Rapid Laboratory Diagnosis of Pulmonary Tuberculosis

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Abstract

Background: Tuberculosis (TB) ranks as the second leading cause of death from an infectious disease worldwide. Early diagnosis of Mycobacterium tuberculosis in clinical samples becomes important in the control of TB both for the treatment of patients and for curbing of disease transmission to others in the community. The study objective was to perform Ziehl–Neelsen (ZN) staining, fluorochrome staining, line probe assay (LPA), and loop-mediated isothermal amplification (LAMP) assay for rapid detection of pulmonary TB (PTB) and to compare the results of LPA and LAMP in terms of sensitivity, specificity, and turnaround time. Methods: A total of 891 sputum samples from clinically diagnosed/suspected cases of TB were subjected to ZN and fluorochrome staining. Smear positive samples were subjected to LPA, and smear negative were cultured on Lowenstein–Jensen media. A total of 177 samples were subjected to liquid culture and LAMP. Conventional culture was considered as “gold standard” for calculation of parameters. Results: Light-emitting diode fluorescence microscopy had the same sensitivity as ZN with similar high specificity. LPA was performed on 548 sputum samples which includes 520 smear positive and 28 smear negative culture positive samples and multidrug-resistant TB was detected in 32.64%. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of TB-LAMP on direct sputum samples was found to be 98.96%, 95%, 96%, and 98.70%, respectively, when compared with ZN smear microscopy. By considering culture as “gold standard,” LAMP showed a sensitivity, specificity, PPV, and NPV of 98.94%, 96.34%, 96.90%, and 98.75%, respectively. The sensitivity and PPV of TB-LAMP were 98.97% and 96%, respectively, when compared with LPA. Conclusions: A successful rapid laboratory diagnosis of PTB is possible when one combines the available methodology of microscopy, culture as well as molecular techniques. The LAMP assay was found to be simple, self-contained, and efficacious for early diagnosis of suspected cases of PTB with advantages of having a high throughput, no requirements of sophisticated equipment, and complex biosafety facilities.

Keywords: Line probe assay, loop-mediated isothermal amplification, multidrug-resistant tuberculosis, tuberculosis

INTRODUCTION

Tuberculosis (TB) ranks as the second leading cause of death from an infectious disease worldwide, after the human immunodeficiency virus. Worldwide, 9.6 million people are estimated to have fallen ill with TB in 2014. Globally, an estimated 3.3% of new TB cases and 20% of previously treated cases have been detected with multidrug-resistant (MDR) TB.[1] Early diagnosis of Mycobacterium tuberculosis (MTB) in clinical samples becomes important in the control of TB, both, for the treatment of patients and for curbing the disease transmission to others in the community.

Smear examination is believed to be simple, cheap, quick, practical, and an effective case finding method for developing countries. Microscopic examination of Ziehl–Neelsen (ZN) or auramine-stained specimen allows detection of most strains in less than an hour. The fluorochrome stain offers the advantage of greater sensitivity compared with carbol-fuchsin method since a significantly larger area of the smear can be scanned per unit time with auramine fluorochrome stain.[2] In addition, a positive fluorescent smear may be restained using the conventional ZN or Kinyoun procedure.

Culture is considered the most accurate test due to its high sensitivity and specificity. However, this technique is both labor intensive and time consuming, requiring 6–8 weeks to achieve maximum sensitivity. In the last few years, various commercial molecular methods of nucleic acid amplification have been introduced for rapid TB diagnosis such as line
probe assay (LPA), GeneXpert, and loop-mediated isothermal amplification (LAMP).

Recent advances in technology have introduced many rapid and reliable methods to differentiate between susceptible and resistant MTB strains; however, due to their high cost and equipment requirement, these new methods are not feasible in the clinical laboratories of developing countries in the diagnosis of TB. Instead, these countries use the proportional method, which is very time consuming. Consequently, physicians base their diagnosis of TB on microscopy results. Therefore, supplementary rapid and reliable methods are highly needed for clinical laboratories with limited resources. In 1995, Yajko et al. used an oxidation-reduction indicator, Alamir Blue, which changes color in response to the growing bacteria. In 2004, Farnia et al. demonstrated the viability of MTB in sputum specimens of TB patients using malachite green indicator dye, a compound routinely used in Lowenstein–Jensen (LJ) medium. Malachite green is a triphenylmethane dye and has a dark green color, which becomes colorless during MTB metabolism.[4]

Kohli et al.’s study was aimed at comparing the indirect nitrate reductase assay (NRA) with the indirect proportion method in terms of speed, cost, ease of performance, and accuracy for drug susceptibility testing (DST) to first-line antitubercular drugs.[5]

Kammoun et al.’s study is to evaluate the performance of the NRA applied directly on microscopy-positive sputum samples from patients with pulmonary TB (PTB) for the detection of resistance to the first-line anti-TB drugs: rifampin (RIF), isoniazid (INH), streptomycin, and ethambutol.[6]

The LPA and GeneXpert also help in detection of drug resistance. The LPA is a DNA strip technology. The INNO-LIPA RIF-TB (Innogenetics, Ghent, Belgium) and GenoType MTBDR (Hain Life-Science, Nehren, Germany) are available commercially. The INNO-LIPA RIF-TB detects rpoB gene and GenoType MTBDR detects rpoB, katG and inhA gene. Both tests are based on reverse hybridization of amplicons to immobilized membrane bound probes, allowing the detection of mutations at the level of the most frequently mutated codons. The presence of a mutation is revealed by the absence of hybridization at the level of the wild-type probes (rpoB WT1 to WT8 and katG-WT and inhAWT1 and 2), with a possible positive hybridization signal at the level of the mutant probes.

Recently, LAMP assay has been developed as a novel technique for nucleic acid amplification. This test is used for the detection of MTB complex which includes the following species: Mycobacterium africanum, Mycobacterium bovis, Mycobacterium canetti, Mycobacterium microti, and MTB. LAMP can amplify DNA with high specificity and efficiency under isothermal conditions using six sets of primers that recognize eight distinct regions on the target sequence. Unlike polymerase chain reaction (PCR), LAMP reaction does not require a denatured DNA template and relies on autocycling strand displacement DNA synthesis by Bst DNA polymerase. The improved sensitivity is due to a large amount of amplicon generated by Bst polymerase. The large amount of DNA is generated in less than an hour, and a positive LAMP reaction can be visualized with the naked eye using a 254 nm wavelength ultraviolet (UV) transilluminator without the need for gel electrophoresis or any other instrumentations.[7]

Janes and O’Grady review outlined how next generation sequencing, through fast and accurate whole genome sequencing and/or shotgun metagenomics sequencing, holds the potential to transform the diagnosis and management of TB both for the individual patient and as an epidemiological tool.[8]

**Methods**

This study was carried out at the Biosafety level III TB laboratory of a tertiary care hospital.

**Study population and sample collection**

A total of 891 patients were enrolled in this study over a period of 18 months. Clinically suspected/diagnosed PTB cases were included in the study while cases of extra PTB were excluded from the study. All sputum samples were received in the TB laboratory from clinically suspected/diagnosed cases of PTB. Two samples per patient as per RNTCP guidelines were taken. These were subjected to direct smear by ZN and auramine staining.

**Ziehl–Neelsen staining**

Direct smears were made from the thick purulent part of sputum specimens, air dried, heat fixed, and stained by ZN staining method. The smears were scanned with an oil immersion objective and graded according to RNTCP guideline.[9]

**Fluorochrome staining**

Fluorescence stained smears were scanned under lower magnification and graded according to the International Union Against Tuberculosis Lung Disease grading using light-emitting diode (LED) fluorescent microscope (FM).

**Sample processing**

The sputum samples were decontaminated and concentrated using NALC–NaOH concentration method. Smear-positive samples were subjected to LPA while smear-negative samples were put up for culture using solid LJ media, and the culture-positive isolates were then subjected to LPA. A total of 177 samples (97 smear positive and 80 smear negative) were subjected to LAMP and liquid culture (mycobacterial growth indicator tube [MGIT]).

**Liquid culture: Mycobacterial growth indicator tube**

The MGIT works on the following principle: The MGIT tube has fluorescent compound embedded in silicone at the bottom of the tube, and this fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially, the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence can be detected. Later, actively respiring microorganisms consume...
the oxygen and allow the fluorescence to be detected. Methodology for MGIT was as per kit literature of BBL™ MGIT™.

**Line probe assay**

LPA is a DNA strip technology using a cellulose acetate membrane strip for the identification of MTB complex and detection of its resistance to RIF and/or INH. The methodology followed for LPA was as per kit literature of GenoType MTBDR plus version 2 kit (Hain Lifescience Germany). It is a qualitative in vitro test for the identification of the MTB complex and its resistance to rifampicin (RMP) and INH from pulmonary smear-positive clinical specimens and culture samples. The LPA involves DNA extraction from clinical specimens or cultured material, multiplex amplification with biotinylated primers and reverse hybridization. Interpretation of results is done using the reference template provided with the kit.[10]

**Loop-mediated isothermal amplification**

Nu-LAMP TB kit is an in vitro diagnostic LAMP test for detection of MTB complex. The assay also uses a set of four primers which recognize six regions on pathogen target which makes the assay highly specific. The target region selected in the pathogen genome is rpoB region of MTB complex. The whole procedure consists of two steps – DNA extraction from clinical specimen or cultured material and the LAMP reaction. The methodology for LAMP was followed as per kit literature using the Nu-LAMP TB kit of RAS Lifesciences Private Limited.[7]

**DNA extraction**

RAS-DNA extraction kit was used for the extraction. A 500 µl of the smear-positive decontaminated sputum specimen was used for the DNA extraction. The DNA extraction kit is a modification of a procedure based on separating contaminating protein from DNA by salt precipitation. It involves digestion of cellular proteins, subsequent removal of proteins by salting out precipitation of DNA with isopropanol, and resuspension of buffer.[11]

**Loop-mediated isothermal amplification reaction**

The Working solution for LAMP reaction was prepared by using RAS-SYBR (fluorescent dye) and RAS-TEB (Dilution buffer for Fluorescent Dye). Separate PCR tubes used for each sample, positive control and negative control (MBGW). The master mix was prepared in a DNA-free room, and the DNA extract was added to the master mix in a PCR hood. Both these procedures were done in separate rooms following the restricted access and unidirectional workflow protocol. PCR tubes were placed in thermocycler and tubes were incubated at 65°C for 60 min. One microliter of working SYBR solution was added into each tube. The results were analyzed by visually observing the color change in the tube against black background and/or by observing the color change in the tube when kept against UV light at short range wavelength (254 nm) using fluorescence visualizer.

**Statistical analysis**

The results were analyzed using Open Epi software. P value was calculated by using Fisher exact test at the <0.05 level of significance. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated using culture as “gold standard.”

**Results**

A total of 891 sputum specimens were received from patients attending the outpatient as well as inpatient departments of a tertiary care hospital from January 2014 to June 2015. Of these, 520 patients’ samples were smear positive. Out of 520 patients, 375 were male and 145 were female. Age group 21–30 years showed maximum smear positivity (29.42%) followed by age group of 31–40 (25.20%).

Out of the total 891 samples, 520 were smear positive and 371 were smear negative by ZN and LED-based fluorescent microscopy, respectively. Out of the 520 smear positive samples, 59, 179, 135, and 147 samples were scanty, 1+, 2+, and 3+, respectively, by ZN staining method, while out of total 891 samples, 371 were smear negative. Fifty-six samples were graded as scanty; 156, 118, and 190 samples were graded as 1+, 2+, and 3+, respectively, by fluorochrome staining.

All 520 smear-positive sputum samples were put up for LPA; results of which are depicted in Table 1.

The 371 smear-negative samples were cultured on LJ media. Of these, 28 were culture positive. These smear-negative culture-positive isolates were subjected to LPA. Of the total 28 strains, 11 were detected as MDR, while 13 were sensitive to both INH and RMP. Three strains were resistant to INH.

Sputum samples from 177 clinically suspected cases of PTB were subjected to LAMP reaction. Of these, 97 were smear-positive and eighty were smear-negative samples.

Comparison of smear microscopy (ZN and fluorochrome staining), liquid culture, LPA, and LAMP is depicted in Table 2.

Out of the total eighty smear-negative samples, three smear negatives were positive by LAMP assay and were subjected to LPA (though smear-negative samples are not endorsed by World Health Organization [WHO] for LPA). MTB complex was detected in two of these, and they were also culture positive.

| **Table 1:** Line probe assay results of Ziehl–Neelsen smear-positive samples (n=520) |
|---|---|
| **LPA results** | **Number of strains** |
| MDR | 162 |
| Pan sensitive (INH + RIF sensitive) | 295 |
| Mono INH resistant | 37 |
| Mono RIF resistant | 9 |
| No tub | 17 |
| Total | 520 |

INH: Isoniazid, RIF: Rifampin, MDR: Multidrug resistant, LPA: Line probe assay
The sensitivity and PPV of LAMP assay were 98.97% and 96%, respectively, when compared with LPA. Specificity and NPV of LAMP assay were not calculated as LPA was not performed on smear-negative samples as per the WHO guidelines [Table 3].

**DISCUSSION**

In our study, 520 out of the total 891 samples were smear positive; total smear-positive samples (58.36%) and majority of the patients (29.42%) were in the age group of 21–30 years, followed by 25.20% being in the age group of 31–40 years with 72.12% being male and 27.88% female patients. According to RNTCP status report 2012,[9] TB primarily affects people in their most reproductive years of life. Our study is in concordance with the same.

The *M. tb* fluoresces as greenish under fluorescent microscope as shown in Figure 1.

In 2010, the WHO recommended conventional FM to be replaced by LED-FM and that LED microscopy be phased in as an alternative for conventional ZN microscopy.[12] In this study, LED-FM had the same sensitivity as compared with ZN and similar specificity. A study by Bonnet et al. also showed that LED-FM had the same sensitivity as ZN with a similar high specificity.[13] In our study, the specificity was not affected by the use of a very sensitive acid-fast bacilli (AFB) cutoff (1–10 AFB/field) to define a positive smear, and the proportion of scanty results was 10.77% among positive smears. Smear reading was faster with LED-FM compared to conventional ZN microscopy. This is consistent with the 25%–66% time saving when using FM compared to ZN microscopy as reported by Marais et al. study.[14] Regarding feasibility aspects, this study confirmed the very good acceptability of the LED-FM, the long working life of the diodes in the conditions of a peripheral laboratory, and the possibility to use LED-FM without a dark room.

In Bhalla et al. study, although differences were not statistically significant, highest sensitivity was achieved with LED-FM with a good overall agreement of 82.6%. A total of 200 patients were included in this study. Sensitivity and specificity for the LED assessment, MVP assessment, and light microscopy were 83.1% and 82.4%, 78.5% and 87.5%, and 81.6% and 83.5%, respectively. Mean reading time was approximately 3 times faster than ZN microscopy. The mean time to read a negative smear was 2 min with FM and 5 min with light microscopy with time savings of 60%.[15]

In our study, LPA was performed on a total of 548 samples which included smear-positive samples and culture-positive isolates. MTB complex was not detected in 18 samples. MDR-TB was detected in 32.64% which was very high.

The LPA is directly performed on clinical samples and offers an enormous advantage to the patients. The turnaround time of LPA (24–48 h) is very short as compared with conventional DST methods, and hence, it would be highly advantageous for high-burden areas.

Various nucleic acid amplification-based methods have been developed to address the need for rapid and sensitive diagnosis of MTB and other mycobacterial infections. These methods require either sophisticated instruments for the amplification or elaborate methods for detection of the amplified products, which are major obstacles.

Application of LAMP to clinical specimens was evaluated by comparing LAMP results with acid-fast smear tests, liquid culture, and LPA. The LAMP showed excellent sensitivity 98.97%, specificity 95%, PPV 96%, and NPV 98.70% when compared with liquid culture by considering it as “gold standard.”

The LAMP reaction results were analysed by observing the color change in the tube when kept against UV light at short

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**Table 2: Comparison of Ziehl–Neelsen, culture, line probe assay, and loop-mediated isothermal amplification (n=177)**

| Results                  | ZN | Culture | LPA | LAMP |
|--------------------------|----|---------|-----|------|
|                          | Positive | Negative | Positive | Negative | Positive | Negative |
| Smear positive           | 97  | 95      | 95  | 2    | 96  | 1       |
| Smear negative           | 80  | 02      | 2   | 78   | 4   | 76      |
| Total                    | 177 | 97      | 80  | 97   | 100 | 77      |

LPA: Line probe assay, LAMP: loop-mediated isothermal amplification, ZN: Ziehl–Neelsen

**Table 3: Comparison of smear microscopy (Ziehl–Neelsen and fluorochrome staining), liquid culture, line probe assay, and loop-mediated isothermal amplification**

|                   | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | P       |
|-------------------|-----------------|-----------------|---------|---------|---------|
| Smear microscopy with liquid culture | 97.93           | 97.5            | 97.93   | 97.5    | <0.05 (significant) |
| LAMP with smear microscopy     | 98.96           | 95              | 96      | 98.7    | <0.05 (significant) |
| LAMP with liquid culture       | 98.97           | 95              | 96      | 98.7    | <0.05 (significant) |
| LAMP with LPA                 | 98.7            | NA              | 96      | NA      | NA      |

PPV: Positive predictive value, NPV: Negative predictive value, LPA: Line probe assay, LAMP: Loop-mediated isothermal amplification, NA: Not available
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The sensitivity of LAMP in smear-positive and culture-positive samples was 98.94% (94/95). Smear-negative culture-positive sample size was very low (n = 2) and both samples were detected by LAMP. Another multicenter evaluation study involving 1061 patients tested in reference laboratories in Vietnam, South Africa, Peru, and Brazil showed that TB-LAMP detected almost 97% of smear-positive/culture-positive patients and 53% of smear-negative/culture-positive patients.\(^{[16]}\)

The detection rate of LPA was found to be 54.80% (97/177) whereas LAMP was found to be 56.50% (100/177). It was 1.7% higher in case of LAMP assay. In a study by Kumar et al., LAMP assay was able to detect 8.5% additional cases over mPCR.\(^{[17]}\) Mitarai et al. reported that the sensitivity of direct LAMP (88.2%) was slightly but not statistically significantly lower than Cobas Amplicor MTB and TRC Rapid MTB.\(^{[18]}\)

In Safi et al. study, sensitivity of LAMP assay for detection of DNA of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) was 4 fg/ll, and the specificity was 100%. This assay successfully detected MAP not only in the bacterial cultures but also in clinical fecal samples and the specificity of both PCR was 100%.\(^{[19]}\)

In Sanker et al. study, Xpert and LPA have a mismatch of up to 20% with MGIT (RIF 0.5 mg/L); the concerned mutations are more frequently encountered toward both ends of rpoB RDR among the tested samples. The increased frequency of mutations associated with “sub-breakpoint low-level resistance,” hetero-resistant bacterial populations, and inherent limitations of the nucleic acid amplification tests (NAATs) and MGIT are probably responsible for the mismatch among samples. Inadequate drug exposure due to low-RIF dosages, poor directly observed therapy, and other issues associated with poor serum drug levels along with the low relative risk (RR) prevalence are the probable reasons. Extensive use of NAATs among such a population without a strictly targeted approach and confirmatory phenotypic tests with multiple RIF concentrations may result in false RR results on a massive scale.\(^{[20]}\)

These results indicate that LAMP is a highly sensitive assay and can detect small quantity of mycobacterial DNA in clinical samples.

LAMP was compared with LPA in terms of turnaround time for detection of MTB complex, wherein LPA takes 24–48 h, whereas LAMP results are obtained within 3 h. The advantages of LAMP assay over LPA are that the LAMP assay does not require the use of sophisticated instruments and helps in rapid detection of MTB.

The only advantage of LPA over LAMP assay is that it gives DST results, whereas the LAMP assay can only detect MTB complex.

**Conclusions**

A successful rapid laboratory diagnosis of PTB is possible when one combines available methodology of microscopy, culture as well as molecular techniques.

LED-based fluorescent microscopy did not have an increased sensitivity or specificity as compared to ZN microscopy, but the simplicity of staining method combined with faster viewing time, long shelf life of the LED bulbs, and the battery backup gives the method an advantage over ZN microscopy.

LPA performed on smear-positive sputum samples was sensitive and specific for detection of PTB, with the added advantage of drug sensitivity testing for RMP and INH. Furthermore, the rapid turnaround time of 48 h helps in immediate initiation of treatment.

LAMP is a rapid molecular test for detection of MTB from pulmonary samples and useful when resources limited, prevalence of TB infection high but prevalence of MDR-TB low. In Mumbai, utility of LAMP is limited as the prevalence of MDR-TB is high (35%–45%) though LAMP has a rapid turnaround time and high sensitivity; LPA was found to be highly sensitive and specific with an advantage of DST within 48 h.

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Conflicts of interest
There are no conflicts of interest.

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