Evaluation of PCR Using TRC$_4$ and IS*6110* Primers in Detection of Tuberculous Meningitis

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We have evaluated a new set of primers (TRC$_4$) in comparison with the IS*6110* primers commonly used in PCR to detect tuberculous meningitis among children. The levels of concordance between the results of IS*6110* PCR and TRC$_4$ PCR with cerebrospinal fluid specimens from patients with clinically confirmed tuberculous meningitis were 80 and 86%, respectively. Results with the two primer sets were concordant for 55 positive and 22 negative specimens ($n = 98$). We conclude that the sensitivity of PCR can be increased by using both IS*6110* and TRC$_4$ primers.

Tuberculosis (TB) is the major cause of death worldwide and is due to a single pathogen (16). Childhood TB in general and TB meningitis in particular owe their existence to unsuspected, undiagnosed, or incompletely treated adults in the community. Among children, mortality due to TB occurs mainly due to the neural form of TB, namely, TB meningitis. About 3% of the pediatric admissions to hospitals in India are due to TB meningitis, with reported mortality ranging from 17 to 71% (2, 14). Prompt diagnosis is critical for initiating appropriate therapy and facilitating measures to prevent dissemination of this highly contagious disease. The prevalence of childhood TB meningitis remains largely underestimated because clinical manifestations are nonspecific in early stages of the disease and bacteriologic confirmation is available only for a small proportion of patients. Also, clinical diagnosis of childhood TB meningitis is difficult due to its varied clinical presentations. Further, routinely used tests employed for clinical diagnosis of TB are inadequate to detect extrapulmonary forms of TB like TB meningitis.

PCR is currently the most sensitive and rapid method to detect extrapulmonary *Mycobacterium tuberculosis* (1, 5, 8, 11, 15). We used as a new target TRC$_4$, which was cloned and characterized previously in our laboratory (10). TRC$_4$ is a conserved repetitive element with specificity for *M. tuberculosis* complex. The aim of this paper was to compare the efficiency of a PCR with a target chosen from this cloned fragment with that of a PCR with the widely used IS*6110* sequence in detecting *M. tuberculosis* in cerebrospinal fluid (CSF) samples from children with meningitis.

CSF specimens from children suffering from meningitis, aged to 12 years, were included in the study if at least four of the following seven indicators of disease were in evidence: first, the presence of clinical features, such as gradual loss of playful activity, irritability, clouding of consciousness, convulsions, neck stiffness, and cranial nerve or motor defects; second, a CSF lymphocyte count at admission greater than 10×10^6/liter; third, a CSF protein level at admission greater than 80 mg/dl; fourth, a CSF glucose/blood glucose ratio at admission of less than 0.5; fifth, contact with an intrafamilial adult positive for pulmonary TB; sixth, induration of 10 mm or more on tuberculin testing with 1 TU of purified protein derivative; and seventh, positive radiological features of primary complex in a chest radiography in the form of hilar lymphadenopathy and/or consolidation with parenchymal infiltrate, segmental collapse, or miliary mottling.

The response to antituberculous treatment was taken as one of the clinical criteria in the final analysis, although CSF samples were collected before initiation of treatment.

CSF cell counting and smear examination of a centrifuged CSF sample were carried out after conventional Ziehl-Neelsen staining. To rule out CSF pyogenic meningitis, we performed Gram staining and culture by inoculation on chocolate and blood agars incubated at 37°C. CSF was centrifuged in a sterile container, and the pellet was resuspended in selective Kirchner liquid medium and processed for culture of mycobacteria in multiple media using standard procedures (7). Mycobacterial DNAs from the samples were amplified using the following primers: IS*6110* a (5′-CCTGCGAGCGTAGGCGTCGG-3′) and IS*6110* b (5′-CTCGTCCAGCGCCGCTTCGG-3′) and TRC$_4$ primer 1 (5′-GACAACGACGTGCGCCTACT-3′) TRC$_4$ primer 2 (5′-GACCGAATTAGCGTAGCTCC-3′).

The IS*6110* primers amplify a fragment with a length of 123 bp, while the 18-mer TRC$_4$ primers amplify a fragment with a length of 173 bp. This fragment is located in open reading frame Rv0697 in the genome of *M. tuberculosis* published by Cole et al. (3) and is a single target of the repetitive element TRC$_4$.

We examined concordance between PCR results and clinical signs of TB meningitis, as well as between the two PCRs, using bivariate two-by-two tables. The level of agreement between the methods was assessed by kappa statistics and disagreement using McNemar's test. Results were considered statistically significant when the P value was less than 0.05.

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TABLE 1. Agreement between clinical diagnosis of TB meningitis and PCR results

Clinical TB meningitis result	Total no. of CSF samples	No. of CSF samples with indicated result by PCR[a] with:			
		IS6110		TRC$_4$	
		Positive	Negative	Positive	Negative
Positive	67	54	13	61	6
Negative	29	6	23	7	22
Total	96	60	36	68	28

[a] The kappa value with IS6110 was 0.56; that with TRC$_4$ was 0.67.

Ninety-eight CSF samples were available, one each from the 98 children included in the study. Of these, clinical data were available for 96 CSF samples. A clinical diagnosis of TB meningitis was made for 67 of the 96 children from whom CSF samples were available. Of the 67 samples, only 20 (21%) led to a confirmed diagnosis of TB meningitis based on culture (17 patients), histopathology (2 patients), and autopsy (1 patient). A clinical diagnosis of non-TB meningitis was made for 29 patients. Among these, only eight patients were confirmed by bacterial culture to have pyogenic meningitis.

Table 1 presents the concordance between PCR results and clinical results confirming TB meningitis. Of the 67 clinically positive samples, 54 (80.5%) were positive by PCR using IS6110 while 61 (91%) were positive by PCR using TRC$_4$. Of the 29 clinically negative samples, 23 (79%) were negative by PCR using IS6110 and 22 (76%) were negative by PCR using TRC$_4$. Six patients whose samples were found to be positive by PCR using IS6110 were clinically assessed to have non-TB meningitis, and 13 patients who were clinically confirmed to have TB meningitis were determined to be negative by PCR using IS6110. The crude agreement was 80%, and the chance-corrected agreement was 55%. There was moderate agreement between the results of PCR using IS6110 and clinical results confirming the disease (kappa = 0.56).

Six patients whose samples were found to be negative by PCR using TRC$_4$ primers were diagnosed as having TB meningitis on clinical grounds. There was 86% crude agreement and 58% chance-corrected agreement between PCR using TRC$_4$ and clinical results confirming the disease. There was substantial concordance between the results of PCR using TRC$_4$ and clinical diagnosis (kappa = 0.67).

Table 2 shows the concordance in diagnoses of the disease by PCRs with the two sets of primers. Fifty-five samples were positive while 22 were negative by both probes. Thus, there was

TABLE 2. Agreement between IS6110 and TRC$_4$ PCR results[a]

Result by IS6110 PCR	No. of CSF samples with indicated result by TRC$_4$ PCR		Total no. of CSF samples
	Positive	Negative	
Positive	55	6	61
Negative	15	22	37
Total	70	28	98

[a] Crude agreement, 79%; chance-corrected agreement, 55%; kappa, 0.53.

concordance between the results of the two PCRs among 77 of the 98 samples. The crude agreement was 79%, and the chance-corrected agreement was 55%. There was moderate agreement between the two methods (kappa = 0.53). However, PCR with TRC$_4$ primers had a higher sensitivity than PCR with IS6110 primers in detecting clinically positive cases.

Of the 20 confirmed TB meningitis cases, PCR using IS6110 primers failed to detect one case while PCR using TRC$_4$ primers was positive in all 20 cases. Of the eight samples from patients with confirmed non-TB meningitis, six were negative by PCR and two were repeatedly positive by PCR using both probes.

While PCR has been previously used to diagnose TB meningitis (6, 9, 12, 13), most researchers have reported using insertion element IS6110 primers. We used a new target for PCR in an area where 40% of the M. tuberculosis organisms carry a single copy of IS6110 and 4% do not carry even a single copy of IS6110 (4). We found that the sensitivity of PCR can be increased by using two probes (IS6110 and TRC$_4$). In our study, there were fewer false-negative results by PCR with TRC$_4$ (9%) than by PCR with IS6110 (22%). The higher false negativity of PCR with IS6110 may in part be due to the absence of IS6110 copies.

The strength of our study is that all 20 of the culture-positive samples were also positive by PCR using TRC$_4$ primers. PCR was positive for one child with undisputed evidence on autopsy. PCR was also positive using both probes for two patients who had lymph node biopsies and skin biopsies positive for TB. Among the eight non-TB meningitis patients who were proven not to have TB by non-acid-fast-bacillus cultures, six were negative by both probes. The remaining two were positive by both probes. In these two cases, the possibility of coinfection with M. tuberculosis cannot be ruled out.

The two samples from patients who were clinically positive and responded to antituberculous therapy were negative by PCR using both probes. The negative PCR results in these cases may have been due to paucibacillary CSF from TB meningitis or due to the effect of an unknown history of prior antituberculous therapy.

The main limitation of our study is that currently there is no diagnostic test to serve as an adequate "gold standard" to evaluate PCR. While culture has low sensitivity, clinical assessment may be subjective. The test still has problems with sensitivity and specificity. The increased sensitivity may come at the expense of decreased specificity.

We found that using one more sets of primers especially from a repetitive element like TRC$_4$ could be advantageous in increasing the sensitivity of PCR. Clinical parameters such as CSF sugar and protein levels, the CSF/blood glucose ratio, a primary tuberculous lesion in the lung, hilar lymphadenopathy, miliary paucification, and calcification are not sufficient in detecting TB meningitis. Cranial imaging computerized axial tomography and magnetic resonance imaging have the potential to study the pathological changes occurring in TB meningitis but are prohibitively expensive for developing countries and continue to be surrounded by uncertainty. Further research is needed to simplify PCR and to convert it into a cost-effective technique that can be included even in an unsophisticated laboratory of a developing country.

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