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Rapid Screening of *Mycobacterium Tuberculosis* Using Lamp PCR

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ABSTRACT:

Tuberculosis is a disease which is spreading like an epidemic. Unless and until something is done to prevent and curtail transmission, be herculean task treating tuberculosis patients and saving the lives.In this project we havereviewed different methods available to detect TB and we made an attempt to do LAMP [loop mediated Isothermalamplification] targeting mycobacterium gene ESAT-6. It is a good method and can easily differentiate other NTM [non–*tuberculosismycobacterium*] from *mycobacterium tuberculosis*. To summarize LAMP can be used as an efficient point of care [POC] detection method to identify the tuberculosis bacteria.

INTRODUCTION:

Mycobacterium tuberculosis is an obligate *[AsunciónMartínez et at,*1999.]pathogenic bacteria species in the family Mycobacteriaceae and the causative agent of most cases of tuberculosis.[*Ryan* et al,2004] First discovered in 1882 by Robert Koch, M. tuberculosis has an unusual, waxy coating on its cell surface (primarily due to the presence of mycolic acid), which makes the cells impervious to Gram staining; M. tuberculosis can appear Gram-negative and Gram-positive in clinical settings. The Ziehl-Neelsen stain, or acid-fast stain, is used instead. The physiology of *M. tuberculosis* is highly aerobic and requires high levels of oxygen. Primarily a pathogen of the mammalian respiratory system, it infects the lungs. The most frequently used diagnostic methods for

One-third of the world's population is thought to be infected with TB. New infections occur in about 1% of the population each year. In 2014, there were 9.6 million cases of active TB which resulted in 1.5 million deaths. More than 95% of deaths occurred in developing countries. The number of new cases each year has decreased since 2000. About 80% of people in many Asian and African countries test positive while 5–10% of people in the United States population tests positive by the tuberculin test.[Kumar et el,2007]. Tuberculosis has been present in humans since ancient times.[Lawn at el ,2011].

MATERIALS AND METHODS:

1.Sputum and bronchial lavage samples from TB patients(20) along with blood samples from ten healthy controls.

2.PCRthermocycler

3.ABI7500FastDx real time PCR

3.Agarose gel electrophoresis units

4.NALC

5.NaOH

6.Nucleospin tissue extraction kit

7.Roche RNA extraction kit

tuberculosis are the tuberculin skin test, acid-fast stain, and chest radiographs [Ryan et al,2004] . Tuberculosis is spread through the air when people who have active TB in their lungs cough, spit, speak, or sneeze. [CDC et al ,2012] People with latent TB do not spread the disease. Active infection occurs more often in people with HIV/AIDS and in those who smoke.Diagnosis of active TB is based on chest X-rays, as well as microscopic examination and culture of body fluids. Diagnosis of latent TB relies on the tuberculin skin test (TST) or blood tests.[Konstantinos ,2010].Prevention of TB involves screening those at high risk, early detection and treatment of cases, and vaccination with the bacillus Calmette-Guérin vaccine. [Harrisa and Randall ,2013] [Hawn Konstantin's ,2010]. Those at high risk include household, workplace, and social contacts of people with active TB. Treatment requires the use of multiple antibiotics over a long period of time. Antibiotic resistance is a growing problem with increasing rates of multiple drug-resistant tuberculosis (MDR-TB).

sample on the surface of the slants. Incubate the slants at 30-35°C with 5-10% CO2 and examine the slants every week up to 8 weeks.Inoculated 200 µl of sediment into plain LJ or antibiotic-containing LJ tubes. Tubes were incubated at 37°C for a maximum of 8 weeks. Cultures showing no growth after 8 weeks of incubation were reported as negative. Liquefied or discolored (dark green) LJ media or LJ slants with colonies of non-acid-fast bacteria were considered contaminated.

Detection: Ziehl-Neelsen acid-fast staining detection procedure

- 10 µl MTB/BCG culture was pipetted on a glass microscope slide and heated on top of a Bunsen flame until it is completely dry to fix the bacteria. The slide was flooded with carbolfuchsin stain (BD kit reagent A). The slide was gently heated until it steams (5 min).
- The carbolfuchsin was poured off.
- The slide was thoroughly washed with tap water (5 min).
- It was then decolorized with acid-alcohol (5 min).

Culturing on Lowenstein Jensen medium:

Composition[Gms\600]

L-asparagine 3.60, Monopotassium phosphate 2.40, Magnesium sulphate 0.24, Magnesium citrate 0.60, Potato starch soluble 30.00, Malachite green 0.40, Glycerol 12.00 ml, Whole Egg Emulsion 1000.00 ml.

L.J. Medium is prepared as per the Jensen's [Jensen,1932] modification of the original formulation of Lowenstein [Lowenstein 1931]. The egg base medium supports a wide variety of Mycobacterium and can also be used for niacin testing [Biosvert,1960]. Glycerol provides fatty acids. Malachite green serves as an inhibitor as well as pH indicator. Formation of blue zones indicates a decrease in pH by Gram positive contaminants (e.g. Streptoccocci) and yellow zones of dye destruction by Gram-negative bacilli. Proteolytic contaminants cause localized or complete digestion of the medium [Nolte. and Methcok,1995].Inoculate the sputum sample previously subjected to decontamination and concentration process or the pure culture of Mycobacteria isolated from a clinical

2. The tube was agitated on a vortex mixer for not more than 20 sec.The tube was inverted so that the NALC-NaOH comes in contact with the entire inner surface of the tube. Excessive agitation was avoided.

3. The tube was allowed to stand for 30min at room temperature (20–25°C) to decontaminate the specimen.

4. The mixture was diluted to a minimum of 20 mL with sterile 0.067 M phosphate buffer (pH 6.8) and inverted several times to mix the contents.

It was centrifuged at 3000 x g for 15min and supernatant was discarded into disinfectant, and the pellet was resuspended in 200ul of sterile 0.067 M phosphate buffer (pH 6.8). (The latter has the added effect of increasing the neutralization activity).

Extraction of DNA:

1.180 µl of Buffer T1 and 25µl of proteinase K solution was added to 200 µl of decontaminated sample.

2.The contents were mixed by vortexing it,(the samples must be completely covered with lysis solution).

- The slide was thoroughly washed with tap water (5 min).
- The slide was flooded with methylene blue (BD kit reagent B) counterstain (1 min).
- The slide was washed with tap water.
- The excess water was blotted and dried in hand over Bunsen flame.
- The slide was then observed under a standard light microscope.

Inoculated 200 µl of sediment into plain LJ or antibiotic-containing LJ tubes. Tubes were incubated at 37°C for a maximum of 8 weeks. Cultures showing no growth after 8 weeks of incubation were reported as negative. Liquefied or discolored (dark green) LJ media or LJ slants with colonies of non-acid-fast bacteria were considered contaminated.

Decontamination of specimens using NALC-NaOH:

1. An equal volume of working NALC-NaOH solution (2% NALC and 0.5 N NaOH, no more than 48hr old) was added to the specimen.

flow-through was discarded and the column was placed in collection tube.

9. 600µl of buffer B5 was added to the column and,centrifuged for 1 min at 11000 xg flow-through was discarded and the column was placed in collection tube.

10.Residual ethanol was removed by centrifuging the column for 1 min at 11000 xg

11.The column was placed into a 1.5ml micro centrifuge tube and 100ul of buffer BE was added.

12.It was incubated at room temperature for 1 min and centrifuged at 11000 xg for 1 min.Thus mycobacterial DNA was obtained.

3.200µl buffer B3 was added to the decontaminated sample and vortexed vigorously.

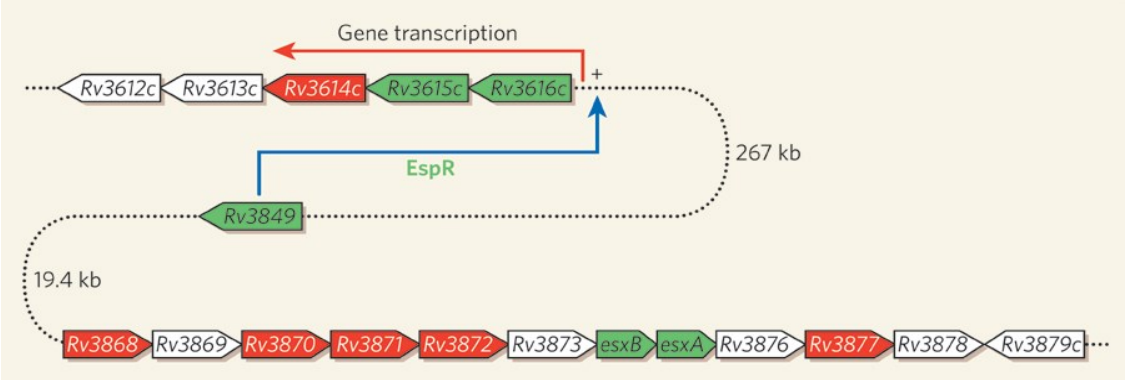
4.This mixture was incubated at 70°C for 10 min and vortexed well.

5.210 µl of ethanol was added and vortexed vigorously.

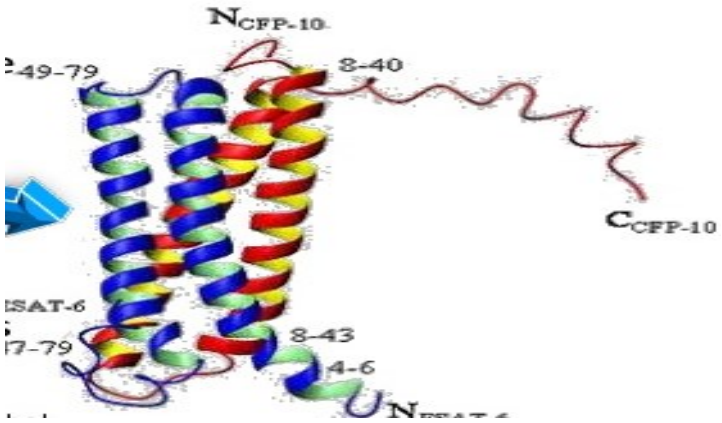
6. A nucleospin tissue column was placed onto a collection tube and sample was added to the column.

7.It was centrifuged for 1 min at 11000 xg and the collection tube with liquid flow- through was discarded and the column was placed in a new collection tube.

8. 500µl of buffer BW was added and centrifuged for 1 min at 11000 xg .The



[Figure -1]ESAT-6 cluster genes



[Figure-2]:ESAT-6 and CFP-10 antigens evoke strong human responses and are the key antigens

mycobacterium virulence and pathogenesis and is present in virulent *M.bovis*strains.The antigens CFP10 and ESAT-6 both evoke strong immune responses and known as key antigens in tuberculosis virulence.

The ESAT -6 gene cluster regions are duplicated 5 times in the genome of mycobacterium tuberculosis.ESAT-6 region1 is the most studied region as it contains Region of difference(RD1).

RD1 is a 9.5 kb deletion region confirmed to be involved in

ESAT-6 gene:

5` ATGACAGAGC AGCAGTGGAA TTTCGCGGGT ATCGAGGCCGCGGCAAGCGC AATCCAGGGA AATGTCACGT CCATTCATTC CCTCCTTGACGAGGGGAAGCAGTCCCTGACCAAGCTCGCAGCGGCCTGGG GCGGTAGCGG TTCGGAAGCG TACCAGGGTG TCCAGCAAAA ATGGGACGCCACGGCTACCGAGCTGAACAACGCGCTGCAGAACCTGGCG C GGACGATCAG CGAAGCCGGT CAGGCAATGG CTTCGACCGA AGGCAACGTC ACTGGGATGT TCGCA 3`

Nucleotide sequences of ESAT 6-LAMP primers.

Primers	Sequence	Length in bp
F3	CAAGCGCAATCCAGGG	16
B3	GCTTCGCTGATCGTCC	16
FIP	CGCTGCGAGCTTGGTCATGTCACGTCCATTCATTCC	36
BIP	TAGCGGTTCGGAGGCGTACGTTGTTCAGCTCGGTAG	36

LAMP assay for the detection of *ESAT-6* was performed using a set of five primers, FIP, BIP, F3, B3 and LF, as designed (Table). LAMP reaction was carried out in 25 µl reaction mixture containing
1.6 µM each of inner primers (FIP and BIP), 0.2 µM*l* each of outer primers (F3 and B3), 0.8 µM of loop primer (LB), 1.4 mM of dNTPs, 0.5 M betain (Sigma, St Louis, MO), 20 mMTris-HCl (pH 8.8), 10 mMKCl, 10 mM (NH4)2SO4, 8 mM MgSO4, 0.2% Tween 20, 8 U *Bst*polymerase large fragment (New England Biolabs, Ipswich, MA), Negative control contained no added DNA and 7.750 µl of sample DNA.[figure-3]

Normal PCR using F3 and B3 primers:

PCR reaction mix was set using the following constituents:

Template DNA-1 µl, FP-0.5 µl, RP-0.5 µl, Master mix(containing dNTPs,Taq polymerase & buffer)-6.25 µl, Nuclease free water-5.25 µl

The PCR conditions were set as follows:

94-10 minutes

94-1 minute
50-1 minute
72-1 minute
} 30 cycles

72-10 minutes

The PCR product was checked on 2 % agarose gel.

defaulted or failed.The samples were cultured on Lowenstein Jensen slants and the positive cultures were stained by carbolfuchsin to identify the tuberculi.The bronchial wash samples are decontaminated as described.The healthy samples underwent the same procedures as the case samples.After decontamination the mycobacterial DNA was extracted from the specimen using Nucleospin kit as described by the manufacturer. The DNA samples were used as template for amplification of ESAT-6.Once the bands were observed,the same DNA templates were used for LAMP reaction.The LAMP was done using the six set of primers as described in methods section. PCR reaction mix was set using the following constituents:

Template DNA-1 ul, FP-0.5 ul, Rp-0.5 ul, Master mix(containing dNTPs,Taq polymerase & buffer)-6.25 ul, Nuclease free water-5.25 ul

The PCR conditions were set as follows:

94-10 minutes

94-1 minute

50-1 minute

30 cycles

Figure 3-Conventional PCR thermocycler

RESULTS:

Twenty bronchial wash samples of TB infected individuals were collected on request from the outpatient clinic of a local hospital .They were clinically TB positive by Acid fast Bacterial(AFB) staining. Along with them ten bronchial wash samples of healthy individuals were also collected to compare the two experimental results.

REGISTRATION GROUP BY OUTCOME OF MOST RECENT TB TREATMENT:

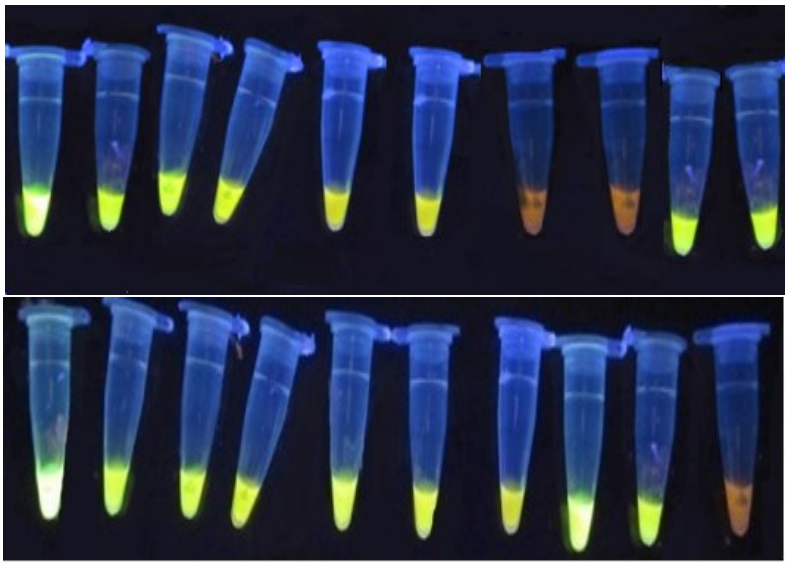
Patients whose sputum is smear-positive at the end of (or returning from) a second or subsequent course of treatment are no longer defined as "chronic". Instead, they should be classified by the outcome of their most recent retreatment course: relapsed,

deg c for 15 min.following this,10 ul of SYBR green dye was added to each tube and seen under blue light.All the positive samples showed fluorescence.

72-1 minute

72-10 minutes

After one hour the reaction was terminated by incubating the mix at 80



[Figure-4]Tubes showing fluorescence indicating amplification of ESAT-6.

Table-Results of AFB staining in comparison with LAMP PCR

	Age	(Gender)	AFB stain	LAMP PCR
Case-1	60	Male	+	+
Case-2	24	Male	+	+
Case-3	41	Male	+	+
Case-4	46	Male	+	+
Case-5	33	Male	+	+
Case-6	39	Male	+	+
Case-7	61	Female	+	–
Case-8	35	Male	+	–
Case-9	53	Female	+	+
Case-10	16	Male	+	+

Case-11	26	Male	+	+
Case-12	27	Male	+	+
Case-13	38	Male	+	+
Case-14	42	Female	+	+
Case-15	14	Male	+	+
Case-16	26	Male	+	+
Case-17	37	Male	+	+
Case-18	57	Female	+	+
Case-19	49	Male	+	+
Case-20	30	Male	+	-
	Age	Gender	AFB stain	LAMP PCR
Control-1	22	Male	_	_
Control-2	36	Male	_	_
Control-3	34	Female	_	_
Control-4	57	Male	_	_
Control-5	43	Female	_	_
Control-6	20	Female	_	_
Control-7	46	Male	_	_
Control-8	27	Female	_	_
Control-9	22	Male	_	_
Control-10	35	Male	_	--

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