International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.6, No.5, pp 1695-1702, Sept-Oct 2014

Development of a PCR Based Nucleic Acid Lateral Flow Assay Device for Detection of *Mycobacterium Tuberculosis* Complex

Thulasi Krishna Haridas[1], S.Thiruvengadam*[1], V.I.Bishor[2]

[1]Department Of Biotechnology, Rajalakshmi Engineering College, Anna University, Chennai, Tamilnadu, India
[2]ubio Biotechnology Systems Pvt. Ltd, Kinfra Biotechnology Incubation Centre, Cochin, Kerala, India

***Corres.author: thiruvengadam.s@rajalakshmi.edu.in.**
Phone:- 044 37181111/12, Fax: 914437181113, Mobile- 09445489066

Abstract: PCR based nucleic acid lateral flow assay is becoming a popular podium for fast, robust, nucleic acid detection with its biomedical application in diagnosis of infectious disease. Focusing on development of a novel nucleic acid lateral flow assay device coated with nano-probes onto nitrocellulose membrane for detection of PCR amplified pathogenic island of *Mycobacterium tuberculosis* complex in a sample.DNA samples were isolated from culture isolates of Mycobacterium species identified as H37Rv which was collected from Cooperative Medical College, Cochin. The PCR amplification of these DNA samples was carried out using primers specifically designed for targeting IS6110 sequence. This sequence is specific for *Mycobacterium tuberculosis* complex organisms. The amplified products were tested on Nucleic Acid Lateral Flow Assay (NALFA) device. NALFA was designed in such a way that it has a nano-probe specific for the PCR product of interest. The PCR product which contained the amplified DNA of Mycobacterium gave a positive result on NALFA thus detecting and confirming the presence of IS6110 sequence. The test results were confirmed with electrophoresis. The NALFA device has to be further optimized and then, it is sure to be a breakthrough product in tuberculosis diagnosis. The test developed is cost effective; time for diagnosis can be drastically reduced and can be produced and used in large numbers.
Keywords : IS6110 Insertion sequence, Nitrocellulose membrane, Streptavidin - Gold conjugate, Biotin labelling

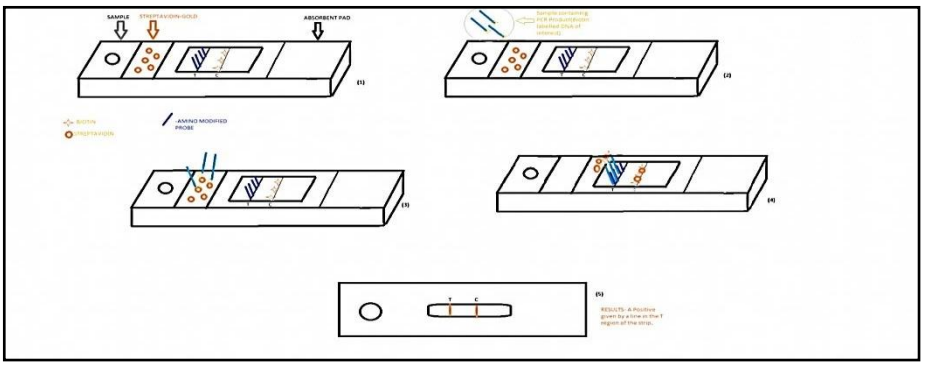
Introduction

Tuberculosis is a treatable airborne infectious disease which takes the lives of about 2 million people every year. The key point in the control of the disease lies in its prevention and also early detection of the disease. Despite all the advances made in the treatment and management, TB still remains as one of the major public health problems, particularly in the developing countries. India accounts for nearly 30 per cent of the global TB burden[1]. The objectives of improved test development for TB diagnostics are to simplify the procedure and reduce the time for diagnosis. PCR has brought new opportunities for the rapid, sensitive, and specific detection of Mycobacteria. The widespread use of PCR has been limited for the detection of Mycobacterium, especially in developing countries with the greatest incidence of the disease, because of, the cost and complexity of the method. IS6110 insertion sequences present in the genome of *Mycobacterium*

tuberculosis is common to all the organisms of Mycobacterium tuberculosis (M.Tb) complex [2,3]. This is a target for identifying the presence of M.Tb complex in a given sample by utilizing the amplification of this sequence by using PCR. Special protocols are available for isolation of mycobacterial DNA from various sources including sputum, liquid culture and solid culture [4-5]. Strong physical treatment, use of a detergent and enzyme for lysis, treatment with Proteinase K, DNA purification step with or without phenol and DNA precipitation in ethanol or isopropanol are essential steps for extraction of mycobacterial DNA from clinical samples [6]. Many works have been reported in which PCR of mycobacterial DNA has been performed [7-13]. Vincent and co-workers [14], has reported the use of different probes specific for different species of TB. This assay employs pan-*Mycobacterium* amplification primers, allowing flexibility in the mycobacterial species that can be identified from a single amplification reaction. In a work reporting PCR based assay for *Mycobacterium* detection, the probes were designed and used to capture the IS6110 PCR products and the 16S rRNA PCR products of Mycobacteria belonging to the M. Tb complex[15]. In a recent work on the development of oligonucleotide lateral flow assays amino modification has been carried out for the oligonucleotides to attach to the nitrocellulose membrane [16]. The same method has been adapted in the present experiment also

A typical LFA device consists of a surface layer which carries the sample from the sample application pad to the conjugate release pad followed by the strip encountering the detection zone up to the absorbent pad[17]. Nucleic acid lateral flow immunoassay (NALFIA) is a method combining molecular biological principle of detection with immunochemical principle of visualisation. The concept of NALFA for detection of M. Tb complex in a sample is illustrated in Figure 1. NALFIA for the simultaneous detection of *Listeria monocytogenes* in particular and the genus Listeria in general, in food, has been reported [18].

Figure 1. Concept of Nucleic Acid Lateral Flow Assay (NALFA) for detection of PCR product present in a sample.



Line probe assay for detection of Mycobacterium complex strains have been developed by Innogenetics Inc. USA and is known as INNO-LiPA Rif.TB® assay which detects the presence of a pathogen belonging to the M. Tb complex by the presence of a specific *M. tuberculosis* complex probe (MTB) on strip. Xpert MTB/RIF® system is also available for detection of Mycobacterial DNA in sputum samples [19]. The system can automatically process the sputum, perform the real time PCR and can give the result as the amount of DNA present in the sample. The cost of the equipment is very high and difficult to be implemented in large numbers. Also, the cartridge is very expensive and is only for single test use. Thus, the need for a low cost diagnostic device, which can be produced in large numbers, is overwhelming. This reason has been the driving force for the development of a nucleic acid lateral flow assay for TB diagnosis.

Materials and Methods

For DNA Isolation the following chemicals were used. Tris Base, Concentrated .HCl, Ethylene diamine tetra acetic Acid (EDTA), Monosodium Glutamate, Sodium dodecyl sulphate(SDS), Sodium chloride, Cetyl Tri-methyl Ammonium Bromide(CTAB), Sodium acetate, Phenol, Chloroform, Isoamyl alcohol, Isopropanol, EDTA, Ethanol, were purchased from Merck, India. The enzymes needed for DNA extraction, Lysozyme, RNase and Proteinase K. were purchased from Sigma Aldrich, India. For PCR of DNA samples, Emerald AMP –GTPCR Mastermix-2X Premix was used. Forward primer-UBFPTB1 (Biotin-5'-GAGGTCTGCTACCCACA-3'), Reverse primer UBRPTB1-(Biotin-5'- GATCGCTGATCCGGCCA-3'), were synthesized by Metabion, Germany. For preparation of the lateral flow assay, Nitrocellulose Membrane(150 CNTH–N-SS40),.Sample Pad (Glass fibre type- GP04), Absorbent Pad (Cellulose AP080) and Conjugate Pad (PTR7 and PTR5) were purchased from MDI-Advanced Micro devices, Ambala, India. The nanoprobes UBPATB1- Amino-

5'TTCGGACCACCAGCACCTAA3'and UBPBTB1-Amino-5'TTAGGTGCTGGTGGTCCGAA3' were synthesized at Metabion, Germany. Other chemicals used for preparation of various buffers, Trisodium Citrate, KCl, K_2HPO_4, Na_2HPO_4, NaH_2PO_4, EDTA, Sucrose, Sodium azide, Sodium carbonate, Sodium bicarbonate, Boric Acid, Sodium tetra borate, Tween 20 and BSA were purchased from Merck, India. The details regarding the preparation of various buffers and enzyme dilutions are given in the supplementary data.

DNA Isolation

A total of ten culture isolates of *Mycobacterium tuberculosis* species identified as H37Rv was collected from Cooperative Medical College, Cochin, Kerala, India .DNA samples for PCR from these ten cultures were isolated using the protocol for DNA isolation [3]. Ten DNA samples were prepared by isolation from solid culture by the following protocol. Culture was taken from a 3 month old LJ culture which was positive *Mycobacterium tuberculosis* and was placed in small glass vial and then covered with aluminium foil. The glass vial was placed at 80°C in hot air oven for 1 hour. This was performed for heat inactivation of the culture. 500 µL of extraction buffer was added to the inactivated culture and solution was poured into a 1.5 mL vial. Sterilized glass beads were added to vials and it was vortexed till the clumps of cells were broken. 10 µL of Lysozyme stock prepared was added (working concentration (WC) is 4 mg/ml of reaction mixture). 2 µL of RNase stock (WC=10 µg/ml) prepared was also added. It was kept for incubation at 37°C for 2 hours with intermittent mixing. 50 µL of Proteinase K buffer and 50 µL of Proteinase K stock (WC=50 µg/ml) were added to the vial. The vial was kept for incubation at 45°C overnight.120 µl of 5M NaCl and CTAB/NaCl solution (pre-warmed at 65^0 C) was added to the vial. It was then vortexed and incubated at 65° C for 10 min. 800 µL of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the vials .The vials were inverted to mix properly. The inversion steps were repeated every 30 min for 2 hours. Centrifugation was done at 3000g for 20 min. The upper aqueous phase was removed and transferred into a new microfuge tube. 800 µL of Chloroform: Isoamyl alcohol (24:1) was added into microfuge tube. It was also mixed by slow inversion steps. Centrifugation of the sample was done at 3000g for 10 min. The upper aqueous phase was transferred to a new microfuge tube. To the aqueous phase volume, 0.1 volume of 3M sodium acetate was added. To that aqueous phase solution 0.6 volume of Ice cold Isopropanol was added. It was mixed gently by inverting 2-4 times. The vial was incubated at -20°C for 30 min. Centrifugation was done at 3000g for 10 min. The supernatant was discarded and 1 ml of 70% ethanol was added. The vial was left for air drying at 55°C.The DNA pellet was then dissolved in 100µL of TE buffer and incubated at 65°C until completely dissolved. It was then stored at -20°C.

Primer and probe design

The target DNA sequence is IS6110 present in the genome of *Mycobacterium tuberculosis.* The PCR product is a 91 bp sequence from this region (Figure.2) The forward and reverse primers and probes for both the sequences were designed using software like Primer BLAST and Oligoanalyzer.

PCR of Mycobacterial DNA

PCR amplification of the samples were done as 25µL total sample volume in which 12.5 µL of Emerald AMP –GTPCR Mastermix-2X premix ,1 µL each of forward and reverse primers ,5 µL of template DNA and 5.5 µL of DNAse free water were used. The thermo cycler used was PTC150- Minicycler. (MJ Research Inc., USA). Forward and reverse primers were designed considering the different parameters for amplification of the target sequence IS6110 (Genbank Accession No.JX627755.1), which is repeated in the genome of the *Mycobacterium* complex organisms. Two different PCR programs were used for trials. Program 1 was of initial denaturation at 95°C for 1 min followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 40 sec and extension at 72°C for 5 sec. The final extension step was at 72°C for 1 minute. Program 2 was of initial denaturation at 98°C for 30 sec, followed by 40 cycles of denaturation at 98°C for 5 sec, annealing at 61°C for 5 sec and extension at 72°C for 5 sec .The final extension step at 72°C for 1 min. The PCR samples were stored at -20°C for future use.

Preparation of Lateral Flow Assay

The lateral flow assay development was performed in the R&D laboratory of ubio Biotechnology Systems, KINFRA Biotech Park, Cochin, India. NALFA was prepared by coating probe A and probe B in test line onto nitrocellulose membrane using lateral flow coating machine. The probe was designed to capture the required PCR product (Figure 2).

Figure 2. The 91 bp PCR product of the IS6110 insertion sequence of the Mycobacterial complex genome. This region is the target for identification. The primer and probe attachment sites are given. Highlighted in yellow is the region where the probes will attach.

The conjugate pad of the NALFA was coated with the streptavidin- gold conjugate using the spray buffer. The NALFA was assembled and ten different trials were performed by varying the parameters for detection in each trial .The details of each trial are given in Table.1.

Table 1. Details of different trials for preparation of nucleic acid lateral flow assay

Trial No.	Type of sample pad	Sample details and volume used	PCR program	Running buffer used	Conjugate pad, spray buffer used	Streptavidin gold: spray buffer concentration	Coating buffer used for coating probe.
Trial 1	Glass fibre pad	PCR product directly 10µL	1	Bicarbonate buffer(pH 11.4)	R7 20% Sucrose	1:3	20X SSC
Trial 2	Glass fibre pad	PCR product after denaturation 10µL	1	Bicarbonate buffer(pH 11.4)	R7 20% Sucrose	1:2	20X SSC
Trial 3	Glass fibre pad	PCR product after denaturation 10µL	1	Bicarbonate buffer(pH 11.4)	R7 20% Sucrose	1:1	20X SSC
Trial 4	Glass fibre pad	PCR product after denaturation 10µL	2	Borate Buffer (pH 8.4) with BSA,Tween 20, Sodium Azide.	R7 20% Sucrose	2:1	1X PBS
Trial 5	Glass fibre pad	PCR product after denaturation 10µL	2	Borate Buffer (pH 8.4) with BSA,Tween 20, Sodium Azide	R7 1ml PBS+ 20 µL of 1% PEG+ 50 µLTween 20	2:1	SSPE
Trial 6	Glass fibre pad	PCR product after denaturation 10µL	1	Borate Buffer without BSA	R5 No spray buffer used	SG directly	20X SSC
Trial 7	Glass fibre pad	PCR product after denaturation 10µL	1	Borate buffer without BSA	R5 50mM Sodium phosphate, 1% BSA,	2:1	20X SSC

					20% sucrose, 1% Sodium azide. pH 7.4		
Trial 8	Glass fibre pad	PCR product after denaturation 10µL	1	Borate buffer without BSA	R5 50mM Sodium phosphate, 1% BSA, 20% sucrose, 1% Sodium azide. pH 7.4	2:1	20X SSC
Trial 9	Glass fibre pad	PCR product after denaturation 10µL	1	Borate buffer without BSA(20 sec time given before adding running buffer)	R5 50mM Sodium phosphate, 1% BSA, 20% sucrose, 1% Sodium azide. pH 7.4	2:1	20X SSC
Trial 10	Glass fibre pad	PCR product after denaturation 5µL	1	Borate buffer without BSA(20 sec time given before adding running buffer)	R5 50mM Sodium phosphate, 1% BSA, 20% sucrose, 1% Sodium azide. pH 7.4	3:1	20X SSC

The PCR products from each of the samples were tested on NALFA. In all the trials except Trial 1, 10 µL of PCR product was subjected to denaturation by adding 2 µL of 1.5 M NaOH and then incubation at 55^{o}C for 30 min. From this 10 µL was used as the sample for detection. In Trial 1 alone 10 µL of PCR product was directly used as sample without denaturation.

Results

The PCR products which contained the amplified DNA gave a positive result on NALFA. Figure 3 shows number of positives versus the trial number .In Trial No. 2, 5, 6, 7 and 10, positive results were obtained. In all of these trials the sample used was denatured PCR product.

Figure 3. Result showing the number of positives obtained versus the trial number.

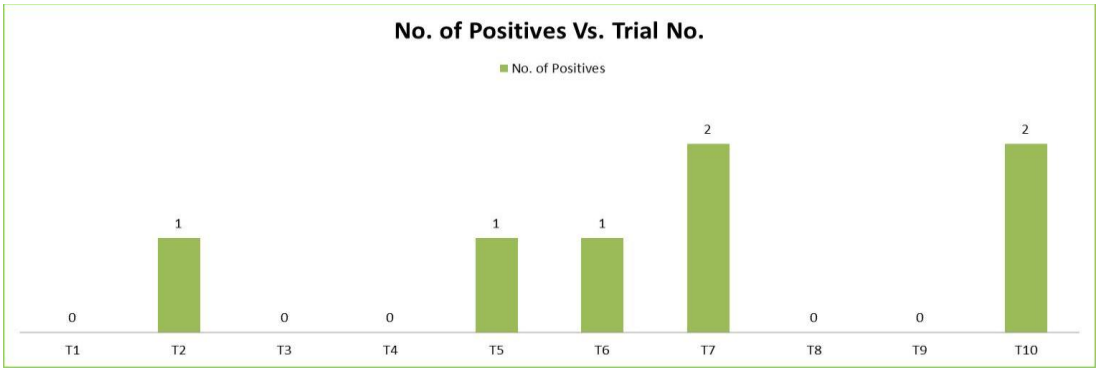


Figure 4. Result showing the total number of positives obtained for each Sample.

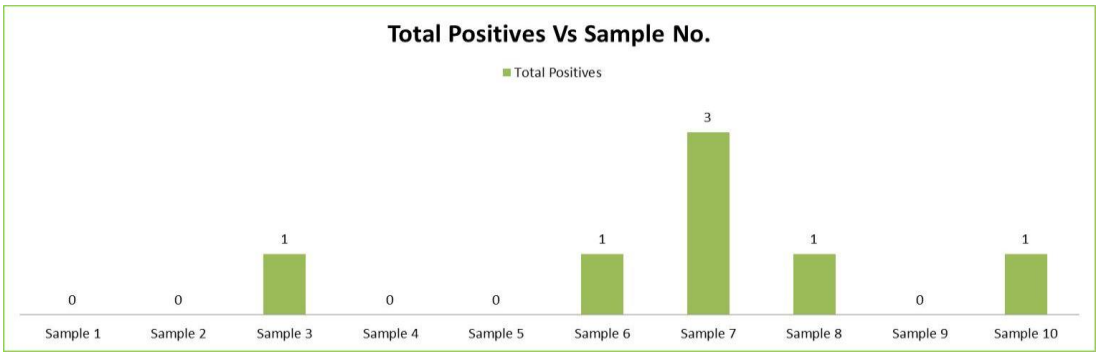
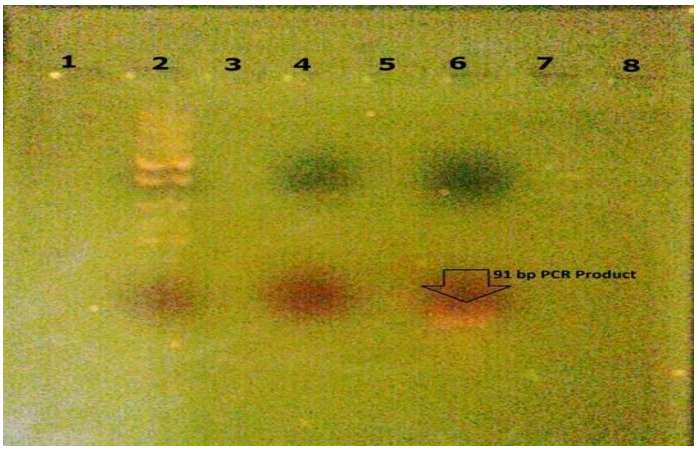


Figure 4 shows total positives obtained versus the sample number. Sample No. 3 showed positive in Trial No. 6. Sample No.6 showed positive in Trial No.5. Sample No.7 showed positive result in Trial No. 2, 7 and 10. Sample No. 8 showed positive in Trial No.7.Sample No.10 showed positive in Trial No. 10. There is variability in the results; however Sample 7 showed maximum number of positive results. The photographs of NALFA with positive results are shown in Figure.5. As maximum number of positives was obtained with Sample No.7, the presence of amplified PCR product in that sample was confirmed by running a 2% gel stained with ethidium bromide. The photographs of the gel showing the presence of amplified DNA in Sample No.7 is shown in Figure.6.

Figure 5. Coloured line formation in (a) Trial 2 Sample 7, (b)- Trial 5 Sample 6,(c) Trial 6 Sample 3, (d) Trial 7 Samples 7 & 8 ,(e) Trial 10 Sample 7 & 10- two lines can be seen in sample 10.

Figure 6. This figure shows the presence of amplified PCR product of length 91 bp which can be seen in the 6th well. The 2nd well is loaded with 1kbp marker. The 6th well was loaded with Sample No.7 amplified by PCR program 1. 5 µL of PCR product containing DNA was loaded using 5 µL of gel loading buffer containing tracking dye bromophenol blue. The DNA was stained using ethidium bromide. 4th well was left as control with no PCR product. The concentration of gel is 2%.



Discussion

The results show that, there is feasibility for the working of the assay. Optimizations have to be done for better and easily detectable signal. The various factors that were important in the trials are discussed here. The chances of missing DNA in the original sample might be one reason for all negative results by certain samples which includes Sample No.1, 2, 4, 5, and 9 The PCR primers, UBFPTB1 and UBRPTB1 and other reaction parameters are working, because certain positives and confirmed PCR product has been obtained with sample No.7. The same sample also gave positive line on NALFA. This suggests that the product can surely detect the PCR product if it has been amplified properly.

The different design parameters for the working of NALFA are discussed next. PCR cycle program 1 was found to be more effective in giving positive results. Probe concentration of 30µM is found to give the best results. Denaturation of PCR products was found to be essential step before adding these samples to NALFA. Running buffer consisting of borate buffer (pH 8.4), Tween 20 and Sodium azide was found to be the buffer with very less nonspecific binding and false positive results. 20X SSC coating buffer was found to be better for coating of the probes. In Trial 10, the probe concentration of 30 µM was used and both the test line containing Probe A and Probe B showed positive results. The streptavidin gold concentration of 3:1 was found to be effective in giving clear line in the same trial.

The positive results were obtained in cases where there was binding of denatured PCR products in the sample with the streptavidin-gold in conjugate pad and then this complex altogether binds to the probe present on test line. The control line can be coated with Biotin-BSA to capture the streptavidin-gold released from the conjugate pad to ensure the test has worked properly. During the final optimization alone the control line can be coated, thus the wastage of the reagents in the trials can be avoided.

Conclusion

The significance of the work is that, a patentable Nucleic Acid Lateral Flow Assay or PCR Lateral Flow Assay for detection of the presence of amplified *Mycobacterium tuberculosis* complex DNA has been developed which is the first of its kind in TB Diagnostic industry. The test developed is cost effective and can be produced and used in large number especially in countries with higher TB burden. Many similar diagnostics assays like the MPT64 antigen detection LFA have been reported. But the present product is unique because this detects the presence of Mycobacterial DNA in a PCR sample without the use of Gel electrophoresis. Also the time required for the diagnosis can be drastically reduced. The result can be seen with naked eyes and without any kind of cumbersome procedure. Also the handling of the device does not need any kinds of expertise and can also be used as a point of care diagnostic device.

Acknowledgements

The funding for the project was provided by All India Council for Technical Education, India in the form of postgraduate scholarship and equal contribution from ubio Biotechnology Systems, Kinfra Biotech Park, Cochin, Kerala, India.

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