Application of polymerase chain reaction for the diagnosis of tuberculous meningitis: a review

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Abstract

In the past five years, a number of reports on polymerase chain reaction (PCR) for the diagnosis of tuberculous meningitis has been published showing that it is a useful and accurate method for the early diagnosis of tuberculous meningitis. The assay has also been proven to be more sensitive over the conventional methods of bacterioloical examination and immunoassay. Sensitivity of the PCR for diagnosis of tuberculous meningitis is around 63-100% and specificity is 88-100%. In addition to the advantage of providing rapid diagnosis, this assay can also be used for the assessment of treatment response in patients. With the expected rapid increase in the number of clinical AIDS patients over the next 10 years, the incidence of tuberculous meningitis is also expected to rise accordingly. Similarly, the importance of PCR as a diagnostic tool.

Key Words: DNA, gene, Mycobacterium tuberculosis, polymerase chain reaction, tuberculous meningitis.

INTRODUCTION

The mortality and morbidity of tuberculous (TB) meningitis remain high, despite the availabliity of effective chemotherapy. Poor prognosis is directly related to delayed diagnosis [1,2]. Early accurate diagnosis is therefore very important in the treatment of TB meningitis. The diagnosis of tuberculous meningitis relies on the identification of Mycobacterium tuberculosis (M. tuberculosis) in the cerebrospinal fluid (CSF) by direct staining or culture. Because of the catastrophic nature of this illness and because effective specific therapy is available, clinicians generally cannot delay treatment. Therapy is often begun on a presumptive basis before a definitive laboratory diagnosis is made. Although there are clinical criteria for the presumptive diagnosis of this disorder (Table 1), the clinical

TABLE 1: Clinical criteria and classification for suspected tuberculous meningitis

Clinical evidence
> fever, headache, and neck stiffness of more than 2 weeks duration

Laboratory evidence
> 1. CSF study (WBC more than 20 cells/mm3, protein more than 100 mg/dl, or glucose less than 45 mg/dl or CSF/blood glucse below 40%)
> 2. Computed tomography scan finding (basal exudate, hydrocephalus, or focal brain abnormalities such as infarction or intracranial tuberculoma)
> 3. Tuberculosis outside the central nervous system or positive PPD skin test
> 4. Hyponatremia or elevated ESR

Definition of the clinically suspected TBM
> met clinical evidence and 1 or more than 1 of 4 laboratory evidence(s)

Classification of clinically suspected TBM
> highly probable TBM : met clinical evidence and 3 or 4 of 4 laboratory evidence(s)
> probable TBM: met clinical evidence and 2 of 4 laboratory evidence(s)
> possible TBM: met clinical evidence and 1 of 4 laboratory evidence(s)

CSF : cerebrospinal fluid
ESR : erythrocyte sedimentation rate
PPD : purified protein derivative

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manifestations of other forms of meningitis may mimic tuberculosis, and some of these other forms demand different kinds of specific therapy. Thus, there remains a major need for additional methods for the rapid diagnosis of TB meningitis.

RAPID DIAGNOSTIC METHODS FOR TB MENINGITIS

There are many methods used for early diagnosis of TB meningitis. Direct smear by acid fast stain is the most simple method and it provides an early and rapid diagnostic procedure. However, the method does not allow identification of the mycobacterium observed. The conventional method of culturing mycobacteria, which require 6-8 weeks is inadequate for the early diagnosis of TB meningitis. Furthermore, there are few organisms in CSF of patients with TB meningitis and diagnostic yield of CSF smear and culture is generally low, ranging from 5-23% [3].

Several new methods for rapidly diagnosing TB meingitis have been proposed. All are based on examination of the CSF. These tests may be divided into: (1) indirect methods, usually measuring a product of the host response to the infection; (2) direct methods, measuring a product of the infecting organism.

Using the indirect methods, adenosine deaminase level, an enzyme produced by T lymphocytes, was elevated in 63-100% of CSF specimens from patients with TB meningitis (4-8), However, a false positive rate was observed in as many as 16% (4-5). Studies detecting the partition of the bromide ion between serum and CSF, reflecting the integrity of the blood-brain-barrier, have revealed the sensitivity and specificity of this test were both approximately 90% in patients with TB meningitis [9-11]. However, the above two indirect methods are not available in most countries and their specificity and sensitivity in diagnosing TB meningitis has not as yet been well established. Another indirect method for diagnosing TB meningitis is the IgG antibody to tuberculin purified-protein derivative in the CSF [12-13]. This assay has however been reported to have a high false positive rate as low levels of sontibody to mycobacterial antigen in the CSF are often found in normal subjects and in patients with pyogenic meningitis from areas with a high prevalence of tuberculosis [14-15]. In addition, low levels of antibody are frequently found in the sera of healthy tuberculin-negative and tuberculin-positive persons.

It is tempting to think that recognizing mycobacterial products or constituents in CSF should be highly specific for TB meningitis. Detection of 3-(2'-ketohexyl)indole by electron-capture gas chromatography [16] and tuberculostearic acid by gas chromatography/mass spectroscopy [17] in CSF have been reported. However, the complex and expensive instrumentation used in the diagnosis of tuberculosis by detecting mycobacterial products causes limitations for use in the clinical setting. Another direct test is however the detection of mycobacterial antigen in CSF [18-19]. Mycobacteria share antigens with other microorganisms and since the antigens are readily detected by sensitive immunoassays, high false positives are frequently found in a high prevalence areas [14, 20].

POLYMERASE CHAIN REACTION (PCR)

The PCR is a versatile and powerful technique for the detection of DNA and it is increasingly being used in the diagnosis of many diseases. The advantage of PCR is that it is able to amplify small quantities of DNA in blood, CSF, biopsy tissue, and formalin fixed tissues.

PCR uses a heat stable DNA polymerase (originally isolated from thermophilic bacterium, Thermus aquaticus [21]) and synthetic DNA primers to amplify DNA sequences of interest. Samples are denatured to allow DNA to separate into single strands. Synthetic primers anneal to complementary sequences flanking the target DNA and the enzyme Taq polymerase synthesizes exact copies of the target DNA. Thirty cycles can be performed in a single tube using an automated thermal cycling machine. This relatively small input in time yields an enormous amplification of product- ideally a 2^{30} (over 1 billion) -fold amplification of a single starting molecule.

There are many infectious diseases of the nervous system which can be diagnosed by PCR [22]. The high sensitivity and specificity of PCR allow an early and accurate detection of occult or latent infections. Moreover, the success of PCR in analyzing CSF may reduce the need for invasive procedures such as a biopsy to establish a tissue diagnosis [22].

APPLICATION PCR FOR DIGNAOSIS OF TUBERCULOUS MENINGITIS

In 1990, Skankar et al. used the PCR to detect M. tuberculosis DNA in CSF specimens from 6

TB meningitis patients with negative mycobacterial cultures [23]. Although only one patient was found to be PCR-positive, the study revealed the possible sensitivity of PCR in the diagnosis of TB meningitis. Subsequently, numerous reports of using PCR in the diagnosis of TB meningitis have been published indicating the assay to be a powerful method for the diagnosis of this disorder [24-34].

Kaneko et al. used PCR to detect M. tuberculosis genome in the CSF of 5 of 6 patients with clinically diagnosed TB meningitis [24]. CSF from 10 patients with other types of meningitis and 10 normal controls all revealed negative results. This study first concluded that the PCR was an extremely rapid and highly sensitive test for the diagnosis of TB meningitis. Shankar et al. also compared PCR in CSF with conventional bacteriology and an enzyme-linked immunosorbent assay (ELISA) for CSF antibodies in the diagnosis of TB meningitis [25]. Results revealed that PCR detected 65% of clinically suspected TB meningitis, but ELISA and culture only detected 44% and 12% of cases respectively. The study of Zhuang et al. [26] showed the positive rates for PCR, detection of acid-fast bacilli, and culture of CSF specimens in 53 patients with clinical diagnosis of TB meningitis was 51.7%, 8.6%, and 1.7%, respectively. Thirty controls in the study were all negative in these three methods. When compared with routine mycobacterial examination in 61 cases of TB meningitis, the study of Pan [27] revealed the positive rate of PCR to be 86.9%, while the positive rate of routine mycobaterial examination (smear and/or culture) to be 13.1% . The same study further analyzed the 53 negative cases on routine mycobacterial examination, and the positive rate of PCR reached 86.8%. The study of Scarpelline et al. [28] revealed that CSF samples obtained before therapy from AIDS patients with TB meningitis was found to be 100% positive with PCR, but only 47% positive with culture technique. In conclusion, application of PCR in CSF to detect M. tuberculosis DNA for a diagnosis of TB meningitis is indeed more sensitive over conventional methods of bacteriology and immunoassay. Moreover, its specificity is also highly reliable.

We have used PCR to detect M. tuberculosis DNA in 20 CSF specimens from patients with clinically suspected TB meningitis and 27 CSF specimens from patients without clinically suspected TB meningitis [29]. Our results revealed that PCR was positive in 8 of 10 highly probable TB meningitis CSF specimens (80%), 4 of 6 probable TB meningitis (67%), and 2 of 4 possible TB meningitis (50%). These patients were diagnosed to have TB meningitis on the basis of clinical manifestations, laboratory findings and response to antituberculous treatment. CSF specimens from controls were all PCR-negative except one from a patient with the initial diagnosis of aseptic meningitis. Finally, the patient had a revised diagnosis of TB meningitis because the culture of CSF was also positive. We, therefore, concluded that PCR is an accurate method for the early diagnosis of TB meningitis. Moreover, studies of Liu et. al. [30], Folgueira et al. [31] and Scarpelline et al. [28] showed that the PCR had a high sensitivity and specificity in the diagnosis of TB meningitis. To summarize the reports in the literature, the sensitivity of PCR for diagnosis of TB meningtis ranges from 63-100% and the specificity from 88-100% (Table 2). Furthermore, PCR has the advantage of being a rapid test and, we believe, it can become a first-line diagnostic test for CSF samples in which tuberculosis investigation is requested.

We also used PCR to monitor M. tuberculosis DNA in the CSF of patients with TB meningitis during treatment [32]. The study showed that PCR was a sensitive test as it was able to detect mycobacterial genome in the CSF of a patient with TB meningitis for up to 3 weeks after the start of antituberculous chemotherapy (78% in sensitivity). The study of Donald et al. [33] revealed that positive results of detecting M. tuberculosis DNA by PCR was obtained up to at least 4 weeks after the start of treatment. Scarpellini et al. [28] demonstrated that PCR-positive CSF converted to M. tuberculosis DNA negative in four AIDS patients that showed improvement during treatment, but remained positive in three AIDS patients who died of disseminated tuberculosis. The CSF study also revealed that PCR became negative during the third or fourth month in patients with severe TB meningoencephalitis, whereas PCR results became negative during the second week of treatment in patient with mild meningeal involvement. The persistence of a positive PCR result was also found in patients with neurosyphilis in whom Treponema pallidum DNA was detectable in the CSF several months after effective treatment [35] and in patients with cerebral toxoplasmosis in whom Toxoplasma gondii in the blood could also be detected for up to 12 days after initiation of treatment [36]. These data indicate that PCR is valuable in the detection of M. tuberculosis DNA in patients with clinically

suspected TB in whom a diagnostic antituberculous therapeutic trial has been started. Antituberculous treatment is usually begun on the basis of the clinical suspicion. Moreover, this assay could be used for assessing treatment response in patients with TB meningitis.

PROBLEM AND SOLUTION

The sensitivity of the PCR in the detection of mycobacterial DNA in CSF specimens of patients with TB meningitis is also its potential drawback. There are 3 mycobacterial DNA sequences commonly used in the PCR protocol; a 123 bp region of the IS6110 insertion element in which multiple copies occur in the mycobacterial genome, a 240 bp region from the MPB 64 protein coding gene, and the 383 bp region of the 65 kDa heat shock protein (HSP) antigen. Lee at al. [37] used these different mycobacterial DNA sequences in their PCR protocol and found that false positive results were 62% using IS6110, 38% in 65 kDa HSP and 10% in MBP 64 protein. The possible cross-contamination of template DNA was a possible explanation for the unacceptably large number of false positives obtained with IS6110. A similar problem was also found in other studies [38-39]. The specificity of the 65 kDa HSP sequence used for PCR protocol was also lacking. The study of Walker et al. [39] showed a 35 % false positive rate in applying this sequence in the PCR protocol. The lowest number of false positive results was obtained with the MBP 64 protein coding gene. The MBP 64 protein gene may be the most specific sequence for the diagnosis of TB meningitis by PCR if extreme care is taken to reduce cross-contamination. In the literature, it was the most common sequence used for the PCR protocol (Table 2).

TABLE 2: Comparison of polymerase chain reaction protocol using different Mycobacterium tuberculosis specific sequences for the diagnosis of tuberculous meningitis.

Regions amplied	Reports	Sensitivity	Specificity	Methods*	Reference
1. MPB64	Kaneko et al.(1990)	5/6 (83%)	20/20 (100%)	PCRH	24
	Skankar et al.(1991)	15/20 (75%)	45/51 (88%)	PCRH	25
	Lee et al.(1994)	6/6 (100%)	19/21 (91%)	PCRH	37
	Liu et al.(1994)	19/21 (90%)	79/79 (100%)	NPCR	30
	Lin et al.(1995)	14/20 (70%)	26/26 (100%)	PCRH	29
2. IS6110	Donald et al.(1993)	27/43 (63%)	24/24 (100%)	PCRR	33
	Lee et al.(1994)	6/6 (100%)	8/21 (38%)†	PCRH	37
	Folgueira et al.(1994)	9/11 (82%)	14/14 (100%)	PCRH	31
	Scarpellin et al.(1995)	17/17 (100%)	24/24 (100%)†	NPCR	28
3. 65kDa HSP	Lee et al.(1994)	6/6 (100%)	13/21 (62%)	PCRH	37
	Machoda et al.(1994)	7/10 (70%)	10/10 (100%)	-	34

* Methods: NPCR = nested PCR
             PCRH = sinlge-step PCR followed by Southern Hybridization
             PCRR = Sinlge step PCR followed by a restricted enzyme SalI digestation
† Study of Lee et al. concluded that the high false positive results caused by the contamination with Mycobacterium tuberculosis DNA of the control CSF specimens .

Many procedures are used to prevent cross-contamination of sample DNA and contamination with amplified DNA products in the laboratory. Preparation of samples in a separate room or in a biosafety hood, autoclaving of solutions, dividing reagents into aliquots to minimize the number of repeated samplings, avoiding splashes, using positive displacement pipettes, and multiple assay blanks with inclusion of positive and negative controls in each run has been reported to prevent false positive with PCR [40-41]. Ultraviolet radiation which can damage DNA has been used as a simple means of "sterilizing" reagents of contaminating DNA molecules prior to their use in PCR [41]. Another approach has been the use of a nucleotide analogue, uracil (dUTP) in the PCR, which does not interfere with the extension or subsequent manipulation of the PCR product [42]. Subsequent reactions are pretreated with the enzyme uracil N-glycosylase (UNG); any carryover PCR contaminants will contain uracil, and are effectively destroyed by UNG, eliminating them from amplification.

One main problem of PCR assay was its sensitivity when testing CSF samples containing small amounts of M. tuberculosis DNA. The study of Hance et al. [43] and Brisson-Noël et al. [44] showed the sensitivity of PCR for detection of M. tuberculosis in clinical specimens could be improved with hybridization with ^{32}P-labelled oligonucleotide. Other studies have also showed that Southern hybridization technique with a radiolabeled probe increased the sensitivity of PCR 10- to 100-fold, although it was more time consuming (3 to 4 days) [45-46]. From our experience, the sensitivity of using one step simple PCR could be improved when the preparation of DNA was from the cell pellet after centrifugation of CSF (unpublished data). The test required 6-8 hours to complete and it is not only convenient but also yields rapid results. Moreover, it does not require radioisotopes in the labeling of DNA probes and less expertise is needed for the DNA hybridization procedure. In both the studies of Kenako et al. [24] as well as ours [29], hybridization after the simple PCR procedure did not yield a further positive result. Nested PCR protocol has been shown to enhance sensitivity by a 1,000-fold for the rapid diagnosis of TB meningitis compared with conventional single-step PCR protocol [47]. Using this method, Liu et al. [30] were able to detect M. tuberculosis genome within 24 hours in the CSF of 19 of 21 patients (90%) with clinically suspected TB meningitis; results were negative in all 79 controls. Scarpelline et al. [28] also used nested PCR protocol to detect M. tuberculosis DNA in CSF from AIDS patients with TB meningitis. The sensitivity was 100% for the 17 CSF samples collected before therapy and all the CSF samples from controls were negative. Although Kaneko et al. [48] claimed that single-step PCR followed by Southern hybridization is almost equally sensitive as in nested PCR, nested PCR protocal is more advantageous and convenient than Southern hybridization protocol in the rapid diagnosis of TB meningitis in clinical practice, because it requires only one working day to complete and does not need radioisotopes. However, precautions to prevent false-positives must be kept in mind while performing the protocol.

CONCLUSION

The early diagnosis of TB meningitis remains a challenge and a rapid diagnostic method has not yet been well established. Although the PCR assay is not a gold standard for the diagnosis of TB meningitis, it still remains a good diagnostic test for patients with clinically suspected TB meningitis. In addition to being highly sensitive and specific, PCR is also a powerful tool for the early and rapid diagnosis of TB meningitis in the routine diagnostic laboratory. It is feasible for use, in particular in developing countries such as Southest Asia, where tuberculosis is pandemic since detection can be achieved by simple agarose gel electrophoresis. Moreover, PCR can also be used for monitoring and assesssing the response to antituberculous treatment in patients with TB meningitis.

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