *M. tuberculosis* DNA copy number and the clinical stage of TBM (Fig. 1C).

DISCUSSION

We have developed an improved WR-QNRT-PCR assay technique for the accurate quantitative detection of *M. tuberculosis* DNA in CSF samples collected from patients with clinically suspected TBM.

In clinical application, the WR-QNRT-PCR assay demonstrated equivalence in sensitivity (95.8%) and specificity (100%) to the OR-QNRT-PCR assay (Table 3). However, for the actual clinical CSF samples, the copy numbers calculated by the OR-QNRT-PCR assay revealed the unfavorable influence of overamplification and the instability of the M-plasmid as the old internal control (Fig. 1A), as well as the preliminary experiment results described in the companion methodology paper (17). In addition, this instability of OR-QNRT-PCR assay was also recognized in the blinded (randomized) assay (Table 4). However, the WR-QNRT-PCR assay results in the blinded assay demonstrated sufficient reproducibility and strengthened the clinical significance of this assay (Table 4). Therefore, the WR-QNRT-PCR assay was considered to be not only more accurate but also a more stable and reliable diagnostic method than conventional assay techniques, including OR-QNRT-PCR. Moreover, it was statistically evaluated using conditional logistic regression analysis whether the specifically diagnostic parameters, including *M. tuberculosis* cultures, conventional single- and nested-PCR assays, and the WR-QNRT-PCR assay for CSF and the period up to initial sample collection associated with risk for a poor prognosis for TBM (i.e., death) (Table 5). The conditional logistic regression analysis results indicated that an *M. tuberculosis* DNA copy number of >8,000 calculated by the WR-QNRT-PCR assay was an independent risk factor for a poor prognosis for TBM, as was *M. tuberculosis* culture positivity (Table 5). Interestingly, a period of more than 2 weeks up to initial sample collection was also one of the most important risk factors for a poor prognosis for TBM (Table 5). This result suggests that a delay in making the appropriate clinical decision, including starting ATT, has a serious influence against the prognosis for TBM. In the present diachronic study, the copy numbers demonstrated statistically significant alterations ($P < 0.01$) during the clinical treatment course for 10 patients (Table 6). In addition, these alterations of *M. tuberculosis* DNA copy numbers were significantly correlated with the patient's clinical condition (stage) and the ATT response (Fig. 1B and C). In cases 3 and 15, although the appropriate ATT was started immediately after admission to our hospital, ATT was noneffective for these two patients and they died due to aggravation of TBM (Table 6). These unsuccessful ATT outcomes may be due not only to the high copy numbers of *M. tuberculosis* DNA in these two patients but also to the delay (more than 2 weeks) in appropriate CSF examination or in starting treatment (Table 2).

We consider that accurate quantitative analysis by WR-QNRT-PCR assay may provide a significantly reliable foundation for appropriate clinical decision, such as the start of ATT, the additional use of corticosteroids and other anti-inflammatory or immune-modulatory adjunctive treatments, and prediction of prognosis in patients with suspected TBM. The present diachronic study results indicate that quantitative analysis by the WR-QNRT-PCR assay is very useful for assessing the clinical course of TBM and ATT response. To our knowledge, there has been no previous study which serially assessed the quantity of DNA or bacterial cell numbers of *M. tuberculosis* in CSF samples throughout the clinical course of TBM patients. Previously (in 2006), to quantitatively detect *M. tuberculosis* DNA in CSF samples, we designed the OR-QNRT-PCR assay (15). However, the OR-QNRT-PCR assay was incomplete and insufficient for massive clinical application and commercial evolution, since this assay technique held the unfavorable influence of overamplification caused by the instability of the M-plasmid, used as the old internal control (15, 17). In this study, the WR-QNRT-PCR assay was developed as a novel improved assay technique for wider use in the clinical practice (17). In actual clinical application, this novel assay technique demonstrated significant accuracy and reliability for the quantitative detection of *M. tuberculosis* DNA in CSF samples due

TABLE 6. Diachronic study results during the clinical course in the 10 patients with suspected TBM[a]

Patient	Serial Clinical Data	Time course												Statistical Analysis *M. Tb* DNA copy number–Time course	ATT Response	Outcome
			Weeks								Months					
		0 (upon admission)	1	2	3	4	5	6	7	8	3	4	5			
		Before treatment	During treatment													
	*Clinical Stage	III	III	III	III	III	III									
	Cells (/µl)	605	1052	834	722	685	951									
	Protein (mg/dl)	434	585	603	787	714	556									
	Glucose (mg/dl)	25	39	33	26	22	18									
	M.Tb culture	+	+		+		−									
	Single PCR assay	−	−		−		−									
Case 3	Nested PCR assay	+	+		+		+								Non-effective	Death
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	22572	23340		18332		20408							p=0.0001		
	Treatment															
	INH (mg/day)	600 →						→								
	RFP (mg/day)	450 →						→								
	PZA (g/day)	2 →						→								
	SM (g/day)	1 →						→								
	*Clinical Stage	II	III	II	II	I	I	0	0	0	0	0				
	Cells (/µl)	418	518	334	283	114	145	83	68	44	33	7				
	Protein (mg/dl)	456	678	786	415	387	247	176	124	106	78	66				
	Glucose (mg/dl)	36	22	38	42	55	53	48	68	72	84	73				
	M.Tb culture	+	+		−		−		−		−	−				
	Single PCR assay	+	−		−		−		−		−	−				
Case 8	Nested PCR assay	+	++		+		*+		−		−	−			Effective	Recovery
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	10532	14980		6248		1312		N.D.		N.D.	N.D.		p<0.0001		
	Treatment															
	INH (mg/day)	600 →							→ 450 →				→			
	RFP (mg/day)	450 →											→			
	PZA (g/day)	2 →									→					
	SM (g/day)	1 →							→							
	*Clinical Stage	II	III	III	III	III	III	II	II	I	0	0	0			
	Cells (/µl)	208	348	130	342	330	474	272	130	59	20	9	19			
	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			
	M.Tb culture	−	−	−		−		−		−	−	−	−			
	Single PCR assay	−	−			−		−		−	−	−	−			
	Nested PCR assay	+	++			+		+		−	−	−	−			
Case 9	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	7892	13408			5020		1624		328	N.D.	N.D.	N.D.	p<0.0001	Effective	Recovery
	Treatment															
	intrathecal administration of INH (100mg: 3times/week)						←				→					
	INH (mg/day)	450 →		800 →								→ 450 →	→			
	RFP (mg/day)	450 →		600 →								→ 450 →	→			
	PZA (g/day)	1.5 →											→			
	EB (mg/day)	750 →										→				
	SM (g/day)			1 →							→					
	*Clinical Stage	I	I	0		0		0								
	Cells (/µl)	107	263	41		14		7								
	Protein (mg/dl)	70	148	31		30		36								
	Glucose (mg/dl)	48	45	40		42		51								
	M.Tb culture	−		−		−		−								
	Single PCR assay	−		−		−		−								
Case 10	Nested PCR assay	+		−		−		−							Effective	Recovery
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	1904		560		N.D.		N.D.						p=0.0001		
	Treatment															
	INH (mg/day)	450 →											→			
	RFP (mg/day)	450 →											→			
	PZA (g/day)	1.5 →						→								
	SM (g/day)	1 →					→									
	*Clinical Stage	II	III	II		I			I		I	I	I			
	Cells (/µl)	18	23	25		19			7		6	10	6			
	Protein (mg/dl)	135	62	46		69			42		33	41	42			
	Glucose (mg/dl)	54	71	78		75			77		71	80	101			
	M.Tb culture	−	−	−		−					−	−	−			
	Single PCR assay	−	−	−		−					−	−	−			
Case 11	Nested PCR assay	+	+	−		−					−	*+	−		Effective	Severe
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	5860	8268	3624		1644					N.D.	104	N.D.	p<0.0001		
	Treatment															
	INH (mg/day)	500 →											→			
	RFP (mg/day)	450 →											→			
	PZA (g/day)	1 →											→			
	SM (g/day)	1 →									→					

Continued on following page

TABLE 6—*Continued*

Patient	Serial Clinical Data	Time course												Statistical Analysis *M. Tb* DNA copy number-Time course	ATT Response	Outcome
		Weeks									Months					
		0 (upon admission)	1	2	3	4	5	6	7	8	3	4	5			
		Before treatment	During treatment													
Case 12	*Clinical Stage	I	I	0		0		0				0			Effective	Recovery
	Cells (/μl)	30	7	9		11		2				4				
	Protein (mg/dl)	25	16	23		29		30				28				
	Glucose (mg/dl)	30	38	40		32		34				40				
	M.Tb culture	−		−		−		−				−				
	Single PCR assay	−		−		−		−				−				
	Nested PCR assay	+		−		−		+				−				
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	5436		2292		N.D.		4720				N.D.		$p<0.0001$		
	Treatment															
	INH (mg/day)	500 →	→	→	→	→	→	→	→	→	→	→	→			
	RFP (mg/day)	450 →	→	→	→	→	→	→	→	→	→	→	→			
	PZA (g/day)	2 →	→	→	→	→	→		1 →	→	→	→				
	SM (g/day)	1 →	→	→	→	→										
Case 13	*Clinical Stage	III	II	I		0		0		0					Effective	Recovery
	Cells (/μl)	60	3	4		3		19		9						
	Protein (mg/dl)	70	68	46		52		62		74						
	Glucose (mg/dl)	52	76	44		42		63		43						
	M.Tb culture	−		−				−		−						
	Single PCR assay	−		−				−		−						
	Nested PCR assay	+		*+				*+		−						
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	9600		1588				496		N.D.				$p<0.0001$		
	Treatment															
	INH (mg/day)	200 →	→	→	→	→	→	→	→	→	→	→	→			
	RFP (mg/day)	225 →	→	→	→	→	→	→	→	→	→	→	→			
	PZA (g/day)	1.5 →	→	→	→	→	→	→	→	→						
Case 14	*Clinical Stage	II	II	II	II	I	I	I	I	I	I	I	I		Effective	Severe
	Cells (/μl)	40	31	24	28	8	15	22	17	7	8	2	1			
	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			
	M.Tb culture	−		−		−			−			−				
	Single PCR assay	−		−		−			−			−				
	Nested PCR assay	+		*+		−			−			−				
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	5112		2292		380			N.D.			N.D.		$p<0.0001$		
	Treatment															
	INH (mg/day)	600 →	→	→	→	→	→	→	→	→	→	→	→			
	RFP (mg/day)	450 →	→	→	→	→	→	→	→	→	→	→	→			
	PZA (g/day)	2 →	→	→	→	→	1 →	→	→	→						
	SM (g/day)	1 →	→	→	→	→										
Case 15	*Clinical Stage	III	III	III	III	III	III	III							Non-ffective	Death
	Cells (/μl)	117	95	34	2	1	1	17								
	Protein (mg/dl)	87	87	104	78	55	47	106								
	Glucose (mg/dl)	48	49	65	55	47	59	51								
	M.Tb culture	−		−			−									
	Single PCR assay	−		−			−									
	Nested PCR assay	+		+			+									
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	8400		7104			8988							$p=0.0054$		
	Treatment															
	INH (mg/day)	500 →	→	→	→	→	→	→	→							
	RFP (mg/day)	450 →	→	→	→	→	→	→	→							
	PZA (g/day)	2 →	→	→	→	→	→	→	→							
	SM (g/day)	1 →	→	→	→	→	→	→	→							
Case 16	*Clinical Stage	III	II	II		I		I		0					Effective	Recovery
	Cells (/μl)	800	592	363		150		75		25						
	Protein (mg/dl)	188	122	88		59		54		42						
	Glucose (mg/dl)	66	73	68		64		52		49						
	M.Tb culture	−	−	−		−		−		−						
	Single PCR assay	−	−	−		−		−		−						
	Nested PCR assay	++	+	*+		−		−		−						
	†*M. Tb* DNA copy number [WR-QNRT-PCR assay]	7052	5264	2424		N.D.		N.D.		N.D.				$p<0.0001$		
	Treatment															
	INH (mg/day)	600 →	→	→	→	→	→	→	→	→	→	→	→			
	RFP (mg/day)	450 →	→	→	→	→	→	→	→	→	→	→	→			
	PZA (g/day)	2 →	→	→	→	→	1.5 →	→								
	SM (g/day)	1 →	→	→	→	→										

[a] *M. Tb*, *M. tuberculosis*; +, positive; ++, strongly positive; *+, slightly positive; −, negative; †, per 1 ml CSF; INH, isoniazid; RFP, rifampin; PZA, pyrazinamide; SM, streptomycin sulfate; EB, ethambutol; N.D., not determined. Clinical stages are defined according to the British Medical Research Council as follows: stage 0, no definite neurologic symptoms; stage I, slight signs of meningeal irritation with slight clouding of consciousness; stage II, moderate signs of meningeal irritation with moderate disturbance of consciousness and neurologic defects; stage III, severe disturbance of consciousness and neurologic defects.

to the development of NM-plasmid, which was used as the new internal control.

The clinical usefulness of the WR-QNRT-PCR assay is based on its capacity for the accurate quantitative detection of *M. tuberculosis* DNA with a wide detection range. However, this assay technique does not have the ability to evaluate the viability of bacteria. Therefore, the copy number of *M. tuberculosis* DNA calculated by WR-QNRT-PCR assay may not necessarily be consistent with the viable bacterial number of *M. tuberculosis* in the CSF sample. At present, the only assay method for detecting viable *M. tuberculosis* in the CSF sample is culture examination. Although the culture for *M. tuberculosis* in CSF samples is the "gold standard" for TBM diagnosis, it is inadequate for early diagnosis due to its poor sensitivity or the long time required (4 to 8 weeks) (3–14, 18). Rapid and accurate diagnosis in the acute phase of TBM and an early start to ATT are the most important factors with regard to the prognosis and the prevention of long-term neurological sequelae (3–19). Based on the present assay results, we considered that the WR-QNRT-PCR assay is a reliable assay technique for assessing ATT response and the clinical course of TBM. Particularly, in clinical practice, the WR-QNRT-PCR assay would demonstrate its capacity in rapid and accurate diagnosis for the difficult cases in which conventional assay methods cannot detect *M. tuberculosis*.

At present, despite an overall decrease in the total numbers of tuberculosis cases in the advanced nations, for example, the United States, a gradual and continuous increase in the proportion of extrapulmonary tuberculosis cases has been reported (1, 2). The causes for the increase of extrapulmonary tuberculosis cases are mainly in the recent rise in immune-compromised patients and in the human immunodeficiency virus/AIDS epidemic (1, 2). Although the overall population-based mortality rate from tuberculosis is low and decreasing, several studies have shown that mortality rates are substantially higher for patients with several forms of extrapulmonary tuberculosis, including CNS tuberculosis or TBM and disseminated disease (1, 2, 8, 18). As the proportion of extrapulmonary tuberculosis cases, including those with CNS tuberculosis, continues to increase, particularly in immune-compromised patients, the WR-QNRT-PCR assay technique may become increasingly important for the rapid and accurate diagnosis of TBM.

Certainly, the WR-QNRT-PCR assay may be inadequate for screening examinations dealing with many samples, since this novel assay technique requires additional complicated experimental procedures. However, in actual clinical practice, definitive diagnosis of TBM is required, and this is not available from present screening examination procedures. Therefore, the WR-QNRT-PCR assay will become a prominently useful assay technique if used for well-defined and appropriate clinical specimens collected from "highly probable" TBM patients. We speculate that if the WR-QNRT-PCR assay is widely and appropriately adopted within clinical practice, it will be a powerful tool for the rapid and accurate diagnosis of TBM.

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