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ASSESSMENT OF PCR AND LAMP TESTS FOR THE DETECTION OF *MYCOBACTERIUM TUBERCULOSIS* IN SPUTUM SAMPLES

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ABSTRACT
Molecular tests such as Loop-mediated Isothermal Amplification (LAMP) beside Polymerase Chain Reaction (PCR) are promising in the diagnosis of infectious diseases. LAMP has several characteristics such as high specificity and sensitivity, rapidity and feasibility. LAMP is promising to be the dominant technique for detection of *M.tuberculosis*, especially in limited resources settings compared with the PCR which is more complex and needs more advanced laboratories.

KEYWORDS: *M. tuberculosis*, LAMP.

INTRODUCTION
Tuberculosis is one of the most important bacterial diseases of human in tropical countries. It is a chronic granulomatous disease affecting mammals. The disease causes high morbidity and mortality rates.[1] Human disease is caused by *Mycobacterium tuberculosis*. The disease is increasingly recognized as a serious disease and worldwide public health concern.[2]

Laboratory diagnosis in developing countries depends on microscopic detection of *Mycobacterium tuberculosis* in the clinical specimens and to achieve this, the number of bacteria should be sufficient.[3,4] Rapid diagnosis and treatment are important for preventing transmission of *M. tuberculosis*.[3] Diagnosis of active TB largely depends upon initial clinical suspicion and radiographic findings, with subsequent laboratory confirmations, by bacteriologic studies.[5,6] Chest X-ray is commonly used for the diagnosis of tuberculosis but has poor sensitivity and specificity.[7] Conventional laboratory methods have a limitation of speed, specificity, and sensitivity. Sputum smear microscopy is the least expensive, simple and relatively easy to perform but has lower sensitivity and specificity rates.[8] Culture is more sensitive and is presently the yardstick for diagnosis, but the time required and frequent negative results in paucibacillary specimens are important limitations.[9] There have been numerous unsuccessful attempts to develop clinically usefulserodiagnostic kits for tuberculosis. A number of proteinatious and non-protein antigens (such as acyltrehalosis and phenolglycolipids) have been explored but they have not been proved to be clinically useful.[10] In the last decads, various molecular methods were introduced such as, polymerase chain reaction (PCR), which have been used for the detection of microorganisms in clinical samples.[11] Although, different PCR protocols are available for detection of *M. tuberculosis* higher laboratory resources required forthe PCR technique makes it disadvantageous. Loop-mediated isothermal amplification (LAMP) assay has been developed as a novel molecular technique for nucleic acid amplification.[12] LAMP can generate detectable DNA copies within limited time and under isothermal condition in low resource facilities by which the results can be visualized with naked eyes.[13]

In this study, we compared LAMP and PCR in the diagnosis of TB to find out reliable, sensitive, and feasible technique particularly under lower facilities.

MATERIALS AND METHODS

Microscopy and Culture: Early morning sputum samples were collected, firstly smears were stained with Ziehl-Neelsen (Z-N) stain (S), then cultured (C) on Löwenstein–Jensen (LJ) media and the results were obtained according to standard methods.[14] A total of 60 smears and culture (S+C+) positive and 30 smears and culture (S-C-) negative sputum samples were taken from individuals referred to the (Hospital of Chest Diseases, Wad Medani, Gazira State, Sudan). Firstly they diagnosed clinically and then laboratory according to previous descriptions with Z-N stained smears[15] and LJ culture technique.[16]

DNA extraction

DNA extraction from sputum samples was done using sucrose method[17] as follows

An equal volume of 4% NaOH was added to the sputum sample, vortexed for 10 minutes until the sample completely homogenized. About 1 ml of treated sputum was overlaid on 1ml of sterile sucrose 50% (wt /vol) in Eppendorf tube 1.8 ml and then centrifuged for 5 minutes. The supernatant was decanted and the pellet was re-suspended in 1.5 ml of Phosphate Buffer Saline (PBS), then vortexed and centrifuged for 10 minutes, then the supernatant was decanted. From DNA solvent 50µl was added to the pellet and placed in a water bath at 95 ˚C for 10 minutes and vortexed every 3 minutes. Samples were kept in -20 ˚C till used.

Polymerase Chain Reaction (PCR): PCR primers targeting *1S6110* gene were used for the detection of *M. tuberculosis* according to a previous report.[18] Presence of 123bp fragment considered as a positive result in comparison with positive control used. Sterile water was used as a negative control.

Loop-mediated isothermal Amplification (LAMP): Set of LAMP primers were selected for detection of *M.tuberculosis* targeting 16s rRNA gene[19] as shown in table 1.

Table. 1: LAMP primers (16s rRNA gene).

Primer	Sequences
FIP (F1c + F2)	CACCCACGTGTTACTCATGCAAGTCGAACGGAAAGGTCT
BIP (B1c + B2)	TCGGGATAAGCCTGGACCACAAGACATGCATCCCTG
FL	GTTCGCCACTCGAGTATCTCCG
BL	GAAACTGGGTCTAATACCGG
F3	CTGGCTCAGGACGAACG
B3	GCTCATCCCACACCGC

LAMP reaction: LAMP reaction was done using a total volume of 25 µl composed of 21µl of the master mix plus 4µl of the DNA template. Master mix for LAMP was include the following contents as in table 2.

Table. 2: LAMP Master Mix.

Mix	Volume µl
Water	3 µl
loopamp buffer	2.5 µl
Betain	5 µl
dNTPs	1 µl
Bst enzyme	1 µl
Primer mix	5 µl
SYBR Green 1	1 µl
Bovine serum albumin	2.5
Total	**21**

The reaction tubes were incubated at 65 ºC in a thermocycler (Esco, Swift MaxProThermal Cycler,Modle: SWT-MXP+SWT-MXP-BLC-1.Gardient 96 X 0.2 ml) for 60 minutes. After amplification, tubes were read under UV light using (High performance ultraviolet Transilluminator, UVP, Ultraviolet Products – Cambridge UK). Results were determined as follows: Tubes with the green solution were considered as positive, while tubes with the colorless solution were considered as negative.

RESULTS

PCR results: A total of 90 samples were examined, 60 samples were S+C+ whereas 30 samples were S-C-. PCR targeting *1S6110* gene was run for positive and negative samples. A total of 54 samples from positives were reacted positively whereas 29 negatives were found to be negative in PCR (figure1). One negative control reacted as false positive.

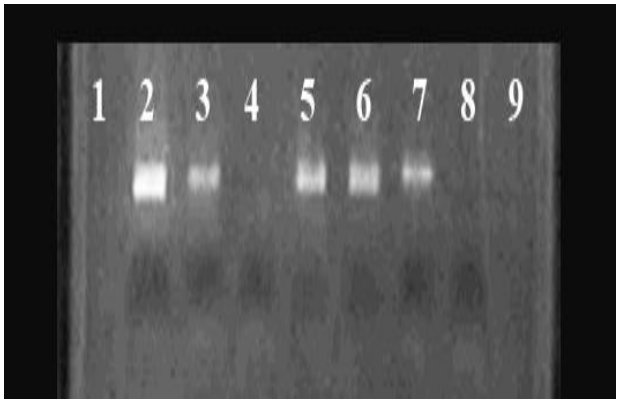

Figure. 1: PCR results. The lane (1) is a negative control; lane (2) is positive control (123bp), and lanes numbers (3, 5, 6, and7) are positive samples, while lanes number (4, 8, and 9) are negative samples.

Sensitivity and specificity, positive predictive value (PPV) and negative predictive value (NPV) parameters were calculated for PCR results obtained in this study (Table 3).

Table. 3: Achievement of PCR assay in (S+C+) and (S-C-) samples.

Sensitivity %	Specificity %	PPV %	NPV %
90.9%	96.8%	98.4%	83.3%

PPV= Positive Predictive Value, NPV= Negative Predictive Value.

Here PCR failed to detect 6 positive samples where 1 sample was reacted false positive.

LAMP results: Sixty positive S+C+ and 30 negative S-C- were examined using the LAMP technique. All negative controls were detected negative by LAMP; whereas 58 out of 60 samples reacted positive (figure 2).

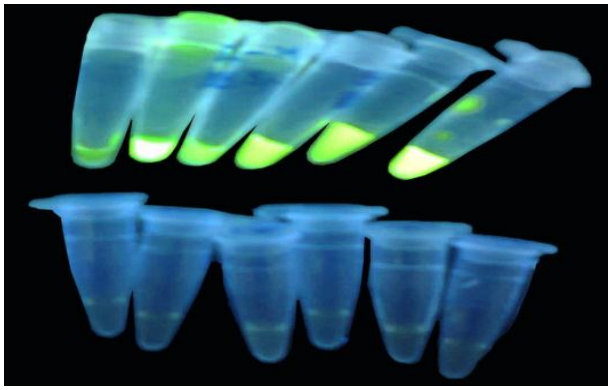

Figure. 2: LAMP results. Samples on the top with green color indicate positive results; and the colorless samples on the bottom indicate negative results.

Results of LAMP compared with Z.N and culture results in order to determine the sensitivity, specificity, PPV and NPV of LAMP are demonstrated in Table 4.

Table. 4: Achievement of LAMP assay in (S+C+) and (S-C-) samples.

Sensitivity %	Specificity %	PPV %	NPV %
96.8%	100%	100%	93.8%

PPV= Positive Predictive Value, NPV= Negative Predictive Value.

LAMP technique shows high sensitivity, specificity, PPV and NPV, as shown in (Table 6). In this study LAMP test was failed to detect just 2 samples.

Comparison between PCR and LAMP: LAMP assay showed higher sensitivity, Specificity, PPV, and NPV (Table 5). LAMP was positive in 58 out of 60 (96.8%) while PCR was positive in 54% (90.9%). In 30 negative control samples LAMP was succeeded to detect all as negative (100%) and PCR just failed to detect one negative sample which reported as a false positive result (96.8%).

Table. 5: Comparison between PCR and LAMP.

Test	Sensitivity	Specificity	PPV	NPV
PCR	90.9%	96.8%	98.4%	83.3%
LAMP	96.8%	100 %	100%	93.8%

DISCUSSION

This study set out with the aim of assessing the importance of rapid, easy, accurate and less expensive test for detection of *M. tuberculosis* in resource-poor settings. Detection of *M. tuberculosis* by LJ culture is taken as a gold standard for diagnosis in this study. Considering positive culture results in this study, PCR technique correctly identified 54 samples out of 60 culture positive samples (90.9%). Six samples were not detected by PCR. In negative culture samples, PCR technique identified 28 samples out of 30 culture negative samples (96.8%). LAMP was detected 58 as positive from 60 positive culture samples (96.8%); whereas 30 samples were tested negative with LAMP technique out of 30 culture samples (100%). Comparison between PCR and LAMP techniques was done (Table 6) the results showed high scores of LAMP technique compared with PCR technique in parameters include sensitivity, specificity, PPV and NPV. These LAMP results match those observed in earlier studies. The study targeted *gyr*B gene, LAMP sensitivity on smear and culture positive (S+C+) were 97.7% and in the same way, other study conducted in Nebal found 96.1% sensitivity in S+C+(19). Similarly, another study carried out in China has shown the sensitivity of LAMP to be 98.6% in S+C+ (20).

Different sensitivity and specificity percentages were obtained by different authors. In comparison with Nagdevet et al (2011), who found the sensitivity of PCR and LAMP were; 52.9 % and 88.23 % respectively, so both studies showed that LAMP technique was more sensitive than the PCR technique. Comparing the result of specificity obtained in the present study with Nagdevet al (2011), who found the specificity of PCR and LAMP were; 90 % and 80 % respectively.[21] This demonstrates the efficiency of LAMP technique in the detection of *M. tuberculosis* than PCR technique.

The following conclusion can be drawn from the present study that both PCR and LAMP are suitable for diagnosis of TB as shown in our S+C+ individuals, however, they are efficient in confirming negative results (S-C-) so far. Considering rapidity and cost-effectiveness LAMP is found to be suitable in low resource settings for TB diagnosis.

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