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Quantitative nested real-time PCR assay for assessing the clinical course of tuberculous meningitis

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Abstract

Although the "gold standard" for diagnosis of tuberculous meningitis (TBM) is bacterial isolation of *Mycobacterium tuberculosis* (*M. Tb*), there are still several complex issues. Recently, in the diagnosis of TBM, the detection of *M. Tb* DNA in cerebrospinal fluid (CSF) samples using PCR has been widely performed as more rapid, sensitive, and specific diagnostic method. Based on Taq Man® PCR, the authors developed a novel technique of internally controlled quantitative nested real-time (QNRT) PCR assay that provided a prominent improvement in detection sensitivity and quantification. Total 43 CSF samples from 8 serial patients with suspected TBM were analyzed. The CSF samples were collected before and during standard anti-tuberculosis treatments (ATT). The QNRT-PCR assay revealed positive results for 24 out of 43 serial CSF samples (55.8%) collected during the treatment course of ATT. Moreover, the bacterial cell (BC) numbers of *M. Tb* analyzed by the QNRT-PCR assay decreased gradually, correlating with the improvements of the patient's clinical conditions. Since the QNRT-PCR assay provides the ability to calculate a numerical value for the initial BC numbers of *M. Tb* in CSF samples, this method is an extremely useful and advanced technique for use in assessing the clinical course of TBM.
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Keywords: Quantitative nested real-time (QNRT) PCR; TaqMan® PCR; Nested PCR; *Mycobacterium tuberculosis*; Tuberculous meningitis; Cerebrospinal fluid

1. Introduction

The diagnosis of tuberculous meningitis (TBM) remains complex issues because the most widely used conventional diagnostic tools, such as direct smear for acid-fast bacilli (AFB) and culture for *Mycobacterium tuberculosis* (*M. Tb*), are unable to rapidly detect *M. Tb* with sufficient sensitivity in the acute phase of TBM [1–8,10–14]. At present, the detection of *M. Tb* DNA in cerebrospinal fluid (CSF) using PCR has been widely used as more rapid, sensitive, and specific diagnostic method [1–8]. Recently, the authors have developed the novel technique of internally controlled quantitative nested real-time (QNRT) PCR assay based on TaqMan® PCR (Applied Biosystems) [14]. This novel technique combines the high sensitivity of nested PCR with the accurate quantification of real-time PCR [14]. In this study, the authors examined the usefulness of the QNRT-PCR assay for assessing the anti-tuberculosis treatment (ATT) response during the clinical course of TBM.

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2. Materials and methods

2.1. Subjects and CSF samples

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

Eight serial patients with clinically suspected TBM and 20 non-TBM control patients were selected from patients who were admitted to our hospital between 1998 and 2005. Total 43 serial CSF samples were collected from these 8 patients, who had follow-ups of more than at least 2 weeks. In addition, the extracted DNA specimen from the *M. Tb* standard strain H37Rv (ATCC 25618) was used as the positive control.

The non-TBM control group consisted of 4 cases of bacterial meningitis, 3 with cryptococcal meningitis, 3 with viral meningitis, 6 with multiple sclerosis, and 1 each with central nervous system (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. Moreover, to determine the analytical specificity and cross-reactivity of our assays, the extracted DNA specimens from 6 additional reference strains of non-*M. Tb* species − *M. bovis* BCG (ATCC 19274), *M. avium* (ATCC 15769), *M. intracellulare* (ATCC 15985) and clinically isolated *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* − were tested.

2.2. Conventional nested PCR assay

The DNA specimens including *M. Tb* DNA were extracted and purified from the 250 μl of CSF samples by previously reported conventional phenol–chloroform method and ethanol precipitation [13,14].

For use in the two subsequent amplification steps of the nested PCR assay, two pairs of primers capable of specifically amplifying the gene sequence encoding the MPB64 protein of *M. Tb* (MPT64; GenBank accession no. NC_000962) were prepared. The sequences of the outer forward (F-1) and reverse (R-1) primers for use in the first step PCR, and the inner forward (F-2) and reverse (R-2) primers for use in the second step PCR are shown in Table 1. In addition, for use as the internal control, a pair of primers that were specific for the human β-globin gene (HBB; GenBank accession no. L48217) was prepared (Table 1). As the template, 2 μl of the extracted DNA specimen at the first step or 2 μl of the first step PCR product at the second step was used. The nested PCR assay conditions were subjected to the previous reported protocol [13,14].

2.3. Preparation of the two types original plasmids

For the quantitative detection of *M. Tb* DNA in CSF samples, two types of original plasmid, "Wild" (W) and "Mutation" (M) plasmids, were created in our laboratory [14].

The original W-plasmid was prepared for use as the standard template to construct the standard curve in the second step of QNRT-PCR assay. It included a 239 base pairs (bp) DNA fragment of the MPT64. The original M-plasmid was created based on the W-plasmid for use as the internal control ("ruler") in the QNRT-PCR assay. It included artificial 22 random nucleotides within the inserted 239 bp DNA fragment of the MPT64. The sequence of the artificial 22 random nucleotides was set so that it had the same nucleotide composition as the wild MRT64. 10^3 copies of the M-plasmid were adopted as the optimum internal control based on the previous reported preliminary experiment results [14].

2.4. Quantitative nested real-time (QNRT) PCR assay

The DNA specimens for use in the QNRT-PCR assay were extracted and purified from the 250 μl of CSF samples added by the 10^3 copies of M-plasmid as the internal control in advance by the phenol–chloroform method and ethanol precipitation [14].

In the first step of the QNRT-PCR assay, 2 μl of the extracted DNA specimen including the internal control as the template was amplified by using the same outer primers F-1 and R-1 under the same assay condition as the nested PCR [13,14].

Table 1
Sequence of primers and TaqMan® probes for PCR assays

Objective		Type	Sequence	PCR product size (bp)
Conventional nested PCR assay	First step PRC	Outer forward primer (F-1)	5′-ATCCGCTGCCAGTCGTCTTCC-3′	239
		Outer reverse primer (R-1)	5′-CTCGCGAGTCTAGGCCAGCAT-3′	
	Second step PCR	Inner forward primer (F-2)	5′-CATTGTGCAAGGTGAACTGAGC-3′	194
		Inner reverse primer (R-2)	5′-AGCATCGAGTCGATCGCGGAA-3′	
	Internal control	Human β-globin forward primer (HBB-F)	5′-GGCAGACTTCTCCTCAGGAGTC-3′	196
		Human β-globin reverse primer (HBB-R)	5′-CTTAGACCTCACCCTGTGGAGC-3′	
Quantitative nested real-time PCR assay (QNRT-PCR assay)		TaqMan® forward primer (TqMn-F)	5′-GTGAACTGAGCAAGCAGACCG-3′	77
		TaqMan® reverse primer (TqMn-R)	5′-GTTCTGATAATTCACCGGGTCC-3′	
		TaqMan® probe-wild-VIC (TqMn-W-VIC)	5′VIC-TATCGATAGCGCCGAATGCCGG-TAMPRA-3′ Total 22 mar, A:5, T:4, G:7, C:6 (GC% 59)	
		TaqMan® probe-mutation-FAM (TqMn-M-FAM)	5′-FAM-ATGGGACGGCTAGCAATCCGTC-TAMRA-3′ Total 22 mar, A:5, T:4, G:7, C:6 (GC% 59)	

Underline: artificial sequence, bp: base pairs.

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The second step of the QNRT-PCR assay was changed to the real-time (TaqMan®) PCR for quantitative analysis. A new pair of inner primers that were also specific for the MPT64 was prepared. The sequences of these new inner primers (TaqMan® forward primer (TqMn-F) and TaqMan® reverse primer (TqMn-R)) are shown in Table 1. In addition, two specific 22-nucleotide TaqMan® probes, which were labeled with the fluorescent reporter dye VIC or 6-carboxyfluorescin (FAM) respectively, were prepared. The sequences of these two TaqMan® probes (TaqMan® probe-wild-VIC (TqMn-W-VIC)

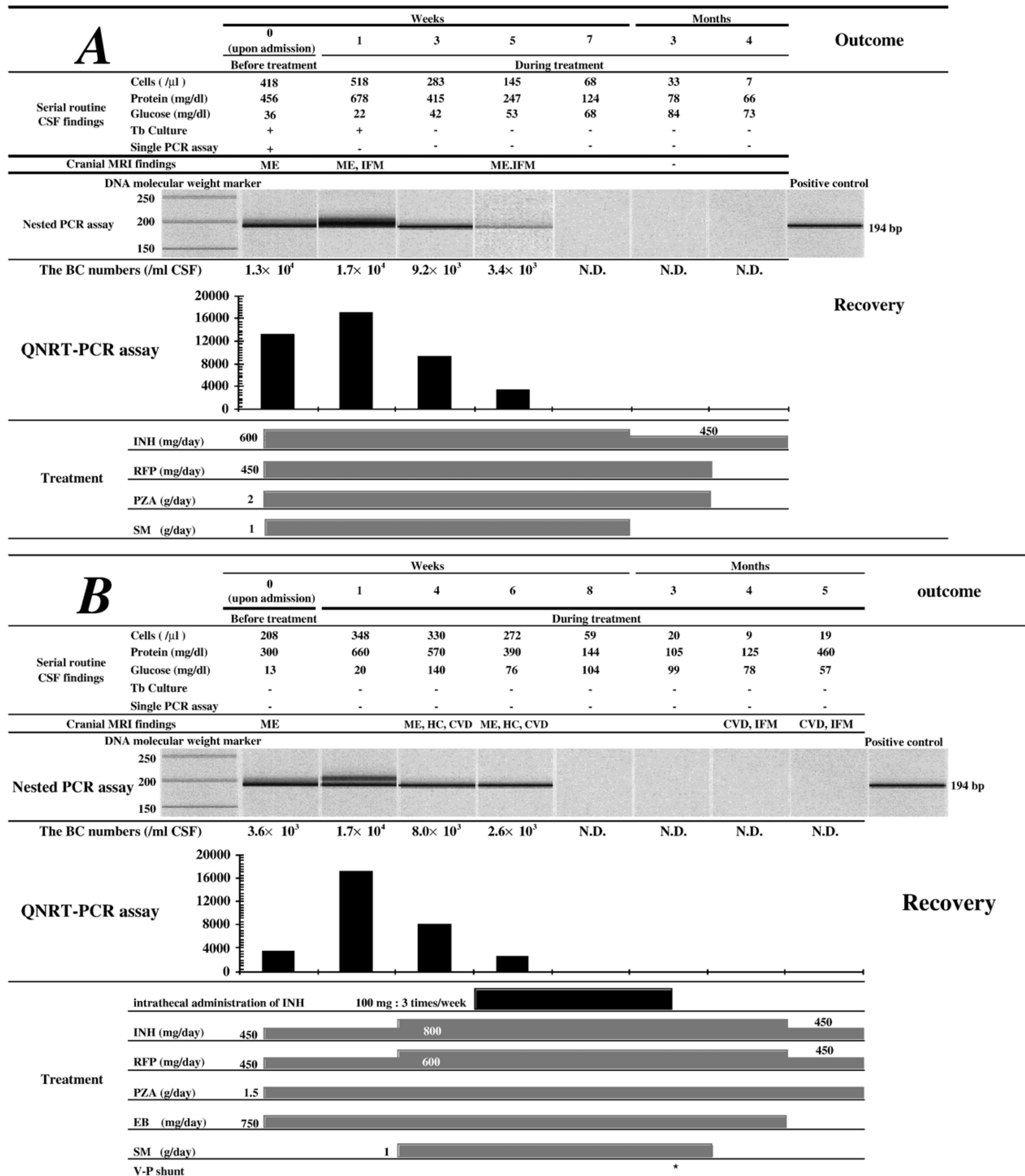

Fig. 1. The summary of the diachronic study results in the 8 patients (one "confirmed" and 7 "highly probable" TBM cases). BC: bacterial cell, ME: meningeal enhancement, HC: hydrocephalus, CVD: cerebrovascular disorder, IFM: intracranial focal mass. A: case 1, B: case 2, C: case 3, D: case 4, E: case 5, F: case 6, G: case 7, H: case 8. The 7 "highly probable" cases (cases 2 to 8) correspond to the cases 3 to 9 in the previous reported paper [see Ref. [4]]. The nested PCR assay results were analyzed by the Agilent 2100 bioanalyzer system™ (Agilent Technologies, Waldbronn, Germany). The QNRT-PCR assay results were analyzed by the ABI PRISM 7700 sequence detector system™ (PE Applied Biosystems, Foster City, CA, USA).

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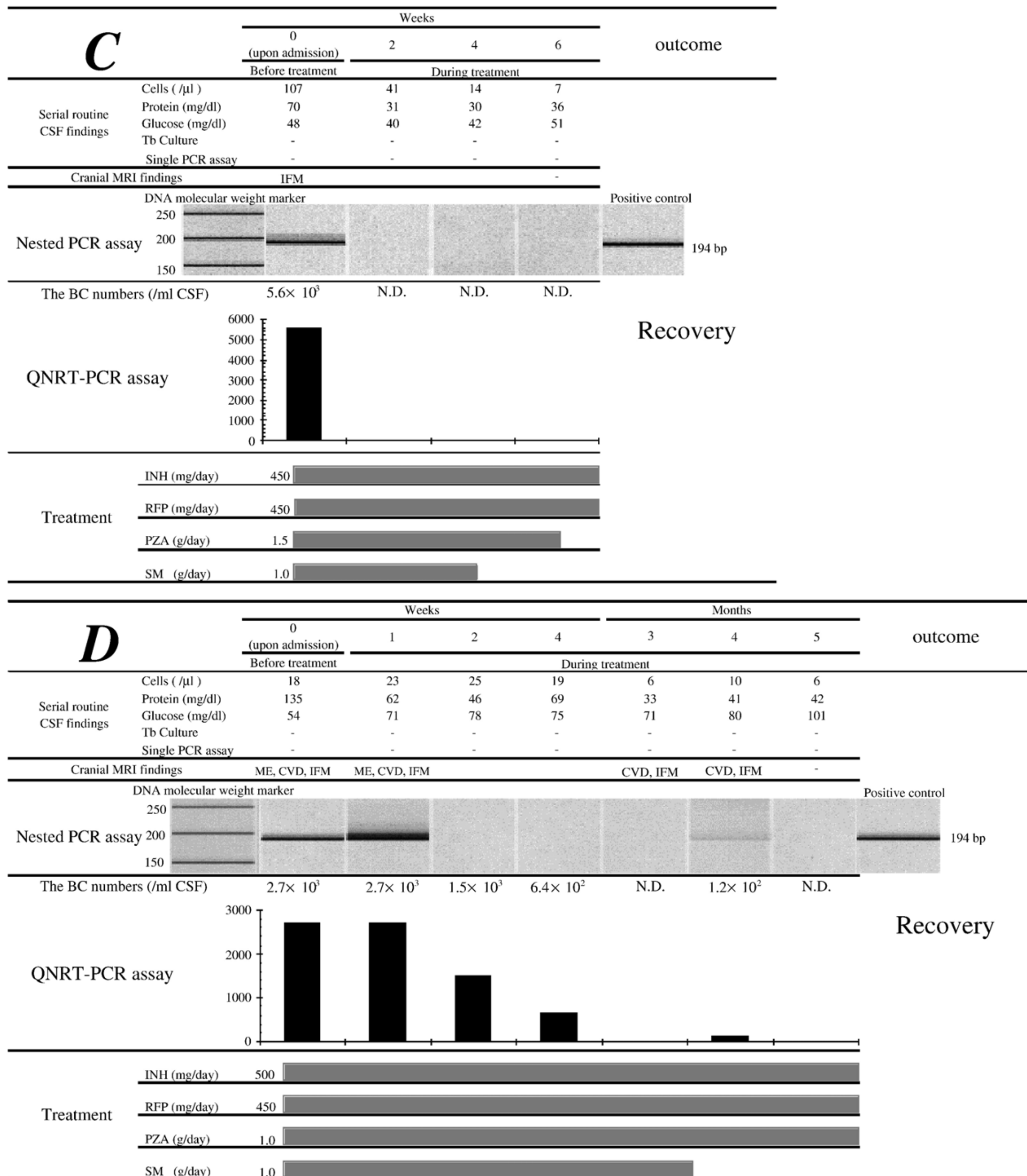

Fig. 1 (*continued*).

and TaqMan® probe-mutation-FAM (TqMn-M-FAM)) are also shown in Table 1. As the template, 2 µl of the first step PCR product was used. Its assay condition was subjected to the previous reported protocol [14] and the procedure used ABI PRISM 7700 sequence detector system™ (PE Applied Biosystems, Foster City, CA, USA).

TqMn-W-VIC specifically anneals to the natural sequence of MPT64 in wild *M. Tb*, whereas TqMn-M-FAM specifically anneals to only the artificial 22 random nucleotides in the M-plasmid for use as the internal control. TqMn-M-FAM has completely the same nucleotide composition as TqMn-W-VIC, but has a different and random sequence (Table 1). Therefore, the annealing efficiency of these two TaqMan® probes to the template can be regarded as the same. In the QNRT-PCR, the procedures of extraction, amplification and detection for both *M. Tb* DNA and the internal control were performed simultaneously by using the same two pairs of primers and the two probes that had equivalent

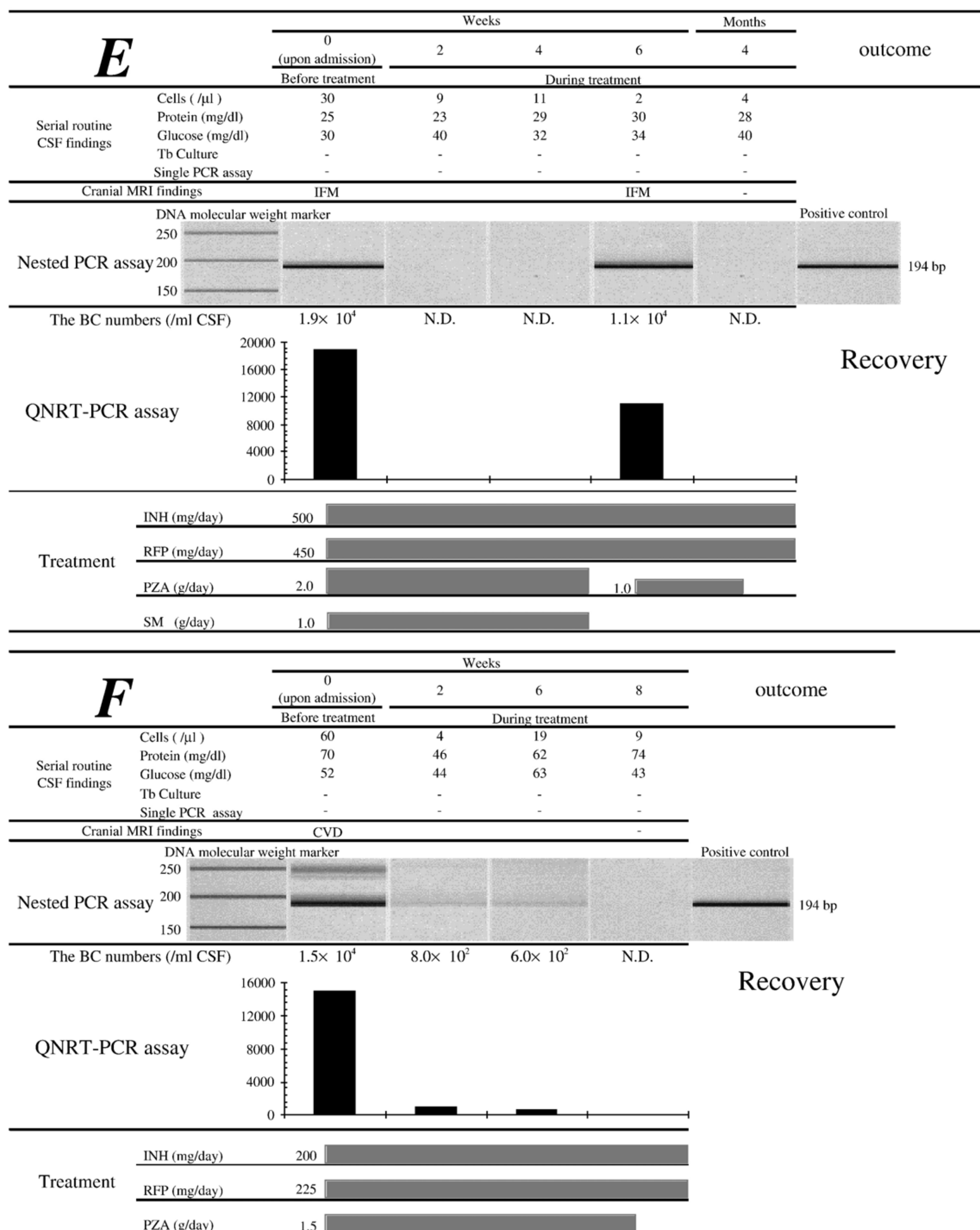

Fig. 1 (*continued*).

annealing efficiency to the templates. Therefore, the initial copy number of *M. Tb* DNA in CSF samples was able to calculate based on the amplification ratio against the internal control (10^3 copies of M plasmid) as a "ruler" [14]. In *M. Tb*, it was universally acceptable that a single copy of MPT64 gene represented one bacterial cell [4,6]. Therefore, we considered that the copy numbers calculated by the QNRT-PCR corresponded to the bacterial cell (BC) numbers of *M. Tb* in CSF samples.

3. Results

3.1. Clinical features of the patients

All 8 patients met the previously established clinical criteria and supporting evidence for TBM [1–3,6–9,13,14], and were classified as one "confirmed" cases (case 1) (positive CSF culture) and 7 "highly probable" cases (cases 2 to 8) (meeting all the clinical criteria and with three positive

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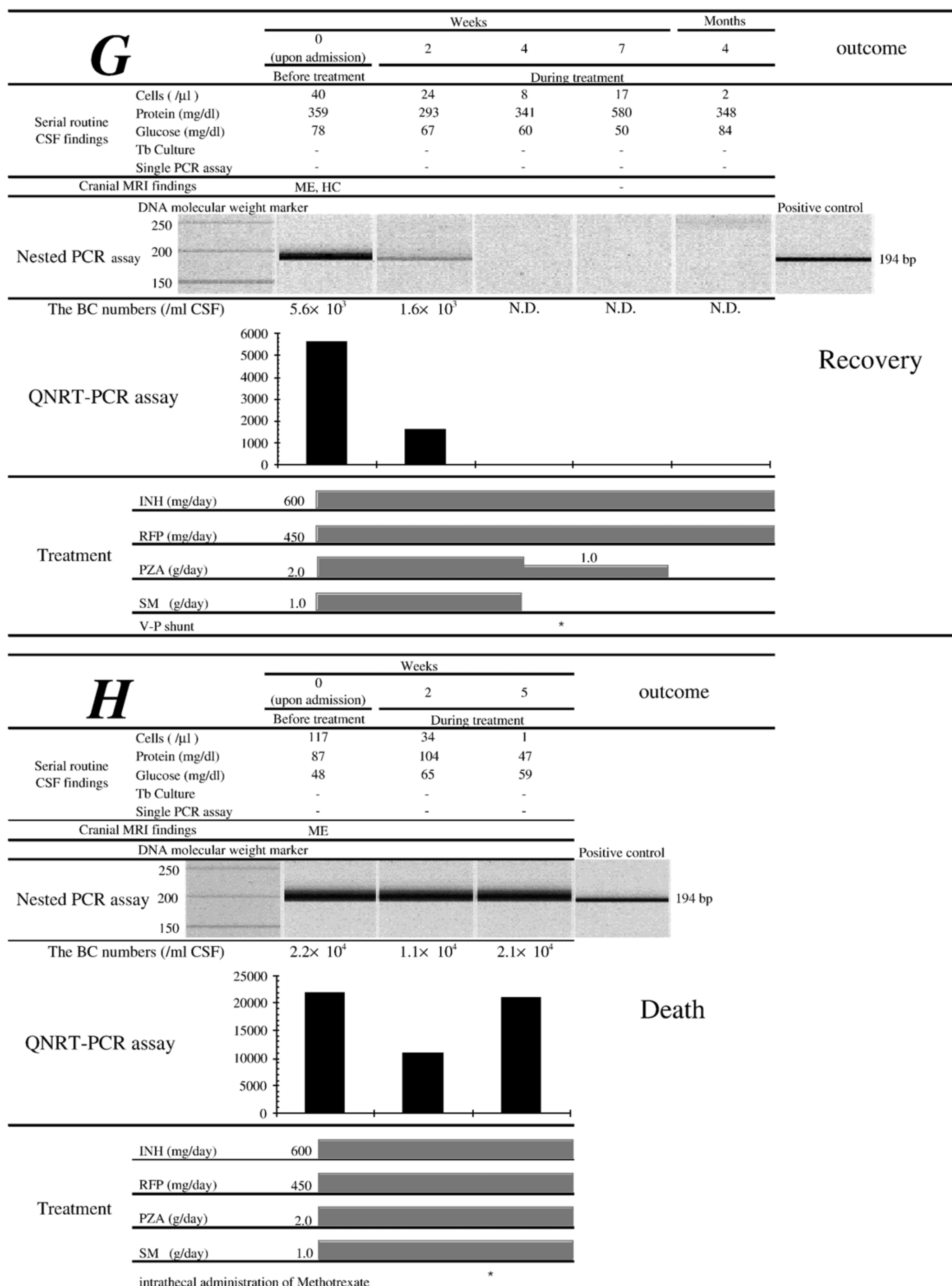

Fig. 1 (*continued*).

supporting evidences, but having no bacterial isolation) [1–3,6–9,13,14]. (These 7 "highly probable" cases (cases 2 to 8) correspond to the cases 3 to 9 in the previous reported paper [see Ref. [14]). Seven patients demonstrated a good response to the ATT, but one patient (case 8) died due to disseminated tuberculosis and aggravation of the malignant background disease (adult T-cell leukemia (ATL)).

3.2. QNRT-PCR assay results during the clinical course; diachronic study

Fig. 1 summarizes the diachronic study results, which include cultures for *M. Tb*, conventional single and nested PCR assays, the QNRT-PCR assay and other routine CSF findings in the 8 patients. The cultures for *M. Tb* and the

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single PCR assays revealed positive results in only case 1 (Fig. 1A) during the clinical treatment course. In contrast, both the nested PCR and QNRT-PCR assays revealed significant alterations during the clinical course in all 8 patients. The nested PCR assay exhibited positive results in 22 out of 43 serial CSF samples (51.2%). In the QNRT-PCR assay, the quantitative detection of *M. Tb* DNA was possible in 24 (55.8%). In addition, both PCR assays revealed all negative results for 20 patients in the non-TBM control group and the 6 reference strains of non-*M. Tb* species (data not shown). While, positive results for the HBB-PCR as the internal control were found in all CSF samples (data not shown).

The BC numbers of *M. Tb* (per 1 ml of CSF) analyzed by the QNRT-PCR assay decreased gradually to below the detection limit in the 7 patients (cases 1 to 7) that demonstrated improvement in both their clinical conditions and routine CSF findings during the ATT. Similarly, the 194 bp positive bands of the nested PCR assay converted from positive to negative in these 7 patients. Particularly, in cases 1, 6 and 7, the BC numbers gradually decreased and these changes were significantly correlated with the reductions in the intensity of the 194 bp bands (Fig. 1A, F and G). In cases 1 and 2, early increases in both the copy numbers and the intensity of the 194 bp band were demonstrated at 1–2 weeks after the initiation of ATT (Fig. 1A and B). This early increase also correlated with the worsening of the patient's clinical conditions and routine CSF findings. In cases 4 and 5, which demonstrated multiple intracranial focal masses (IFMs) on MRI (Fig. 1D and E), after conversion to negative, both the nested PCR and QNRT-PCR assays revealed transiently positive once again during the course of the ATT (Fig. 1D and E). Each of the BC numbers at 4 months (case 4) and at 6 weeks (case 5) after the initiation of ATT reflected the intensity of the transient 194 bp bands. In case 8, who died due to aggravation of ATL, the BC numbers were continually at a high degree throughout the clinical course (Fig. 1H). Similar results were demonstrated for this patient in the nested PCR assay.

4. Discussion

At present, TBM has decreased markedly due to advancement and prevalence of available anti-tuberculosis drugs [5,10–15]. However, TBM still remains a serious clinical and social problem, with increasing the number of immunocompromised hosts caused by the recent prevalence of AIDS, increasing numbers of older people, the wider use of immunosuppressive agents, and so on [5,10–15]. In TBM, accurate and rapid diagnosis and early starting ATT are the most important factors with regard to the prognosis and the prevention of long-term neurological sequelae [1–8,10–15]. Now, although the "gold standard" for TBM diagnosis is bacterial isolation such as direct smear for AFB and culture for *M. Tb* in CSF samples, they are inadequate for early diagnosis, owing to the poor sensitivity or the long time required (4–8 weeks) [1–9,13,14]. Therefore, in place of conventional bacteriological examinations, a new reliable diagnostic tool for TBM is needed [1–8,10–14].

In this diachronic study, both the nested PCR and QNRT-PCR assay results revealed prominent alterations during the clinical course, and these alterations were significantly correlated with the improvement of the patient's clinical conditions and routine CSF findings due to ATT. In contrast, conventional methods such as cultures and single PCR assays were completely inadequate for use in assessing the clinical course of TBM patients. This study was strictly adopted from the previous reported precautions to avoid sample contamination throughout the entire experimental procedure [14]. Therefore, there was no false positive due to sample contamination in this study, and the authors considered that the nested PCR and QNRT-PCR assay results are reliable. Previously, the authors reported a diachronic study that examined the usefulness of the nested PCR assay for assessing the clinical course of TBM [13]. Since the QNRT-PCR assay has the great advantage of being able to calculate the initial BC numbers of *M. Tb* in CSF samples as a numerical value, it is a more useful and superior method for assessing the clinical course and the ATT response of TBM patients as compared to the conventional nested PCR assay. For example in case 2, in which serious hydrocephalus occurred as a complication of TBM due to severe meningeal adhesion, there was no effect from the standard ATT in early stage. The early increase of the BC numbers in 1–2 weeks well reflected the early worsening of the patient's clinical conditions and the lack of effect of the standard ATT. The clinical conditions and routine CSF findings of this patient improved for the first time due to the intrathecal administration of isoniazid (INH). Interestingly, the BC numbers decreased gradually to below the detection limit and this reduction was significantly correlated with the successful intrathecal administration of INH. In addition, the authors speculate that the transient detections of *M. Tb* DNA by the nested PCR and QNRT-PCR assays in cases 4 and 5 during the ATT were correlated with the multiple IFMs on MRI. In these two cases, the IFMs disappeared at 5 and 3 months after the initiation of ATT respectively, during the follow-up MRIs (Fig. 1D and E). Both the nested PCR and QNRT-PCR assay results became completely negative with the disappearance of the IFMs. Since a biopsy to the IFM was not performed in both cases, it was impossible to prove the presence of *M. Tb* in the IFMs. However, it is possible that a small amount of *M. Tb* might have survived within these IFMs. These results may suggest that the standard ATT regimen should be reevaluated and modified based on the QNRT-PCR assay results during the clinical course of TBM (i.e., drug types, dosage, administration route (e.g., intrathecal administration of INH), etc.). However, assessing the clinical course of TBM is difficult, since TBM has many complications such as hydrocephalus and vasculopathy, which are really not dependant upon the presence of bacterial protein or DNA in the CNS but rather on the immunological reaction of the host [1–8,13,14].

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In conclusion, the QNRT-PCR assay is a novel and advanced technique that combines the high sensitivity of nested PCR and the accurate quantification of real-time PCR. To establish the superiority of the QNRT-PCR assay for assessing the clinical course of TBM versus other conventional methods, it will be necessary to accumulate data from a larger number of patients with suspected TBM. The authors speculate that if the QNRT-PCR assay is widely adopted within clinical practice, it will be a powerful tool for the rapid and accurate diagnosis of TBM.

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