Development of a Loop-Mediated Isothermal Amplification Assay Targeting the *mpb64* Gene for Diagnosis of Intraocular Tuberculosis

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A loop-mediated isothermal amplification (LAMP) assay targeting the *mpb64* gene for the diagnosis of intraocular tuberculosis was highly specific (100%), sensitive (85.7%), rapid, and easy to perform. The LAMP assay can be an alternative to conventional PCR for the diagnosis of ocular tuberculosis in resource-limited settings.

Intraocular tuberculosis (TB) is a disease with an extremely paucibacillary nature that is difficult to diagnose with conventional tests such as microscopy and culture. Molecular techniques such as PCR are vital for definitive diagnosis of this condition (1). However, PCR requires expensive instrumentation, which hinders its widespread use. The loop-mediated isothermal amplification (LAMP) assay relies on autocycling strand displacement DNA synthesis in the presence of Bst DNA polymerase under isothermal conditions and can be conducted in the laboratory water bath or heating block (2). We report the application of a LAMP assay targeting the *mpb64* gene for rapid detection of intraocular tuberculosis.

The study was approved by the institutional review board. A set of six LAMP primers (Table 1) targeting the *mpb64* gene in the *Mycobacterium tuberculosis* H37Rv strain (GenBank accession number NC_018143) were designed using primer explorer V4 software (<http://primerexplorer.jp/elamp4.0.0/index.html>). The *mpb64* gene was chosen since significant number of clinical iso-

lates of *M. tuberculosis* in India may have low numbers of copies (1, 2) or no copies (3) of the IS6110 gene element. Also, the sensitivity of *mpb64* detection was found to be much higher than that of IS6110 detection during PCR analysis of ocular samples in our laboratory (unpublished data). The LAMP assay was standardized according to a previously published protocol (4), except that 2.0 μ l instead of 1.5 μ l of 50 mM MgSO₄ was used. Nondenatured DNA was used as the template. The reaction mixture was incubated at 65°C for 1 h and heated at 80°C for 2 min to terminate the reaction in a thermal cycler (Veriti; Applied Biosystems), though the same could be achieved with a water bath. *M. tuberculosis* H37Rv DNA was used as the positive control and double-autoclaved Milli-Q water as the negative control. LAMP-positive amplicons were confirmed by adding 1 μ l of (1,000 $\times$) SYBR green I dye (Sigma-Aldrich) to each reaction tube and incubating in the dark for 10 min, as previously described (5). A yellowish green color (under natural light conditions) indicated a positive reaction, while reddish orange indicated a negative reaction (Fig. 1). LAMP products were verified by 2% agarose gel electrophoresis with UV light transillumination. The analytical sensitivity of the LAMP assay was found to be 10 fg per reaction (2 copies/reaction),

TABLE 1 Primers used for *M. tuberculosis* LAMP assay targeting the *mpb64* gene

Primer name	Primer sequence (5'–3')
MTB-F3	CCCCCGGGTTGAAGAAGA
MTB-B3	GGCCTATCGCAAGCCAAT
MTB-FIP	GTATCGATAGCGCGAATGCCGT
	TTTCGTTTCGTGACTGCGAAGT
MTB-BIP	TGCTTGCTCAGTTCACCTTGCAT
	TTTCACCTATGACACGCTGTGG
MTB-FLP	GCTTGACCCGGTGAATTATCAGA
MTB-BLP	AGCGGATCGGTGTCAGCCT

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FIG 1 (a) Visual detection of LAMP-amplified products. Positive reactions indicated by a yellowish green color are seen in tubes S1, S2, and S3 (patient samples) and tube PC (positive control), while a reddish orange color indicates a negative reaction in tube NC (negative control). (b) Agarose gel electrophoresis (2%) picture of LAMP assays showing DNA amplification (ladder-like banding pattern) in lanes S1, S2, and S3 (patient samples) and lane PC (positive control). No amplification was detected in lane NC (negative control). MW (molecular weight), 100-bp DNA ladder.

TABLE 2 Results of multitarget PCR and LAMP assay

Sample no.	Clinical diagnosis	Sample type	Multitarget PCR result	LAMP result
1	Anterior uveitis	Aqueous	Negative	Negative
2	Panuveitis	Vitreous	Positive	Positive
3	Retinal vasculitis	Aqueous	Negative	Positive
4	Panuveitis	Aqueous	Positive	Positive
5	Panuveitis	Aqueous	Positive	Positive
6	Anterior uveitis	Aqueous	Negative	Positive
7	Retinal vasculitis	Aqueous	Negative	Positive
8	Multifocal serpiginoid choroiditis	Aqueous	Negative	Negative
9	Intermediate uveitis	Vitreous	Positive	Positive
10	Retinal vasculitis	Aqueous	Positive	Positive
11	Retinal vasculitis	Vitreous	Positive	Positive
12	Retinal vasculitis	Vitreous	Positive	Positive
13	Panuveitis	Aqueous	Positive	Positive
14	Multifocal serpiginoid choroiditis	Aqueous	Positive	Positive

while analytical specificity was confirmed using DNA from different microorganisms, including *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, and clinical isolates of *Streptococcus pneumoniae*, *Klebsiella pneumoniae*, *Nocardia asteroides*, *Corynebacterium striatum*, *Mycobacterium fortuitum*, *Fusarium solani*, *Acanthamoeba* species, herpes simplex virus 1, cytomegalovirus, human adenovirus (kindly provided by Clinical Virology Laboratory, Christian Medical College, Vellore, India), and varicella zoster virus Oka strain (Varilix live attenuated vaccine; Smith Kline Beecham, Belgium) and human leukocytes.

For clinical verification, we included 14 intraocular samples (10 aqueous humor and 4 vitreous humor) collected from patients with presumed ocular TB (criteria described previously) (1). Twenty aqueous humor samples from patients undergoing cataract surgery were used as negative controls. DNA was extracted from all the samples using a QIAamp DNA minikit (Qiagen, Germany) and tested for *M. tuberculosis* DNA by the LAMP assay and multitarget PCR as described earlier (6). Of the 14 tested samples from patients with presumed ocular TB, 12 (85.7%) were positive by the LAMP assay and 9 (64.3%) by multitarget PCR (Table 2). The distribution of results between multitarget PCR and LAMP (PCR/LAMP) was as follows: positive/positive, 9; negative/negative, 2; positive/negative, 0; and

negative/positive, 3. Considering clinical presentation as the gold standard, the sensitivity, specificity, and positive and negative predictive values of the LAMP assay were 85.7%, 100%, 100%, and 90.9%, respectively. No amplification was observed in 20 negative controls.

LAMP assays have been used for detection of several types of infections, including ocular infections (4, 7). Our report is the first on its application in diagnosis of intraocular TB. We believe that LAMP assays can have wide application in cases of ocular TB, considering its extreme paucibacillary nature and the limited access to thermal cyclers in most countries where TB is endemic.

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