Efficient diagnosis of tuberculous meningitis by detection of *Mycobacterium tuberculosis* DNA in cerebrospinal fluid filtrates using PCR

Sagarika Haldar,[1] Neera Sharma,[2] V. K. Gupta[2] and Jaya Sivaswami Tyagi[1]

Correspondence
Jaya Sivaswami Tyagi
jstyagi@aiims.ac.in

[1]Department of Biotechnology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India

[2]Department of Biochemistry and Department of Pediatrics, Dr Ram Manohar Lohia Hospital, New Delhi 110001, India

Tuberculous meningitis (TBM) is the most devastating form of meningitis and prompt diagnosis holds the key to its management. Conventional microbiology has limited utility and nucleic acid-based methods have not been widely accepted for various reasons. In view of the paucibacillary nature of cerebrospinal fluid (CSF) and the recent demonstration of free *Mycobacterium tuberculosis* DNA in clinical specimens, the present study was designed to evaluate the utility of CSF 'filtrates' for the diagnosis of TBM using PCR. One hundred and sixty-seven CSF samples were analysed from patients with 'suspected' TBM ($n=81$) and a control group including other cases of meningitis or neurological disorders ($n=86$). CSF 'sediments' and 'filtrates' were analysed individually for *M. tuberculosis* DNA by quantitative real-time PCR (qRT-PCR) and conventional PCR. Receiver-operating characteristic curves were generated from qRT-PCR data and cut-off values of 84 and 30 were selected for calling a 'filtrate' or 'sediment' sample positive, respectively. Based on these, TBM was diagnosed with 87.6 % and 53.1 % sensitivity ($P < 0.001$) in 'filtrates' and 'sediments', respectively, and with 92 % specificity each. Conventional *devR* and IS*6110* PCR were also significantly more sensitive in 'filtrates' versus 'sediments' (sensitivity of 87.6 % and 85.2 % vs 31 % and 39.5 %, respectively; $P < 0.001$). The qRT-PCR test yielded a positive likelihood ratio of 11 and 6.6 by analysing 'filtrate' and 'sediment' fractions, respectively, which establishes the superiority of the 'filtrate'-based assay over the 'sediment' assay. PCR findings were separately verified in 10 confirmed cases of TBM, where *M. tuberculosis* DNA was detected using *devR* PCR assays in 'sediment' and 'filtrate' fractions of all samples. From this study, we conclude that (i) CSF 'filtrates' contain a substantial amount of *M. tuberculosis* DNA and (ii) 'filtrates' and not 'sediments' are likely to reliably provide a PCR-based diagnosis in 'suspected' TBM patients.

INTRODUCTION

Tuberculosis (TB) is a devastating disease with approximately 8.8 million new cases and 1.6 million deaths each year worldwide (WHO, 2006). While pulmonary disease is the most common manifestation of TB, the involvement of the central nervous system (CNS) is associated with the most severe form of disease, namely tuberculous meningitis (TBM). Prompt diagnosis is crucial for successful disease management; the case fatality rate for untreated TBM is almost 100 %, and delay in treatment often leads to permanent neurological damage (Bonington *et al.*, 2000; Katrak *et al.*, 2000). In developing countries, TBM primarily afflicts children; the highest incidence is noted in the first 3 years of life (Tandon, 1978). It is the most commonly dreaded complication and cause of death in childhood tuberculosis and 2–4 % of all hospital paediatric admissions in India were diagnosed as TBM (Udani *et al.*, 1971). In a study of 292 autopsy-proven cases of TB, 200 were in the paediatric age group (<15 years), and CNS involvement was observed in 60 % of children compared to 31.5 % of adults (Vithalani & Udani, 1982).

Abbreviations: CIIMS, Central India Institute of Medical Sciences; CNS, central nervous system; CSF, cerebrospinal fluid; NIND, non-infectious neurological disorders; NTIM, non-tuberculous infectious meningitis; OIND, other infectious neurological disorders; qRT-PCR, quantitative real-time PCR; RML, Dr Ram Manohar Lohia Hospital; ROC, receiver-operating characteristic; TB, tuberculosis; TBM, tuberculous meningitis; USP, universal sample processing.

Supplementary tables are available with the online version of this paper.

The prompt and accurate diagnosis of TBM is a daunting challenge especially in paediatric subjects (Kumar *et al.*, 1999; Jatana *et al.*, 2000; Narayanan *et al.*, 2001) because of the difficulty in obtaining a precise history and collecting an adequate volume of cerebrospinal fluid (CSF) for laboratory investigation. Definitive diagnosis requires detection of tubercle bacilli in CSF. Smear microscopy is inexpensive and rapid but insensitive (0–20 %), while culture techniques are unacceptably slow and insensitive. Nucleic acid-based amplification (NAA) tests have emerged as potentially important tools for diagnosing TBM though no commercial test is licensed for use in non-respiratory specimens. A meta-analysis of NAA tests used in the diagnosis of TBM concluded that commercial tests yielded results with high specificity but low sensitivity while heterogeneity and low diagnostic accuracy were a concern with in-house PCR tests (Pai *et al.*, 2003). The overall test sensitivities ranged from ~2 to 100 % and specificities ranged from 75 to 100 % (Pai *et al.*, 2003; Hooker *et al.*, 2003; Jonsson & Ridell, 2003; Kulkarni *et al.*, 2005; Bhigjee *et al.*, 2007; Rafi *et al.*, 2007a, b), highlighting the need for an improved NAA-based test for diagnosing TBM.

We recently demonstrated the presence of both *Mycobacterium tuberculosis* DNA and intact bacilli in freshly collected sputum samples and suggested that amplification of free DNA may improve diagnostic accuracy in paucibacillary specimens (Pathak *et al.*, 2007). This hypothesis was tested for the PCR-based detection of *M. tuberculosis* DNA in CSF taken predominantly from paediatric subjects. DNA-based diagnosis was compared with the currently used TBM diagnostic criteria.

METHODS

Patients and samples. The approval of the Institutional Ethics Committee and informed consent of the patients was obtained for this diagnostic study, which included 167 CSF samples from 167 patients, predominantly from the paediatric age group, which were collected from Dr Ram Manohar Lohia Hospital (RML), New Delhi. CSF was subjected to biochemical and cytological analysis and the remaining sample aliquots were stored at 4 °C and subjected to PCR testing within the next 6–36 h without knowledge of the clinical diagnosis (Fig. 1). All CSF samples were collected prior to the administration of antitubercular treatment. The diagnosis of TBM was established on the basis of CSF culture, biochemistry and cytology, clinical findings, neuroimaging findings from CT/MRI scans and the occurrence of extraneural TB as described in Supplementary Table S1 in JMM Online. Neuroimaging was performed only of selected subjects under clinical suspicion of TBM. After PCR analysis, patients were classified into 'highly probable' TBM ($n=2$), 'probable' TBM ($n=10$), 'possible' TBM ($n=69$) and non-TBM ($n=86$). No culture-positive definite TBM was diagnosed. The 'highly probable', 'probable' and 'possible' TBM groups were grouped together as 'suspected' TBM ($n=81$) for analysis. The diagnoses for non-TBM control cases ($n=86$) and their classification into non-tuberculous infectious meningitis (NTIM), other infectious neurological disorders (OIND) and non-infectious neurological disorders (NIND) groups were based on clinical and laboratory findings compatible with the respective disease conditions for the individual groups (Table 1).

After analysis of 167 samples (RML, New Delhi), the performance of the PCR tests in 'filtrates' and 'sediments' was validated in CSF from 10 known culture-positive cases of TBM ('definite' TBM) from the Central India Institute of Medical Sciences (CIIMS), Nagpur.

Specimen processing and DNA isolation. CSF specimens were processed by universal sample processing (USP) methodology as outlined in Fig. 1. The utility of USP methodology to efficiently detect *M. tuberculosis* DNA by PCR in multibacillary and paucibacillary samples of pulmonary and extrapulmonary origin has been previously established (Chakravorty & Tyagi, 2005; Chakravorty *et al.*, 2005a, b, 2006; Haldar *et al.*, 2005, 2007; Pathak *et al.*, 2007). Briefly, CSF was filtered through a 0.22 μm membrane filter to yield 'filtrate' and 'sediment' fractions. Filtration concentrated and entrapped intact mycobacteria on the membrane (Kumar *et al.*, 2008). The membrane ('sediment' fraction) was divided into two halves: one half was suspended in 200 μl 0.05 % Tween 80 and inoculated on Löwenstein–Jensen medium while the other half was placed in 500 μl USP solution and subjected to processing as described by Chakravorty & Tyagi (2005). Briefly, after incubation with USP solution for 10–15 min, the contents were centrifuged at 20 000 *g* for 20 min. The sediment was rinsed twice with water and resuspended in 0.1 % Triton X-100. Smears were prepared and microscopically examined after Ziehl–Neelsen staining. The remaining sediment was incubated at 90 °C for 40 min for lysis of mycobacteria and DNA isolation. The lysate was centrifuged at 15 000 *g* for 10 min and an aliquot was used in PCR. 'Filtrate' fractions were extracted with an equal volume of phenol/chloroform (1 : 1, v/v) and chloroform and the DNA was precipitated overnight with ethanol. The DNA pellet was suspended in sterile water and an aliquot was used for PCR. Two different methods were employed to isolate DNA since 'filtrates' contain free DNA and 'sediments' contain intact bacteria. DNA from 'filtrate' requires purification, which was done using the conventional phenol/chloroform extraction method. Purified DNA from 'sediments' was obtained after bacterial lysis using USP methodology described above.

PCR. DNA from 'sediment' and 'filtrate' fractions of all CSF samples was subjected to *devR* quantitative real-time PCR (qRT-PCR), gel-based *devR* and IS*6110* PCR assays. The qRT-PCR assay amplified a 144 bp region of *devR* (Haldar *et al.*, 2007). Fifty microlitre reactions contained 0.5 μM each primer, PCR buffer, 3 mM MgCl₂, 0.2 mM dNTPs, 0.5× SYBR Green (Amresco), 2.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and specimen DNA in an I-Cycler (Bio-Rad). The tubes were incubated for 10 min at 94 °C and 40 cycles each of 1 min at 94 °C, 1 min at 52 °C and 30 s at 72 °C. A standard curve was generated under identical amplification conditions by plotting increasing dilutions of input *M. tuberculosis* DNA (ranging from 1.4 to 1.4×10^7 genome equivalents) versus threshold cycle (C_t) and was used to determine the bacterial DNA load in the sample volume analysed. The C_t was determined from SYBR Green fluorescence measured at 72 °C in the extension stage of each thermal cycle.

The gel-based *devR* PCR assay amplified a 162 bp sequence with primers devRf3 (5′-ATCTGTTGTCCCGCATGCC-3′) and devRr3 (5′-GTCCAGCGCCCACATCTTT-3′). The assay contained 0.5 μM each primer, PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase and specimen DNA. The tubes were subjected to 10 min at 94 °C, 45 cycles each of 1 min at 94 °C, 1 min at 52 °C and 30 s at 72 °C, and finally 7 min at 72 °C. Volumes of 'sediment' and 'filtrate' fractions added to PCR were normalized; since only half of the membrane filter was processed for 'sediment' PCR 10 μl 'sediment' DNA was used versus 5 μl 'filtrate' DNA. IS*6110* gel-based PCR was performed as described by Haldar *et al.* (2007). A positive control, having *M. tuberculosis* DNA, and two negative control reactions, lacking DNA, were always included. The amplification products in gel-based assays were detected by ethidium bromide staining and visualization under UV light after electrophor-
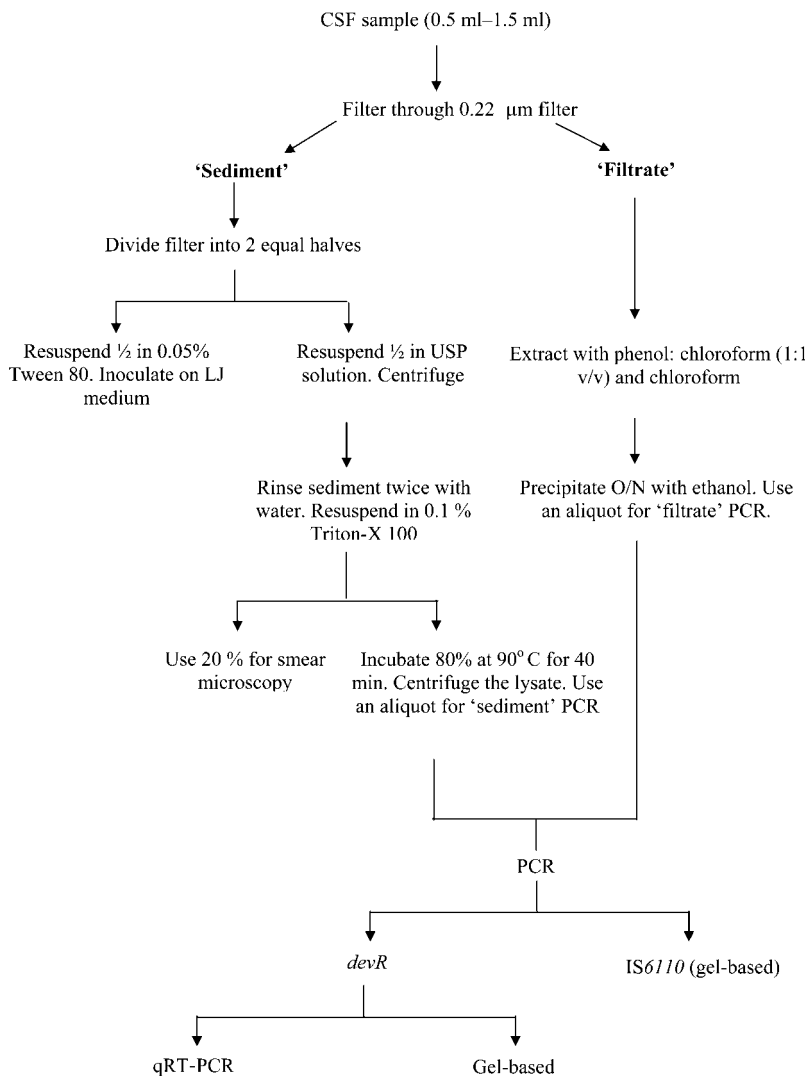
Fig. 1. Flow chart of CSF sample processing.

esis on a 2.3 % agarose gel. The absence of PCR inhibitors (residual amounts of guanidinium or phenol from the sample preparation step) in 'sediment' and 'filtrate' samples was established by inhibitor check reactions that were spiked with *M. tuberculosis* DNA.

Statistical analysis. The diagnostic performance of PCR was evaluated against that of clinical diagnosis as gold standard. The various performance characteristics were calculated as described by Altman (1991). The diagnostic accuracy was calculated as $[Tp + Tn/N] \times 100$, where $N=167$ (Tp and Tn represent true positives and true negatives). Significance of differences between various PCR assays was calculated by the chi-squared test. Receiver-operating characteristic (ROC) curves were plotted using the STATA 9.0 software (College Station, Texas, USA) and cut-off values of DNA genome equivalents were selected that conferred a high sensitivity (~ 88 %) without compromising on specificity (92 %). The cut-off values were used to call a sample positive or negative by qRT-PCR.

RESULTS AND DISCUSSION

This study evaluated 167 CSF samples for the diagnosis of TBM using PCR. After the PCR assays were completed, the subjects were grouped as 'suspected' TBM ($n=81$) and control subjects having NTIM ($n=22$), OIND ($n=21$) and NIND ($n=43$). Approximately 91 % of the subjects belonged to the paediatric age group (0–16 years), of which ~36 % were ≤3 years of age.

Conventional CSF examination is of limited utility

There were no significant clinical differences between patients from the various groups except that seizures were associated more frequently with the NIND group and positive CT/MRI findings were generally associated with 'suspected' TBM patients. Seven paediatric subjects in the 'suspected' TBM category had associated pulmonary and abdominal tuberculosis (Table 1). All patients that were clinically diagnosed with 'suspected' TBM ($n=81$) were administered antitubercular treatment and were included in the analysis. Of these, 61 subjects responded and information on 20 subjects was not available as they were lost to follow up.

Table 1. Clinical features in TBM and non-TBM patients

	Suspected TBM* (n=81)	NTIM* (n=22)	OIND* (n=21)	NIND* (n=43)	Definite TBM† (n=10)
Clinical findings					
Fever	48	19	17	29	5
Headache	18	4	3	3	3
Vomiting	22	13	8	6	1
Altered sensorium	9	2	2	1	1
Seizures	17	8	4	29	2
Focal signs	1	0	0	0	0
Neck stiffness	20	8	0	4	0
Nausea	2	0	0	0	0
Convulsions	7	4	2	5	0
Head injury	3	1	0	2	1
Nerve palsy	10	0	0	0	0
History of contact	7	0	3	1	0
CT/MRI					
Basal exudates	4	0	0	0	0
Hydrocephalus	30	1	0	3	3
Ring enhancing lesions	5	0	0	0	1
Infarcts	4	0	0	0	1
Tuberculoma	3	0	0	0	0
Pulmonary TB	5	0	0	0	0
Abdominal TB	2	0	0	0	0

*From RML, New Delhi (n=167), 'suspected' TBM category includes 'highly probable' (n=2), 'probable' (n=10) and 'possible' TBM (n=69) cases as defined in Supplementary Table S1; the NTIM group (n=22) includes nine cases of bacterial meningitis and 13 cases of viral meningitis; the OIND group (n=21) includes four cases of cerebral malaria, three cases each of bronchopneumonia, chicken pox and viral fever, and two cases each of acute gastroenteritis, sepsis, pneumonitis and enteric fever; the NIND group (n=43) includes 29 cases of febrile seizures, three cases of head injury, two cases each of Guillain–Barré syndrome, metabolic encephalopathy, intracranial space occupying lesion and brain stem glioma, and one case each of CNS lupus, CNS malignant lymphoma and hypocalcaemic seizures.

†Ten culture-positive samples of definite TBM from CIIMS, Nagpur.

All the specimens were subjected to biochemical analysis, cytology examination, USP smear microscopy, culture and PCR. Approximately 41 % of the 'suspected' TBM patients had protein levels >100 mg%. Only 6.7 % of the non-TBM group had an elevated protein level of >100 mg%, most of whom were of the NTIM category. With reference to CSF glucose levels, ~55 % of the 'suspected' TBM patients had levels <60 mg% as compared to 46.4 % of the non-TBM patients. A clear-cut difference in the biochemical characteristics of CSF samples between the various patient groups was not apparent (Supplementary Table S2). However, the CSF lymphocytes versus polymorphs response was greater in the case of 'suspected' TBM patients, ~62 % of whom had >60 % lymphocytes compared to only 15 % of non-TBM patients, most of whom were placed in the NTIM group. None of the samples were positive by microscopy in spite of using concentrated smears. Culture yield was also nil. Enhanced culture positivity could be obtained by using sediments from larger volumes of CSF (Thwaites *et al.*, 2004) and using liquid culture media versus Löwenstein–Jensen slopes. However, it is to be noted that it is extremely difficult to obtain CSF from paediatric subjects in volumes reported to give reasonably good culture yield.

devR qRT-PCR

A standard curve was generated in eight independent experiments for real-time detection of amplified *M. tuberculosis* DNA over an 8-logarithmic range of genome equivalents based on C_t data (Fig. 2a). The variation between the standard curves was marginal and demonstrated the reproducibility of the qRT-PCR assay (Fig. 2a). *M. tuberculosis* DNA was quantified in 167 CSF samples and ROC curves were generated to establish cut-offs that distinguished between TBM and non-TBM samples. The DNA load in each sample was quantified by extrapolation from the standard curve generated for the same batch of assayed samples. To construct ROC curves, DNA loads from 'suspected' TBM were considered true-positive values and those from control subjects as true-negative values. A combined ROC curve for 'filtrate' and 'sediment' samples was first constructed. However, a common threshold value that provided reasonably favourable specificity and sensiti-
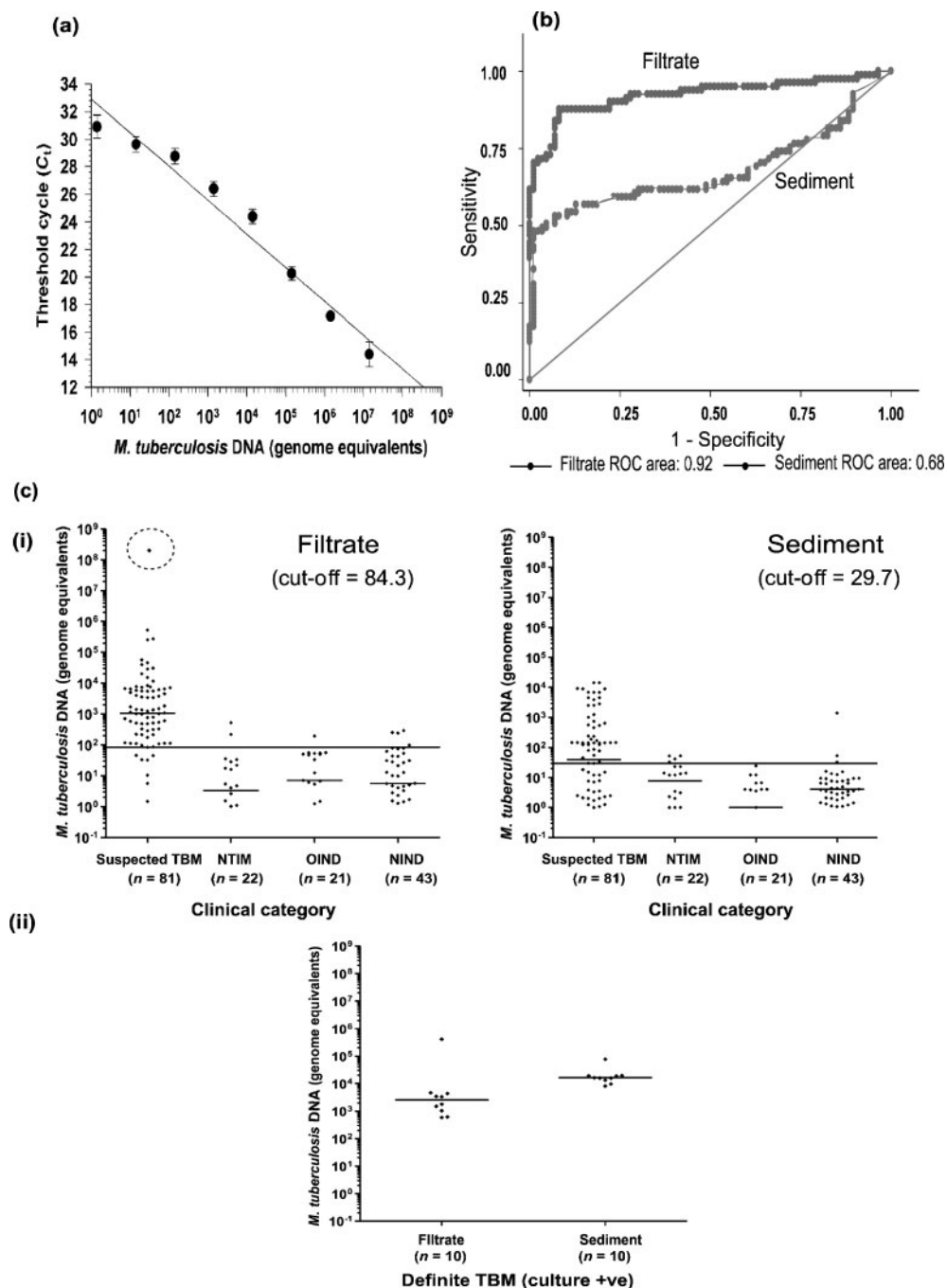
Fig. 2. (a) A qRT-PCR standard curve was generated using *M. tuberculosis* DNA over a range of $1.4-1.4\times10^7$ genome equivalents. The standard curve data from eight independent experiments (mean ± SD) are shown. C_t (threshold cycle) is plotted versus logarithm of the number of *M. tuberculosis* genome equivalents added to each tube at the start of the reaction. (b) ROC curves were generated from bacterial DNA load quantified in 167 'filtrate' and 'sediment' fractions. (c) Scatter plot showing bacterial DNA load in individual CSF 'filtrate' and 'sediment' fractions of (i) 167 samples from RML, New Delhi, and (ii) 10 definite TBM samples from CIIMS, Nagpur. The encircled value was excluded for determination of mean DNA load. The horizontal bar drawn across each dataset denotes the median value. DNA negative values are not depicted in the scatter plot. The horizontal line across the 'filtrate' and 'sediment' plots denotes the cut-off points determined by ROC curve analysis.

vity ($>\sim85\%$) was not attainable. Therefore, two separate ROC curves were constructed and values of 0.92 and 0.68 for areas under the ROC curves for 'filtrate' and 'sediment', respectively, were obtained (Fig. 2b). A 0.92 value for 'filtrate' implies that a randomly selected DNA value from the TBM group has a test value larger than that from a
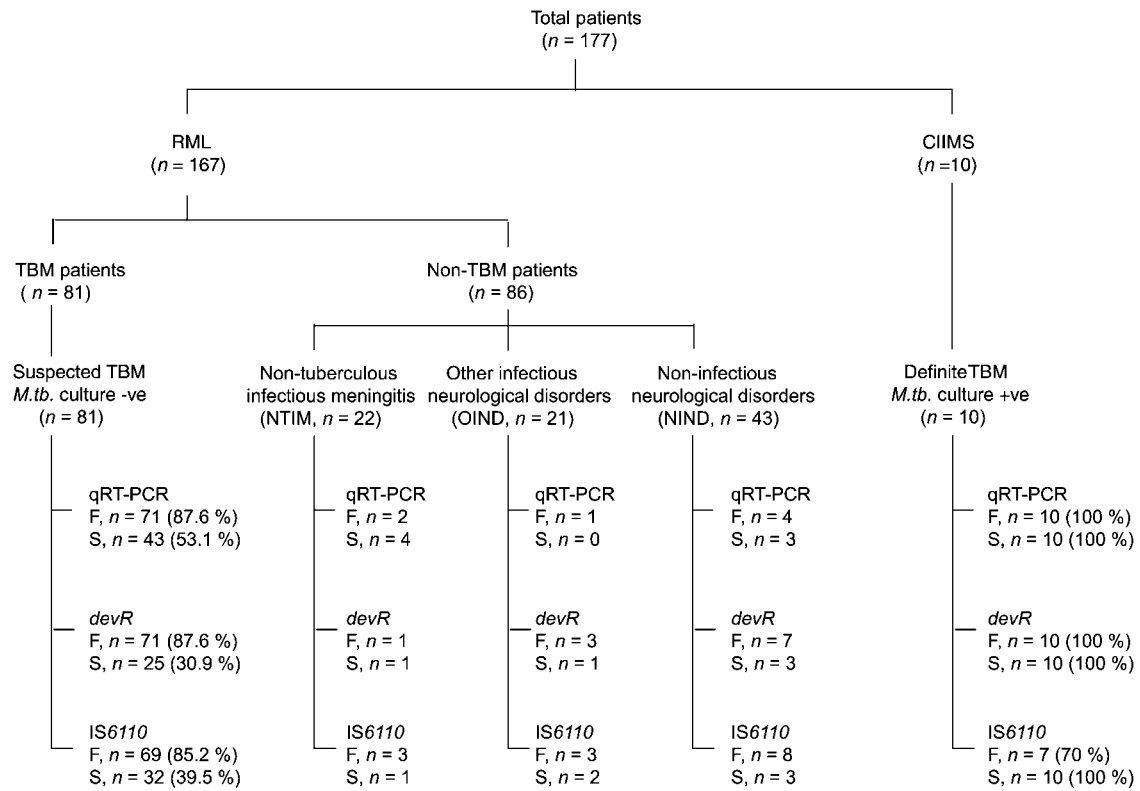
Fig. 3. Summary of PCR results obtained in various patient groups. F and S denote 'filtrate' and 'sediment', respectively. *devR* and IS*6110* are gel-based assays. Values in parentheses are % positivity.

randomly chosen individual from the control group 92 % of the time. Likewise, a 0.68 value for 'sediment' indicates that test values were larger than that from a randomly chosen individual of the control group only 68 % of the time (Zweig & Campbell, 1993). Thus to attain a specificity of 92 %, a cut-off value of 84 genome equivalents was selected for calling a CSF 'filtrate' TBM-positive. To call a 'sediment' TBM-positive at equivalent specificity, a threshold of 30 was selected. On applying these cut-offs to the study samples

(n=167), the 'filtrate' assay sensitivity was ~88 % while that of 'sediment' PCR was ~53 % (Figs 2c, 3).

Conventional gel-based PCR assays

devR and IS*6110* gel-based assays were performed on all 'filtrate' and 'sediment' fractions. PCR results are summarized in Fig. 3 and Table 2. Sensitivities of 87.6 % and 85.2 % were obtained with 'filtrate' in contrast to 31 % and 39.5 %

Table 2. Performance of the various PCR assays

Based on analysis of 167 CSF specimens from RML, New Delhi.

PCR assay	Sensitivity* (%)	Specificity (%)	Diagnostic accuracy (%)	LR+	LR−
Filtrate					
qRT-PCR	87.6	92.0	90.0	11	0.14
devR†	87.6	87.2	87.4	6.8	0.14
IS*6110*†	85.2	83.7	84.4	5.2	0.18
Sediment					
qRT-PCR	53.1	92.0	73.1	6.6	0.51
devR†	31.0	94.2	63.5	5.3	0.73
IS*6110*†	39.5	93.0	67.1	5.6	0.65

*P <0.001 for all 'filtrate' versus 'sediment' assays.
†Conventional gel-based assays.

with the 'sediment' fraction by *devR* and IS*6110* assays, respectively. Thus PCR positivity was ~2 to 3 times more in the 'filtrate' fraction versus 'sediment' fraction.

Performance of the various PCR assays

'Filtrate'-based qRT-PCR was adjudged the single best assay with sensitivity and specificity of 87.6 % and 92 %, respectively. PCR sensitivity was enhanced by 6–11 % when the results of any two 'filtrate'-based assays were considered; however, it was compromised by a 7–10 % decrease in specificity. In contrast, all the 'sediment'-based assays performed at unacceptable levels of sensitivity (~31–53.1 %; Table 2) and a large number of false-negatives were obtained (Fig. 3). It may be argued that USP treatment lowered DNA yields in 'sediments'. However, this possibility is excluded as DNA recovery was not adversely affected by USP treatment (Chakravorty & Tyagi, 2005). We conclude that restricting PCR analysis to CSF 'sediments' may be a contributory factor for poor test sensitivity in addition to other previously cited reasons such as low bacterial load, insufficient sample volume, inefficient DNA extraction and incomplete removal of inhibitory substances (Gascoyne-Binzi & Hawkey, 1999).

Most studies have used IS*6110* as a target for PCR-based diagnosis of TBM with varying degrees of success (Miörner *et al.*, 1995; Kox *et al.*, 1995; Nguyen *et al.*, 1996; Jatana *et al.*, 2000; Narayanan *et al.*, 2001; Bhigjee *et al.*, 2007; Rafi *et al.*, 2007a, b). However, this insertion element is absent in a proportion of *M. tuberculosis* isolates from India (Narayanan *et al.*, 2001; Radhakrishnan *et al.*, 2001), which argues against its utility as a sole target for gene amplification. In contrast, *devR* sequences (Dasgupta *et al.*, 2000) were universally detected in *M. tuberculosis* strains and PCR tests based on this target have been useful for pulmonary and extrapulmonary tuberculosis diagnosis (Chakravorty & Tyagi, 2005; Chakravorty *et al.*, 2005a, b, 2006; Haldar *et al.*, 2005, 2007; Pathak *et al.*, 2007). Only few studies have assessed amplification of multiple gene targets from the same CSF sample (Bhigjee *et al.*, 2007; Rafi *et al.*, 2007b). An increased sensitivity was noted when all three assays were analysed together: the sensitivity of 'filtrate' assays increased to ~99 % and that of 'sediment' assays increased to ~74 %. However, in another study, an increase in diagnostic yield was not noted on adding a second or a third target (Bhigjee *et al.*, 2007). A ~22 % increase in sensitivity was observed with qRT-PCR versus conventional PCR in 'sediment' assays. This is consistent with a recent report of ~16 % improvement in sensitivity with real-time PCR compared to conventional PCR tests when applied to CSF deposits (Bhigjee *et al.*, 2007).

A limited number of false-positive results were obtained in spite of physical separation of areas used for sample processing, PCR-setup and analysis of amplification products, meticulous handling procedures and unambiguous negative results with control PCRs lacking *M. tuberculosis* DNA. We believe that assay specificity can be further improved by use of the dUTP-uracil glycosylase system. In the present study, qRT-PCR gave the highest specificity (92 %) and was associated with the lowest false-positivity rate (7 samples were false-positive compared to the gel-based assays which detected 11 and 14 samples as false-positives; Fig. 3), which is consistent with the suggestion that cross-over contamination can be minimized in real-time PCR that employs a closed tube system.

CSF 'filtrates' are superior to 'sediments' for PCR

The highlight of this study was the demonstration of a substantial quantity of *M. tuberculosis* DNA in CSF 'filtrates' from 'suspected' TBM subjects (*n*=81, mean load 18 066 genome equivalents; Fig. 2c). The detection of a large quantity of DNA in 'filtrate' argues against the commonly held view that CSF is paucibacillary per se. Several studies have reported the use of real-time PCR for *M. tuberculosis* detection (Wada *et al.*, 2004; Aldous *et al.*, 2005; Takahashi & Nakayama, 2006; Takahashi *et al.*, 2007; Bhigjee *et al.*, 2007) but its quantification in CSF has been documented in a limited number of samples (Takahashi & Nakayama, 2006; Takahashi *et al.*, 2007). The DNA content estimated in the present study broadly agreed with that of recent reports (Takahashi & Nakayama, 2006; Takahashi *et al.*, 2007) but substantially exceeded an earlier estimation of <100 organisms ml^{-1} (Davis *et al.*, 1993). By contrast, lower amounts of DNA were recovered from 'sediments' of the same samples (mean load 1303 genome equivalents; Fig. 2c). Not a single sample that was positive by 'sediment' PCR was negative by 'filtrate' PCR. 'Sediment'-based PCR was significantly less sensitive than the 'filtrate' assays (*P* <0.001, Table 2).

The outcome of microbiological tests in CSF is reported to be volume-dependent (Thwaites *et al.*, 2004). In the present study, relatively small volumes of CSF (500 µl–1.5 ml) were adequate to achieve a sensitivity of ~88 % using 'filtrate' PCR. PCR positivity of CSF 'filtrates' indicates the occurrence of bacterial lysis, which may be an outcome of cell-mediated immune response in TBM (Thwaites *et al.*, 2000) or a consequence of antitubercular treatment (Kox *et al.*, 1995). However, the latter possibility is excluded since the samples were collected before treatment was initiated. An additional advantage of 'filtrate' PCR is that the entire 'sediment' fraction can be used for conventional microbiology.

PCR analysis of CSF from 'definite' TBM subjects

All 10 culture-positive CSF samples were also positive by qRT-PCR in both 'filtrate' and the 'sediment' fractions. Higher DNA loads were detected in individual 'sediment' versus 'filtrate' fractions, which was consistent with culture positivity of these samples; 9/10 samples had higher DNA load in 'sediments' (mean load, 21 299 genome equivalents; Fig. 2c) as compared to the corresponding 'filtrates' (mean load, 2343 genome equivalents; Fig. 2c). Amongst the

conventional assays, PCR was positive in both fractions with both targets in all CSF samples, except for an IS*6110* PCR-negative result in three 'filtrates'.

Comparison of PCR results with conventional diagnostic parameters

Since culture yield was nil in the present study, we compared individual TBM-associated clinical and laboratory criteria proposed by Ahuja *et al.* (1994) to PCR positivity. The commonly observed clinical symptoms were not discriminatory enough for TBM diagnosis. Pleocytosis appeared to be the most informative laboratory parameter for ruling in cases of infectious meningitis that included 'suspected' TBM and NTIM groups. Approximately 62 % (50/81) of 'suspected' TBM patients had a lymphocytic predominance versus only ~23 % (5/22) of NTIM patients. Therefore, by corollary, a CSF sample with predominantly neutrophils was unlikely to indicate TBM. Amongst 50 subjects of the 'suspected' TBM category with a lymphocytic predominance, 46 were positive by two or more PCR assays; by contrast, amongst five subjects of the NTIM group with a lymphocytic predominance, only one CSF sample was positive by one PCR test. However, Kumar *et al.* (1999) reported that 36 % of paediatric subjects with TBM had a predominant polymorphonuclear type of CSF pleocytosis. These results point to limitations in the use of CSF cytology in an individual subject. Imaging techniques such as CT/MRI are being increasingly used in conjunction with conventional tests to diagnose TBM. In our study, CT/MRI analysis was performed on 44/81 'suspected' TBM patients and ~91 % (40/44) were positive by two or more PCR assays. The evidence of extraneural TB is also a parameter used to diagnose TBM. In our study, CSF PCR in all seven subjects having extraneural TB was positive by two or more assays.

Utility of 'filtrate' PCR in TBM diagnosis

The two *devR*-based 'filtrate'assays matched in sensitivity (87.6 %) and were significantly superior to the corresponding 'sediment' assays (Table 2; $P < 0.001$). qRT-PCR yielded a positive likelihood ratio of 11 for the 'filtrate' assay versus 6.6 for the 'sediment' assay, which suggests that a positive qRT-PCR result will be associated with the presence of the disease. A test result is linked with the presence of the disease when the likelihood ratio is >10 whereas a test result is associated with the absence of disease when the likelihood ratio is <1. The further likelihood ratios are from 1, the stronger the evidence for the presence or absence of disease; thus likelihood ratios >10 and below 0.1 are considered to provide strong evidence to rule in or rule out disease in most situations (Deeks & Altman, 2004). Our findings indicate that the 'filtrate' assay (LR+ =11) would be useful to rule in TBM. However, due to the poor sensitivity of the 'sediment' assay, a negative test result does not mean that the subject does not have TBM. A somewhat lower PCR specificity was noted in the present study compared to that of the

commercial tests (92 % vs 98 %; Pai *et al.*, 2003) while the sensitivity was substantially higher (~88 % vs 56 %; Pai *et al.*, 2003). Note that no commercial test is licensed for diagnosis of TBM or any other form of extrapulmonary TB. Therefore, rapid tests are required with better diagnostic accuracy, and the present report is a step in this direction.

The *devR* gel-based assay (87 %) was equally sensitive as qRT-PCR (87.6 %) and would be a useful tool for clinicians in settings that do not have access to real-time PCR instrumentation. In summary, an excellent diagnostic accuracy of 90 % was achieved using limited volumes of CSF in 'filtrate'-based *devR* PCR. These tests hold promise as valuable aids in the rapid diagnosis of TBM, particularly in the paediatric age group. Lastly, the presence of lysed bacteria in CSF is likely to have implications for detecting DNA of other CNS pathogens which are often difficult to culture.

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