Loop-Mediated Isothermal Amplification for Rapid and Reliable Diagnosis of Tuberculous Meningitis

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Diagnosis of tuberculous meningitis (TBM) is often difficult. A reliable, simple, and rapid diagnostic test that can be performed in any standard laboratory could be helpful in TBM diagnosis. In this study, a loop-mediated isothermal amplification assay (LAMP) was evaluated to rapidly detect and diagnose TBM infection and was compared to the performance of nested PCR. Six specific primers were used to recognize the IS6110 genomic sequence from Mycobacterium tuberculosis, which included one forward outer primer, one reverse outer primer, two respective inner primers, and two loop primers. The optimum reaction temperature and time were 63°C and 60 min, respectively. Nested PCR was performed targeting the IS6110 region from M. tuberculosis using a commercial kit. The LAMP method yielded a sensitivity of 88.23% and a specificity of 80%, compared to the nested-PCR assay, which yielded a sensitivity of 52.9% and a specificity of 90% for TBM diagnosis. Comparative experiments showed that the LAMP assay is a rapid, sensitive, and specific method to detect TBM infection and that it is superior to the nested-PCR assay. LAMP is very simple, and it can be performed in any laboratory and in rural settings.

Tuberculous meningitis (TBM) is a fatal complication of the central nervous system (CNS) (10, 29). A major obstacle in treatment of TBM lies in the initial delay of treatment. This delay is caused by poor disease diagnosis at the initial onset of symptoms. Diagnosis of TBM relies on detection of Mycobacterium tuberculosis in cerebrospinal fluid (CSF) by acid-fast bacillus (AFB) staining and culturing (9). However, AFB staining of CSF is not very sensitive. Although for diagnosis of TBM in culture it has better sensitivity than AFB staining, it takes 3 to 5 weeks and is thus unable to provide the appropriate and timely diagnosis required for proper patient management (32).

During the past decade, molecular methods, such as PCR, have been widely evaluated in TBM diagnosis (4, 17, 26). In addition to conventional PCR, recent advanced technologies, like nested PCR and real-time PCR, have been used for early and rapid detection of TBM (27, 28, 31). Although nested PCR and real-time PCR are beneficial, they both require expensive equipment, as well as a huge amount of space in routine diagnostic laboratories, limiting their use to highly sophisticated facilities. These methods can be technically difficult, and they require considerable expertise, which can be a major hindrance in providing correct diagnosis of the patient.

To overcome the limitations of current molecular techniques, a new molecular-biological technique, known as loop-mediated isothermal amplification (LAMP), was developed by Notomi et al. (19). This technique has many merits. It is highly sensitive and specific, due to the fact that all six primer pairs recognize eight distinct regions in the target DNA. A large amount of product is formed, due to the autodisplacement activity of the enzyme, and because of this property, identification of a positive reaction does not require any special processing or electrophoresis (13). It can be detected by a color change of the reaction mixture in ambient light when a DNA binding dye is used. Another advantage of the assay is that the whole reaction takes about 1 h, saving a considerable amount of time (16). The most important benefit of LAMP is that it can be conducted under isothermal conditions (-ranging from 60 to 65°C), eliminating the need for specialized equipment or expertise.

Due to all these characteristics, LAMP has the potential to be adopted by any laboratory and can be used as a near-patient test. LAMP is increasingly used by various investigators to rapidly detect and type mycobacteria in pulmonary samples (1, 2, 3, 8, 12, 21, 25, 33, 34). To the best of our knowledge, there are currently no studies regarding the efficacy of the LAMP assay in diagnosing TBM infections. In the present study, a one-step, single-tube, real-time, accelerated LAMP assay targeting the IS6110 region was evaluated in diagnosis of TBM infection. Since nested PCR is the method of choice for molecular diagnosis of TBM, we compared the LAMP assay results to the results obtained by nested PCR.

MATERIALS AND METHODS

Clinical samples. A total of 27 CSF specimens were evaluated retrospectively using the LAMP assay, and the results were compared to the nested-PCR assay results. All specimens were collected from patients who were admitted to the Central India Institute of Medical Sciences (CIIMS), Nagpur, India, between September 2009 and December 2009. The patients included 17 cases of TBM (17 clinically suspected with complete clinical findings) and 10 non-TBM disorder cases (4 infectious and 6 noninfectious neurological disorders). The ages of the patients with TBM ranged from 6 to 73 years, and there were 17 males and 10...

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females. CSF samples were obtained from almost all TBM patients before initiation of anti-Koch's treatment (AKT) and were stored at −20°C until they were tested. The Institutional Ethics Committee of CHIM, Nagpur, India, approved the study.

Inclusion and exclusion criteria. This study included patients who were suspected to be infected with M. tuberculosis based on their clinical characteristics and for whom the follow-up in response to treatment was available. Patients were excluded if there was microbiological evidence of another CNS infection.

The clinical diagnosis of these groups was based on the criteria described below.

(i) TBM group (n = 17). TBM diagnosis was based on clinical features, including subacute or chronic fever and signs of meningeal irritation with or without other features of CNS abnormality. CSF findings in these patients could be described as increased protein levels, decreased glucose levels (CSF/blood glucose ratio, ≤0.5), and pleocytosis with lymphocyte predominance. Patients for whom AFB was demonstrated by smear and/or cultures were considered “confirmed” cases of TBM. In the remaining cases, evidence of tuberculosis (TB) meningitis from both computed tomography (CT) and magnetic resonance imaging (MRI), response to AKT, and high adenosine deaminase activity (ADA) values were used as the criteria for the “suspected/probable” cases of TBM diagnosis.

(ii) Non-TBM group (n = 10). Three female patients and seven male patients with a mean age of 39.25 years (age range, 19 to 73 years) with other infectious diseases, in which the CNS was involved as a concomitant viral meningitis in infections with a typical self-limited clinical course, slightly elevated CSF protein concentrations, and increased cell counts dominated by lymphocytes; 1 had cryptococcosis meningitis verified with an India ink preparation; and 5 had other noninfectious neurological diseases, i.e., 2 cases of polyneuritis, 2 cases of cerebral infarction, and 1 case of pituitary adenoma.

Microbiological investigations. CSF samples of approximately 3 to 4 ml were initially available. A total of 2 ml of CSF samples was used for routine biochemical and microbiological tests. Briefly, 2 ml of CSF was centrifuged, and a portion of the pellet was examined by Gram, India ink, and Ziehl-Neelsen stains. The remaining portion of the pellet was cultured on blood and chocolate agar plates for bacteria and fungi and on Lowenstein-Jensen medium (Becton Dickinson) and in liquid 7H9 medium (Mycobacterium Growth Indicator Tubes; Becton Dickinson) for mycobacteria. CSF cultures were incubated at 37°C for 12 weeks and examined weekly for growth. The supernatant was used in routine biochemical tests.

Phenol chloroform-based DNA extraction. Approximately 1 to 1.5 ml of sample was used to extract DNA. The DNA extracted was further used in the LAMP and the nested-PCR assays as previously described (4). Briefly, 100 μl of pellet suspensions of CSF samples were subjected to cell lysis using detergents and then purified by phenol chloroform extraction. The resulting DNA was ethanol precipitated and dissolved in 50 μl of Tris-EDTA (TE) buffer.

PCR designs. The LAMP reaction was designed using six primers targeting the M. tuberculosis IS6110 gene as previously described by Aryan et al. (2): a forward inner primer (FIP), a reverse inner primer (BIP), two outer primers (F3 and B3), and two loop primers (FLP and BLP). FIP consists of a complementary F1 sequence and an F2 sense sequence, BIP consists of a complementary F3 sequence and an F2 sense sequence, BIP consists of a complementary B3 sequence and a B2 sense sequence, and the two loop primers (the forward loop primer [FLP] and the reverse loop primer [BLP]) were designed to accelerate the amplification reaction. The primer sequences are as follows: F3, 5'-AGACCTCACCATTAGTGTCGTA-3'; B3, 5'-TCCTGGTAACCGGGATCGA-3'; FIP, 5'-ATGGAGGGTGTGGCGATGGAGCTACGTGCGTGGCCCGTTGTACGAC-3'; BIP, 5'-AGGCCATCGGACCGCGAACCCATCACGTTAGGATGCTGTCAC-3'; FLP, 5'-AGGATCTGCCAGGCTGTAG-3'; and BLP, 5'-AAGAAGCGCTACTCGAGCTGCCT-3'.

LAMP reaction. LAMP was carried out in a 25-μl reaction mixture containing 50 pmol each of the FIP and BIP primers, 5 pmol each of the outer F3 and B3 primers, 25 pmol each of the loop primers F and B, and 8 μl of the large bSt DNA polymerase fragment (New England BioLabs, Beverly, MA) in 20 mM Tris-EDTA (pH 8.8), 10 mM KCl, 8 mM MgSO4, 10 mM (NH4)2SO4, 0.1% Tween 20, 0.8 M betaine (Sigma, St. Louis, MO), 1.4 mM each dNTP, and the template DNA. The LAMP assay was monitored in real time by incubating the reaction mixture at 63°C for 60 min in a Loopamp real-time turbidimeter (LA-200; Teramecs, Japan). Positive and negative controls were included in each run, and all precautions to prevent cross-contamination were observed.

Detection of the amplification products. Three detection methods were used to analyze false-positive and false-negative results: real-time turbidity detection, agarose gel analysis, and visual detection. Changes in absorbance at 400 nm were measured in real time using a Loopamp real-time turbidimeter (LA-200) to detect changes in turbidity.

RESULTS

Figure 1 shows the ROC curve for determining the cutoff value, which was determined to be 0.0024. Specimens with absorbance equal to or greater than the cutoff value were considered positive cases.

The TBM and non-TBM CSF LAMP assay results are listed in Table 1. The samples were considered to be positive or negative on the basis of the turbidometric results. In 17 of the clinically suspected TBM patients tested using the LAMP assay, 88.23% (15/17) were positive for TBM. Clinical diagnosis of the two false-negative cases revealed that one case had HIV encephalopathy with TBM, while the other case had TBM with pyogenic meningitis. Twenty percent (2/10) of the non-TBM patients scored positive in the LAMP assay. One case was diagnosed with encephalitis, but the patient had suffered from
pulmonary TB 5 years prior to the test, raising the possibility of TB infection. The other case was diagnosed as being in a multi-infarction state. Since AKT was not initiated in either of these cases, they were considered to be non-TBM samples. Thus, the specificity of the LAMP assay was 80%. The test PPV and NPV were 88.23% and 80%, respectively.

The nested-PCR assay results for the TBM and non-TBM CSF samples are listed in Table 2. In 52.9% (9/17) of cases, the nested-PCR result was positive, while the specificity of the nested PCR was about 90%. The test PPV and NPV were 90% and 52.94%, respectively.

Table 3 shows the concordance of TB diagnosis using both LAMP and nested PCR. Eight samples were positive, while one sample was negative in both tests. Thus, there was concordance between the two test results in 9 of 17 samples, and agreement was found to be 52% (κ = 0.014). Similarly, 7 of 10 samples in the non-TBM group were negative in both tests, yielding a concordance of 80% (κ = 0.375). Two samples from the non-TBM infectious-disorder group were positive using the LAMP assay, and one of these samples was also positive using the nested-PCR assay.

To understand the reason for the high percentage of positive samples using the LAMP assay compared to that using nested PCR, we analyzed the respective OD values obtained from the LAMP assay. Table 4 lists the nested-PCR and LAMP assay results for the clinically suspected TB group. In subjects who had positive nested-PCR results, the LAMP OD values were considerably higher than for samples that had negative nested-PCR results. There was one exception that had a positive nested-PCR result and an OD value of −0.081, and this sample was therefore negative in the LAMP assay. Another negative sample in the LAMP assay had an OD value of −0.004 and was also negative in the nested-PCR assay.

Comparative analysis was performed between the three methods used to detect LAMP products: turbidometric analysis, visual detection by the naked eye, and a gel-based detection assay. Turbidometric analysis was performed as described above. Visual detection was performed with the naked eye using SYBR green I, which turns green in the presence of amplified DNA. A sample was considered positive when the reaction mixture turned green after the addition of SYBR green I dye. The endpoint determination for a positive sample by agarose gel-based detection was done by observing a typical ladder pattern. The results obtained using SYBR green I were not truly consistent with the results from the real-time turbidimeter. Figure 2 shows that all three systems were equally sensitive in detecting highly positive and negative cases. However, CSF samples from the clinically suspected TB group, in which the OD for the LAMP assay was only slightly above the set threshold limit, were not easily discriminated as positive or negative using the SYBR green assay. In all these cases, the typical ladder pattern was detected on the gel.

**DISCUSSION**

Therapy for TB treatment is usually initiated empirically on the basis of strong clinical suspicion and radiological, CSF cytological and biochemical, and ADA findings (11, 15, 30). Newer diagnostic modalities that can rapidly aid in confirming the clinical suspicion of TB are still needed. The present study in this context describes the diagnostic utility of LAMP in

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**TABLE 1. Sensitivity, specificity, positive predictive value, and negative predictive value for LAMP and nested-PCR in clinically suspected TBM and non-TBM-classified groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of clinically suspected TBM cases (n = 17)</th>
<th>No. of non-TBM cases (n = 10)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMP positive</td>
<td>15</td>
<td>2</td>
<td>88.23</td>
<td>80</td>
<td>88.23</td>
<td>80.00</td>
</tr>
<tr>
<td>LAMP negative</td>
<td>2</td>
<td>8</td>
<td>88.23</td>
<td>80</td>
<td>88.23</td>
<td>80.00</td>
</tr>
</tbody>
</table>

**TABLE 2. Sensitivity, specificity, positive predictive value, and negative predictive value for the nested PCR assay in clinically suspected TBM and non-TBM-classified groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of clinically suspected TBM cases (n = 17)</th>
<th>No. of non-TBM cases (n = 10)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR positive</td>
<td>9</td>
<td>1</td>
<td>52.9</td>
<td>90</td>
<td>90</td>
<td>52.94</td>
</tr>
<tr>
<td>PCR negative</td>
<td>8</td>
<td>9</td>
<td>52.9</td>
<td>90</td>
<td>90</td>
<td>52.94</td>
</tr>
</tbody>
</table>

**TABLE 3. Concordance between PCR and LAMP results for TBM and non-TBM patients.**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>LAMP result</th>
<th>PCR result</th>
<th>Concordance (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBM (n = 17)</td>
<td>LAMP (+15)</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>LAMP (-02)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Non-TBM (n = 10)</td>
<td>LAMP (+02)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

**TABLE 4. Nested PCR and corresponding LAMP results in individual patients from the clinically suspected TB group.**

<table>
<thead>
<tr>
<th>No. of clinically suspected TBM patients</th>
<th>Nested PCR result</th>
<th>LAMP result</th>
<th>Corresponding LAMP OD* value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive</td>
<td>Positive</td>
<td>0.21</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>Positive</td>
<td>0.081</td>
</tr>
<tr>
<td>3</td>
<td>Positive</td>
<td>Positive</td>
<td>0.255</td>
</tr>
<tr>
<td>4</td>
<td>Positive</td>
<td>Positive</td>
<td>0.257</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>Positive</td>
<td>0.095</td>
</tr>
<tr>
<td>6</td>
<td>Positive</td>
<td>Positive</td>
<td>0.089</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>Positive</td>
<td>0.312</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>Positive</td>
<td>0.378</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>Negative</td>
<td>−0.081</td>
</tr>
<tr>
<td>10</td>
<td>Negative</td>
<td>Positive</td>
<td>0.003</td>
</tr>
<tr>
<td>11</td>
<td>Negative</td>
<td>Positive</td>
<td>0.025</td>
</tr>
<tr>
<td>12</td>
<td>Negative</td>
<td>Positive</td>
<td>0.003</td>
</tr>
<tr>
<td>13</td>
<td>Negative</td>
<td>Positive</td>
<td>0.034</td>
</tr>
<tr>
<td>14</td>
<td>Negative</td>
<td>Positive</td>
<td>0.051</td>
</tr>
<tr>
<td>15</td>
<td>Negative</td>
<td>Positive</td>
<td>0.003</td>
</tr>
<tr>
<td>16</td>
<td>Negative</td>
<td>Positive</td>
<td>0.007</td>
</tr>
<tr>
<td>17</td>
<td>Negative</td>
<td>Negative</td>
<td>−0.004</td>
</tr>
</tbody>
</table>

* OD, optical density.
The high sensitivity in our study may be due to three factors. First, the sample tested was CSF, which has less inhibition than other biological samples. The second factor is the selection of primers targeting a high-copy-number gene, i.e., IS6110. By designing primers for this region, 5 fg of M. tuberculosis DNA (equivalent to 1 copy) could be detected, which was better than when other primers were used (2). We also performed experiments on serial dilutions by spiking a control CSF sample with known concentrations of M. tuberculosis and obtained a high analytical sensitivity of about 10 CFU/100 μl, demonstrating that by using the IS6110 primers, LAMP can also be effective in CSF samples. The second important point that we have taken into account is the determination of an appropriate cutoff, or threshold value, that can be helpful in discriminating between TB and non-TB samples, even with a very low load. This differs from the standard threshold value of 0.1 that had been determined earlier by several workers (7, 14, 20, 22). With this OD, a large number of samples would have proven to be false negatives. Therefore, we calculated a different threshold value, 0.0024, which was more appropriate for CSF samples. This was similar to the work done by Han et al., who used different threshold values to differentiate between different species of plasmid (6).

LAMP performance was also compared to that of nested PCR in the same set of samples. Nested PCR is the molecular method of choice for diagnosis of TB infection in samples harboring a low microbial load (27, 28). In accordance with a few earlier published reports (23, 24), in our study, the LAMP assay was more sensitive than nested PCR. The possible reason for the lower sensitivity could have been inhibition of the nested PCR, as mentioned in a previous study (5). However, in our study, this was ruled out, because all the internal controls in nested PCR yielded positive results. Even the clinical sensitivity of the test was high in our previous studies (18). The reason that we could postulate for the discrepancy between the two tests was the analytical sensitivity of the nested-PCR kit. By performing the spiking experiments in CSF, the analytical sensitivity of nested PCR was found to be 20 CFU/100 μl, which is lower than the analytical sensitivity of LAMP. It may be due to this fact that some samples with very low bacterial load have proven to be LAMP positive but nested-PCR negative. The further observation that these LAMP-positive but nested-PCR-negative samples have a low OD value with LAMP strongly suggests the presence of low bacterial loads in the samples. Further analysis of these LAMP-positive samples verified that they were positive samples, which was determined through patient follow-up. This suggests that LAMP can be useful for detecting TB cases at an early stage of disease that can be missed by other diagnostic tests.

Despite the encouraging results obtained using LAMP, there were some issues of major concern. Two of the clinically suspected TB cases appeared to be LAMP negative. Despite confirmatory observations, this issue needs to be further studied by incorporating internal amplification controls. This will be helpful in determining whether any kind of inhibition is occurring in these reactions that may be responsible for such results. Since the turbidity assay is carried out in a closed system, the risk of contamination is lower than when agarose gel electrophoresis is used, providing an additional advantage of the LAMP assay in clinical use. However, we still obtained two false-positive results using this assay. Analysis of false-positive reactions using sequencing and restriction enzyme analysis would easily distinguish between false positives and contamination. To reduce the chances of contamination, we took the necessary precautions for avoiding any discrepancies in the results; however, technical limitations were possibly responsible for erroneous results.

One of the great advantages of the LAMP assay is that...
amplification can be monitored with the naked eye using SYBR green I dye. Boehme et al. have found that this type of detection system is easy and helpful in discriminating TB and non-TB samples, and they have shown an interreader variability of only 0.4% (3). However, in our case, some samples were not easily discriminated by visual detection. Although gel electrophoresis was sensitive, real-time monitoring using an inexpensive turbidimeter was the most suitable method for interpretation of results because it was able to resolve small variabilities in sample concentrations.

The LAMP technique can be a useful option for detecting TBM infection in low-copy-number CSF samples. However, a greater number of samples have to be analyzed to compare it with other commonly used methods. Based on our study, we propose the importance of setting appropriate threshold limits, which should vary according to the biological fluid analyzed and the microbial load it harbors. For instance, it may be necessary to have a lower threshold limit for CSF and a higher threshold limit for sputum samples for the same infection, such as TB, for the assays to be clinically meaningful.

Conclusion. The LAMP assay can be beneficial in confirming TBM infection in clinically suspicious cases when small mycobacterial loads are present. It is more sensitive than nested PCR. In addition, it takes only 60 min, compared to 3 to 4 h for other molecular tests, making it beneficial for tertiary health care centers that require quick results.

ACKNOWLEDGMENT

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REFERENCES