Biologicals 69 (2021) 59–65

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Research paper

Development of a visible loop mediated isothermal amplification assay for rapid detection of *Bacillus anthracis*

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ARTICLE INFO

Keywords:
Bacillus anthracis
Pag gene
LAMP

ABSTRACT

Distressing effects on animal and human health with lethal progression, being used as bioweapon and shared features with non-pathogenic bacteria demands sensitive, specific, safe, cost effective and rapid detection methods for anthrax causing organisms. Conventional microbiology based diagnostics for anthrax are time consuming and need sophisticated equipment, while molecular diagnostics require less time and labor. The Loop mediated isothermal amplification assay (LAMP) is rapid, sensitive and specific assay and requires no specialized equipment. In the present study, we developed a LAMP assay for rapid as well as specific detection of *Bacillus anthracis*. The optimized assay produced positive results with the Sterne strain and one field isolate of *B. anthracis* and, negative results with other bacteria of the same and different genera within 2 h. Sensitivity was 500 fg of total DNA of *B. anthracis*, which was 100 times more sensitive than conventional PCR. The present study also demonstrated that the simple method of total DNA extraction by repeated boiling and freezing will not adversely affect the LAMP results. In conclusion, the optimized LAMP assay is a promising tool for the specific, sensitive, less time-consuming diagnosis for anthrax causing bacteria and also, for detecting the virulence of suspected *B. anthracis* cultures.

1. Introduction

Anthrax usually caused by *Bacillus anthracis* is well known since antiquity for its serious devastating effect on animal as well as on human health [1]. Due to its dormant spores that can survive even the extreme environmental conditions, anthrax has also received much attention as a biological weapon [2]. The quick onset and rapid lethal progression in anthrax cases necessitate development of rapid, reliable, sensitive and specific methods for the detection of organisms causing anthrax. Conventional microbiological methods for this purpose require several days and are less sensitive [3]. Moreover, the numerous phenotypic and genotypic characteristics of *B. anthracis* were reported as common with other bacteria of the same and different genera especially with *B. cereus* group which are unable to produce anthrax [3–5]. Likewise, non-pathogenic isolates of *B. anthracis* lacking plasmid encoding anthrax toxin was reported [6,7]. Furthermore, *B. cereus* strains causing anthrax by the production of anthrax toxin were also isolated [8]. All these facts point out the need for an anthrax toxin based diagnostic methods for the specific identification of anthrax causing organisms.

Consequently, several nucleic acid-based assays targeting anthrax toxin genes have been optimized for the reliable, quick, specific and sensitive identification of organisms causing anthrax. However, these require expertise, expensive devices, heavy infrastructure laboratories and complicated protocols [9], thus are unsuitable for routine laboratories. The Loop-mediated isothermal amplification (LAMP) assay is preferred over other nucleic acid-based assays because it is fast, less labor-intensive and does not require any sophisticated equipment or skilled personnel, and is suitable for the applications at field level and routine laboratories [10]. Therefore, in the present study we have optimize anthrax toxin specific LAMP-PCR as a specific, sensitive, time and cost-effective method for the detection of anthrax causing bacteria.

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https://doi.org/10.1016/j.biologicals.2020.11.004
Received 28 March 2020; Received in revised form 9 November 2020; Accepted 17 November 2020
Available online 10 December 2020
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Table 1
Different bacteria used in the study.

Bacteria	Source
Bacillus anthracis Sterne's strain	Division of Biological Standardization, IVRI (Indian veterinary Research Institute), Izatnagar
B. anthracis IVRI strain	Division of Biological Standardization, IVRI, Izatnagar
B. cereus MTCC 7409	Microbial Type Culture Collection (MTCC), Chandigarh
B. subtilis MTCC 1133	MTCC, Chandigarh
B. megaterium MTCC 1684	MTCC, Chandigarh
Salmonella Typhimurium E2393	National Salmonella Centre, IVRI, Izatnagar
Brucella abortus strain 19	Division of Biological Products, IVRI, Izatnagar
Pasteurella multocida strain P52	Division of Biological Products, IVRI, Izatnagar
Escherichia coli M15	Division of Biological Products, IVRI, Izatnagar
E. coli DH5α	Division of Biological Products, IVRI, Izatnagar
Clostridium perfringens	Division of Veterinary Bacteriology and Mycology, IVRI, Izatnagar
Campylobacter jejuni	Division of Veterinary Public Health, IVRI, Izatnagar
Arcobacter sp.	Division of Veterinary Public Health, IVRI, Izatnagar
Leptospira sp.	Division of Veterinary Bacteriology and Mycology, IVRI, Izatnagar
Mycoplasma capri	Division of Veterinary Bacteriology and Mycology, IVRI, Izatnagar
Listeria monocytogenes	Division of Veterinary Bacteriology and Mycology, IVRI, Izatnagar
Bordetella bronchiseptica	General Bacteriology Lab, CADRAD

2. Materials and methods

2.1. Bacterial cultures

Different bacteria used in the study are given in Table 1. Out of these, two strains of *B. anthracis*, virulent IVRI strain (originally attained from Bacteriological Laboratory, ICAR-Indian Veterinary Research Institute, Mukteswar), another non-capsulated Sterne vaccine strain were obtained from Type Culture Laboratory, Division of Biological Standardization, ICAR-IVRI and used for the optimization of LAMP-PCR.

2.2. Primer designing

Based on the information gathered from GenBank and Blast database

Table 3
Optimized LAMP-PCR conditions.

Components	Concentration in final reaction	Time	Temperature
10X Thermopol buffer	1X	90 min without loop primer	Reaction temperature was 64 °C and enzyme inactivation was done at 80 °C for 5 min
dNTP (10 mM)	1.2 mM		
PAG-F3 (5 pM)	5 pM		
PAG-B3 (5 pM)	5 pM		
PAG-FIP (50 pM)	40 pM		
PAG-BIP (50 pM)	40 pM		
PAG-LoopF (50 pM)	20 pM	60 min with loop primers	
PAG-LoopR (50 pM)	20 pM		
Betaine	1 M		
$MgSO_4$	6 mM		
NFW	To make up to 23 μl		
Template	1 μl		
Bst DNA polymerase	1 μl		

Table 2
Details of primers used in the study.

Primer Name	Sequence (5'————————3')	Reference
PAG-Full-Upper	GAAGTTAAACAGGAGAACCGGTTA	In this study
PAG-Full-Lower	TTATCCTATCTCATAGCCTTTTTTAGAAA	
PAG-F3	AGGATCAATCCACACAGAATAC	
PAG-B3	GGTAACACGTTGTAGATTGGA	
PAG-FIP(F1c + F2)	CTGCAGATACACTCCCACCAATGGACACATACTAGTGAAGTACA	
PAG-BIP(B1c + B2)	ACGGTCGCAATTGATCATTCACCAGCGGTATTTAAACCCATTG	
PAG-LoopF	GAACGACGCATGCACTTC	
PAG-LoopR	GGGAAAGAACTTGGGCTGA	

Fig. 1. Schematic diagram on the position of different primers used in LAMP assay.

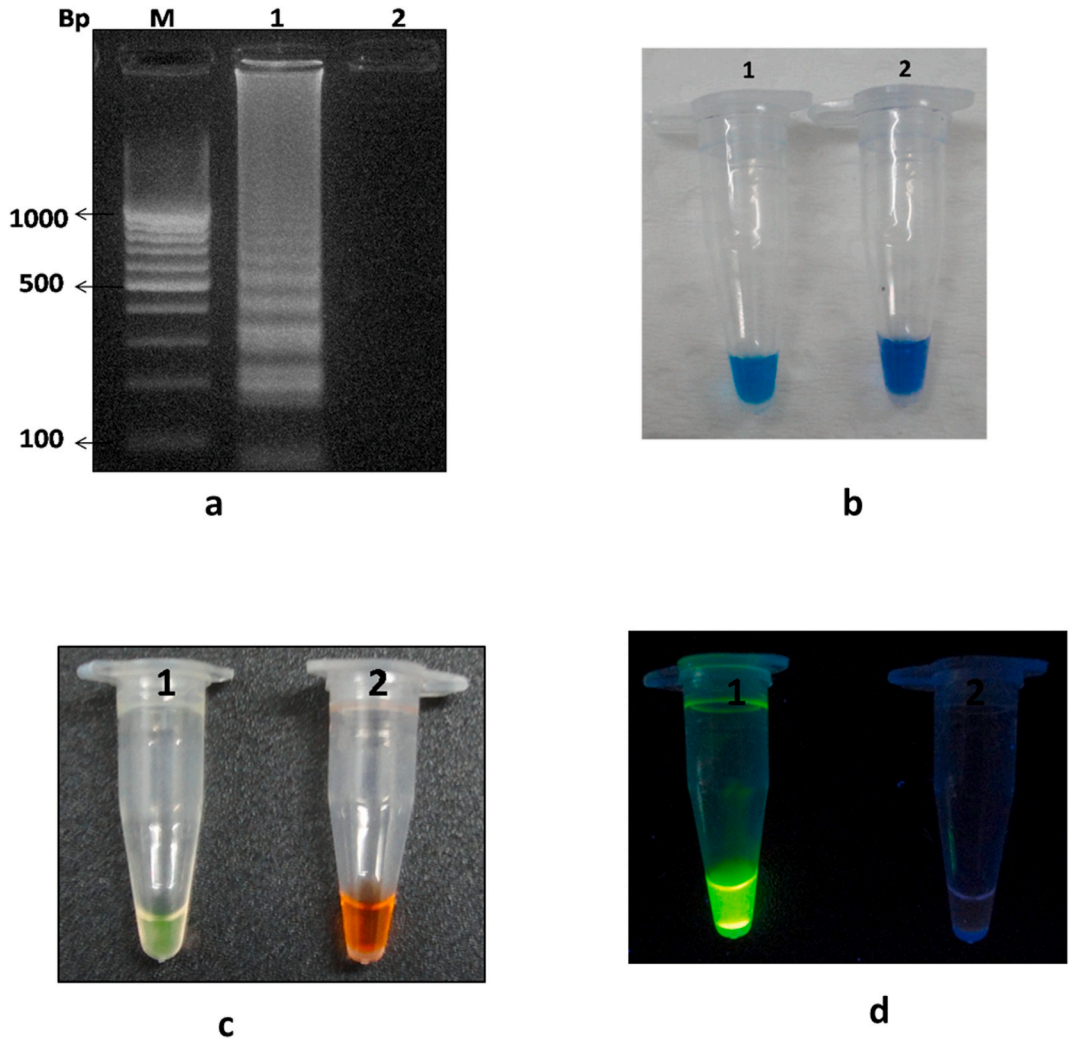

Fig. 2. Interpretation of LAMP-PCR results by (a) Agarose gel electrophoresis (b) HNB dye based detection under white light (c) SYBR Green I dye based detection under white light (d). SYBR Green I dye based detection under UV light: **Lane M**: 100 bp Ladder; **Lane/Tube 1**: Positive; **Lane/Tube 2**: Negative

of NCBI, LAMP primers specific for *pag* gene (Accession Number KJ631748) [encoding Protective Antigen (PA) portion of anthrax toxin] were designed using Primer Explorer V4 online software (http://primerexplorer.jp/e) (Table 2; Fig. 1). The primers were custom synthesized by Integrated DNA Technologies (Coralville, Iowa).

2.3. Total DNA extraction

A simple method for total DNA extraction *having* the minimal risk for biohazard/laboratory acquired infection [11] was used in the present study. Simultaneously plasmid was isolated from *B. anthracis* Sterne strain using QIAGEN maxi kit (M/s Helden, Germany) with minor modifications as *pag* encoding PA is located on pXO1 plasmid.

2.4. Conventional PCR

Conventional PCR for *pag* gene was carried out as described earlier [12]. Briefly the reaction was perform in 0.2 mL tube, with 10XPfx buffer; 3.75 µl (1.5X), 50 mM MgSO4; 1 µl (2 mM), 10 mM dNTP; 0.5 µl (200 µM), PAG Forward primer; 0.75 µl (50 pmol), PAG Reverse primer; 0.75 µl (50 pmol), PX01 plasmid; 1 µl, Platinum *Pfx* polymerase; 0.25 µl and Nuclease free water up to 25 µl. The following cycling conditions were used: Initial denaturation of 94 °C for 5 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 68 °C for 2 min and a final extension at 68 °C for 10min. The amplified products were confirmed for the expected size (1683 bp) in 1.0% agarose gel.

2.5. LAMP-PCR

LAMP-PCR was first carried out in 25 µl reaction volume as previously described [13] and the reaction was terminated by incubating at 80 °C for 5 min. Subsequently the reaction was optimized by varying reaction conditions and components such as temperature (58^0C-66 °C), $MgSO_4$ (2–8 mM), dNTP (0.4–1.4 mM), betaine (0.6–1.4 M) and reaction time (10–90 min) for identifying optimized ladder pattern. A positive control (plasmid of *B. anthracis*) and a negative control (nuclease free water) were also included in every reaction. The final reaction mixture of the standardized LAMP PCR is tabulated in Table 3

Components	Concentration or volume	Time	Temperature
10X Thermopol buffer	2.5 µl	90 min without loop primer	Reaction temperature was 64 °C and Enzyme inactivation at 80 °C for 5 min.
dNTP (10 mM)	1.2 mM		
F3 (5 pM)	5 pM		
B3 (5 pM)	5 p.m.		
FIP (50 pM)	40 pM		
BIP (50 pM)	40 pM		
LF (50 pM)	20 pM		

(*continued on next page*)

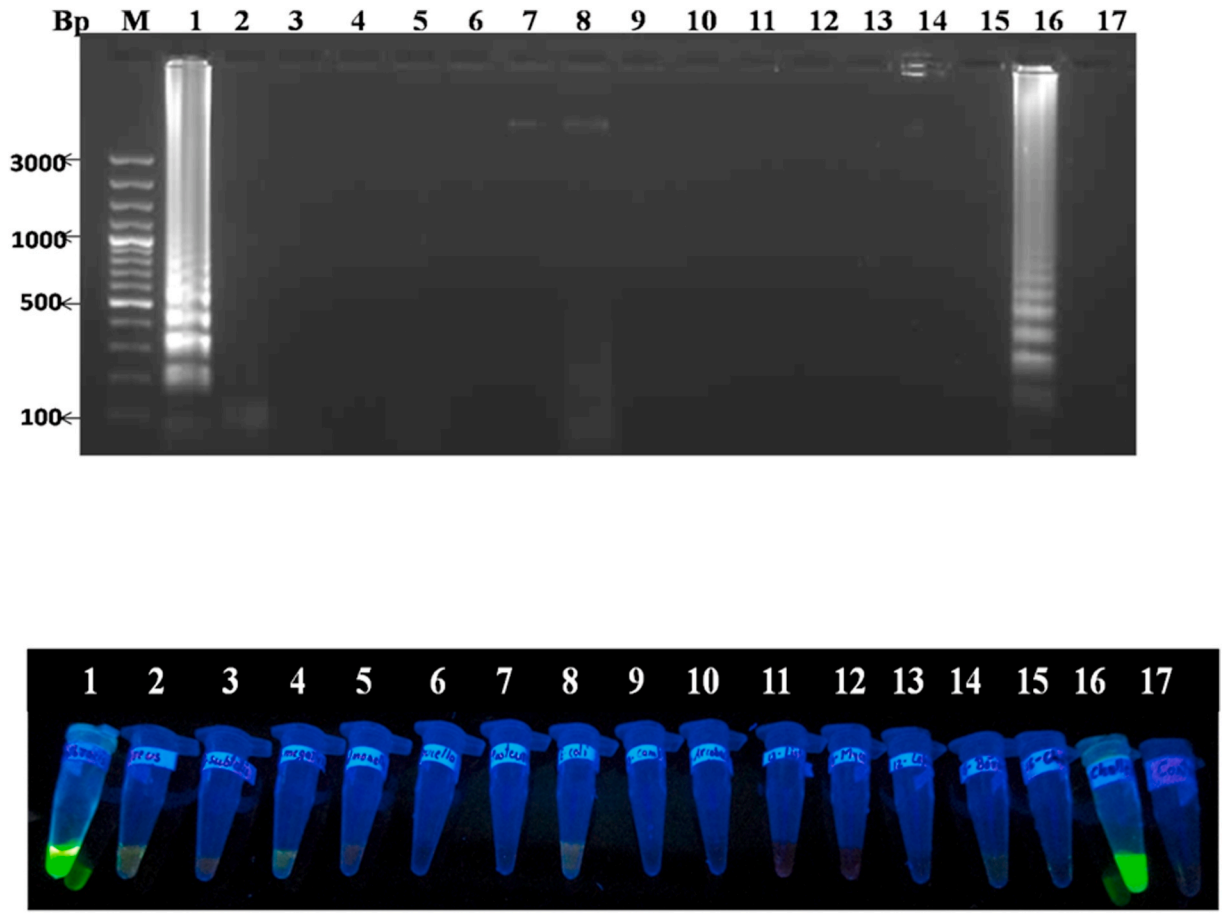

Fig. 3. Specificity evaluation of LAMP-PCR by agarose gel electrophoresis and SYBR Green I dye based detection under UV light: **Lane M**: GeneRuler™100 bp Plus DNA Ladder; **Lane/Tube 1**: *B. anthracis* Sterne 34F2; **Lane/Tube 2**: *B. cereus*; **Lane/Tube 3**: *B. subtilis*; **Lane/Tube 4**: *B. megaterium*; **Lane/Tube 5**: *Salmonella* Typhimurium; **Lane/Tube 6**: *Brucella abortus* strain 19; **Lane/Tube 7**: *Pasteurella multocida* B:2; **Lane/Tube 8***: E. coli*; **Lane/Tube 9:** *Campylobacter jejuni*; **Lane/Tube 10**: *Arcobacter* sp.; **Lane/Tube 11**: *Leptospira* sp.; **Lane/Tube 12**: *Mycoplasma* sp.; **Lane/Tube 13**: *Listeria monocytogenes;* **Lane/Tube 14**: *Bordetella bronchoseptica*; **Lane/Tube 15**: *Clostridium perfringens*; **Lane/Tube 16:** *B. anthracis* IVRI strain; **Lane/Tube 17**: Negative control

(*continued*)

Components	Concentration or volume	Time	Temperature
LR (50 pM)	20 pM	60 min with loop primers	
Betaine	1 M		
$MgSO_4$	6 mM		
NFW	To make up to 23 μl		
Template	1 μl		
Bst DNA polymerase	1 μl		

2.6. Detection of amplified LAMP products

The amplified LAMP products were analyzed by naked eye as well as by agarose gel electrophoresis. The color difference between the positive and negative reactions after adding either 0.12 mM hydroxynaphthol blue (HNB) dye (M/s Sigma Aldrich, USA) pre-amplification, or 1 μl of 1000X SYBR Green I (M/s Invitrogen, USA) post-amplification was also then checked. For the confirmation of a successful assay, the products were then run on 2% w/v agarose in Tris–acetate-EDTA buffer containing 0.5 μg/mL ethidium bromide and checked under ultraviolet illumination.

2.7. Detection of specificity and sensitivity

Specificity was checked by employing the total DNA extracted from various bacteria as template (Table 1). Sensitivity was determined by limiting dilution method and compared with conventional PCR. Briefly, serial tenfold dilutions of total DNA from *B. anthracis* was prepared and 2 μl from each dilution was used as template for LAMP-PCR and conventional PCR. The highest dilution producing distinct positive reaction was found out. DNA quantification of the undiluted supernatant was performed using Nanodrop® (Thermo Scientific, USA).

3. Results and discussion

The present paper describes the optimization of a LAMP-PCR for the specific and sensitive identification of anthrax causing bacteria in a user friendly platform. The main targets for diagnosis of anthrax are plasmid-located virulence genes [14] which are considered as hallmark genetic feature of *B. anthracis* [15]. There are 2 virulent plasmids as pXO1 encoding 'toxin' [16] and pXO2 encoding 'capsule' [15]. However, pXO1 encoding anthrax toxin will be a more apt target, as the loss of pXO1 in environmental samples is infrequent while the loss of pXO2 is common [17]. Furthermore, The Sterne strain which lacks pXO2 has some virulence for humans [18] and certain animal species [19]. At the same time, *B. anthracis* lacking pXO1 is reported to be non-pathogenic [6]. Besides, some *B. cereus* strains causing anthrax through anthrax toxin were isolated [8], showing the need for pXO1 based assays.

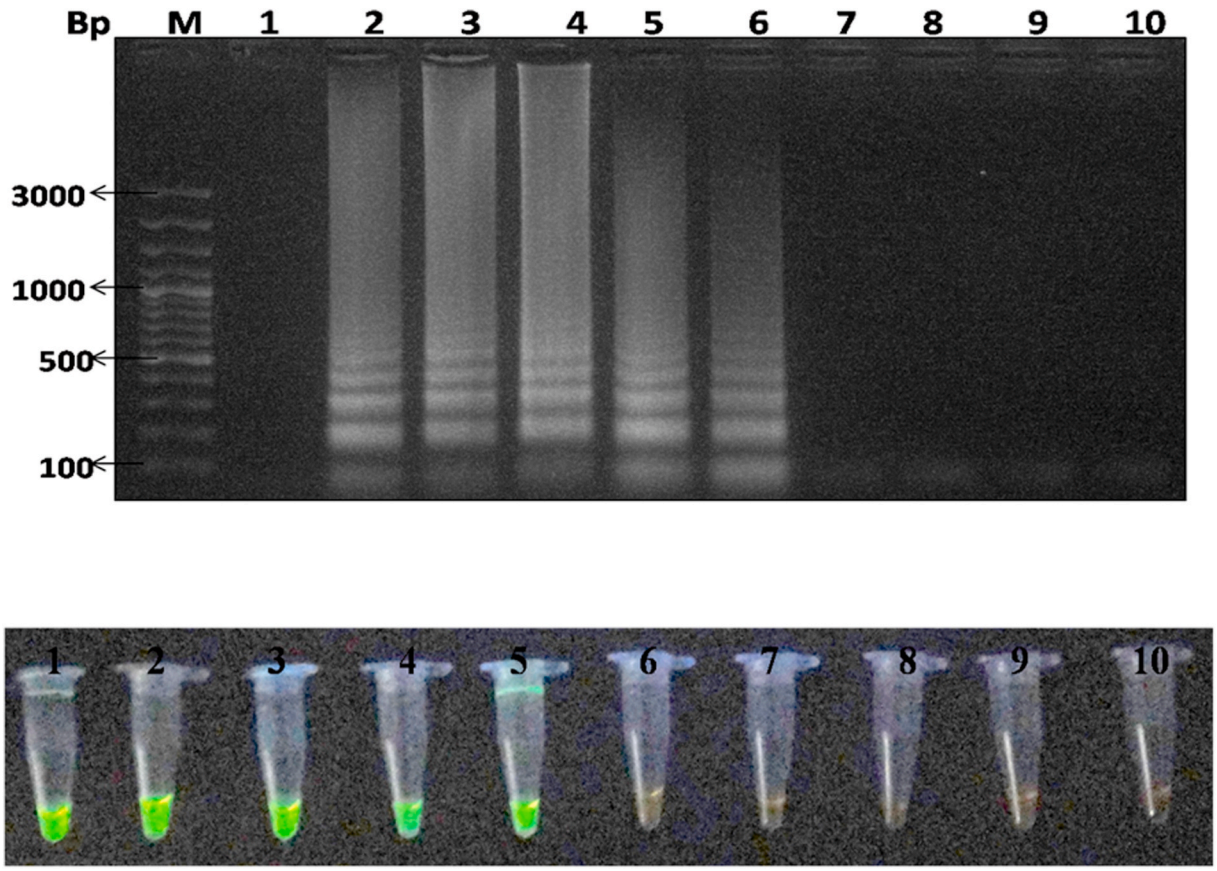

Fig: 4. Sensitivity evaluation of LAMP-PCR without loop primers by agarose gel electrophoresis and SYBR Green I dye based detection under UV light: **Lane M**: GeneRuler™ 100 bp plus DNA ladder; **Lane 1:** Negative control; **Lane 2/Tube 1:** 500 ng/μl; **Lane 3/Tube 2:** 50 ng/μl; **Lane 4/Tube 3:** 5 ng/μl; **Lane 5/Tube 4:** 500 pg/μl; **Lane 6/Tube 5:** 50 pg/μl; **Lane 7/Tube 6:** 5 pg/μl; **Lane 8/Tube 7:** 500 fg/μl; **Lane 9/Tube 8:** 50 fg/μl; **Lane 10/Tube 9:** 5 fg/μl; **Tube10:** Negative control

Accordingly, for identifying all bacteria that can produce anthrax together with the Sterne strain, *pag* encoding protective antigen (PA), the most essential component of anthrax toxin in pathogenesis was chosen as the target. As the virulence of all bacteria causing anthrax is mainly due to their toxin [20], detection methods aiming at *pag* have extra benefit for finding out the virulence of suspected organisms.

In LAMP-PCR reactions four primers recognizing six distinct regions of the target has extremely high specificity [21] and inclusion of two loop primers in the reaction mixture accelerate the amplification reaction [22]. Consequently, six LAMP primers including two loop primers were designed using Primer Explorer V4 online software, to recognize six specific regions on the highly conserved sequences of *pag* gene (302 bp to 614 bp) of *B. anthracis* (GenBank Accession number JQ798178).

As temperature is a critical factor for any successful LAMP reaction, the optimization assay was done at different temperatures starting from 58 °C to 66 °C. Although amplification happened at all these temperatures, the typical ladder like pattern with good intensity was seen at 64 °C. Further, optimization of different concentration of dNTPs, $MgSO_4$ and betaine was carried out and the best amplification was found with 1.2 mM, 6 mM and 1 M, respectively. Similarly, optimal reaction time was determined by analyzing the LAMP products after different periods of incubation. Although the amplified products could be detected after 45 min; the maximum amplification was observed after 60 min. In order to investigate the differences in reaction time in the presence orabsence of loop primers, the optimal reaction time without loop primers was determined. In the absence of loop primers the amplified products could be detected after 60 min and the maximum amplification was observed after 90 min. Thus, the results showed that inclusion of loop primers greatly reduced the reaction time from 90 min to 60 min as already reported [23]. Briefly, the optimized assay conditions were: 5 pmol of F3 and B3, 40 pmol of FIP and BIP, 20 pmol of LF and LR, 1.0 M betaine, 6 mM $MgSO_4$, 1.2 mM of dNTPs mix, 8U *Bst* DNA polymerase, 2.5 μl of 10X ThermoPol reaction buffer and 1 μl of target DNA in 25 μl reaction volume. The assay was carried out at 64 °C for 60 min and 90 min with loop primers and without loop primers respectively, followed by termination at 80 °C for 5 min.

The interpretation of positive and negative results was done by four different methods. In the first method simple visual observation for the presence of turbidity was carried out as accumulation of reaction by-product magnesium pyrophosphate in positive LAMP reactions can produce turbidity [21]. However, detection of turbidity requires some skill to distinguish between positive and negative LAMP results, so that other methods were also tried. In the second method LAMP product was run on 2% agarose gel which revealed a clear ladder like pattern (Fig. 2a) due to the formation of large stem loop cauliflower like structures [12] while no amplification and laddering pattern was observed with the negative control. However, in order to analyze this optimized LAMP assay without any specialized equipment and expertise, visual detection by addition of either SYBR Green I [10] or HNB dye [24] were also tried. With HNB dye, the purple color of the reaction mixture transformed to sky blue post-amplification in positive test (Fig. 2b) which could be detected by naked eye which is in accordance with the findings of Goto et al., 2018 (28) with the use of HNB dye. With SYBR Green I, the amplified products in positive assay gave green color while negative reaction developed orange color (Fig. 2c) and, under UV illumination at 475 nm positive LAMP reaction gave bright green fluorescence (Fig. 2d). Therefore, both dyes helped in the identification of positive LAMP reactions. However, HNB was more sensitive than SYBR green I for distinguishing the positive and negative reactions. Additionally, since HNB was incorporated into the reaction mixture prior to

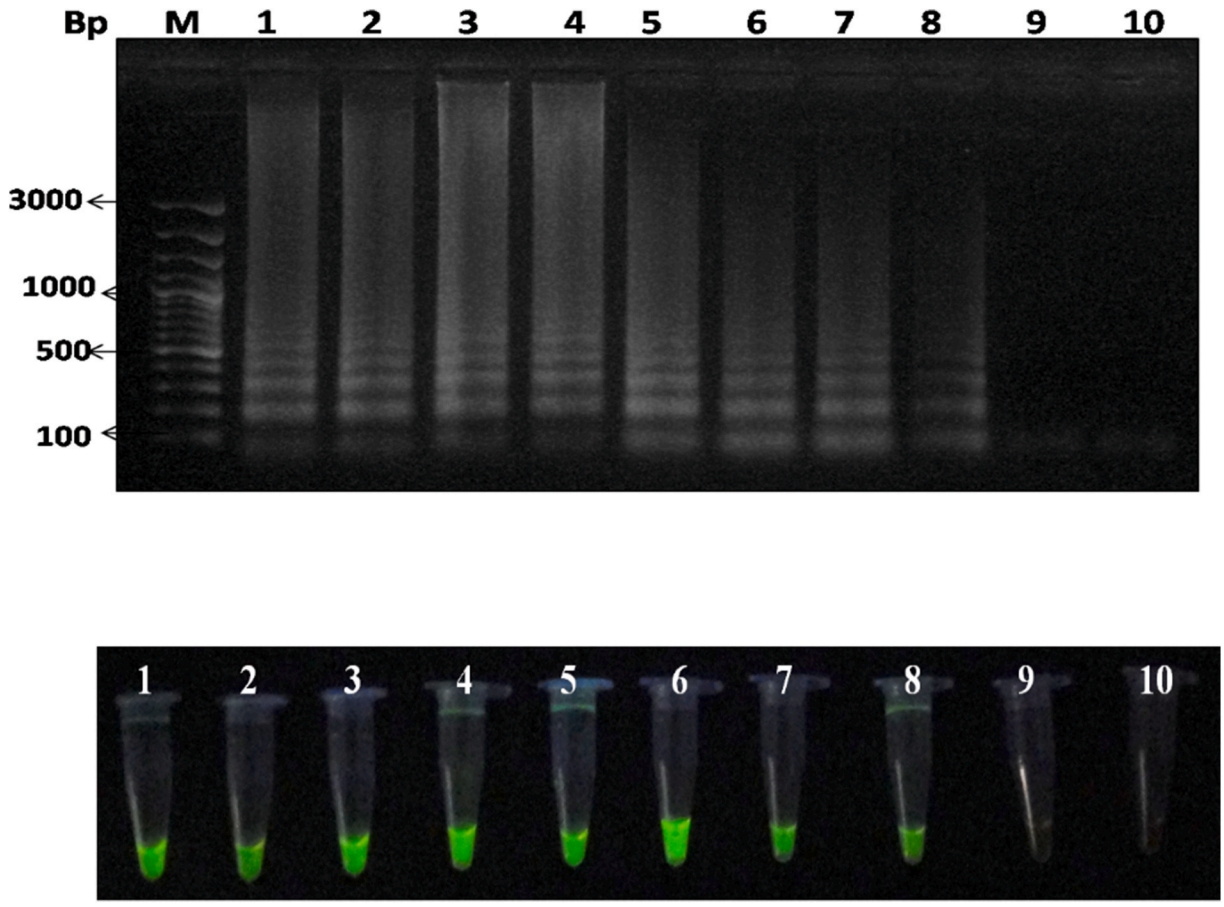

Fig: 5. Sensitivity evaluation of LAMP-PCR with loop primers by agarose gel electrophoresis and SYBR Green I dye based detection under UV light: **Lane M**: GeneRuler™ 100 bp Plus DNA Ladder; **Lane/Tube 1**: 500 ng/μl; **Lane/Tube 2**: 50 ng/μl; **Lane/Tube 3**: 5 ng/μl; **Lane/Tube 4**: 500 pg/μl; **Lane/Tube 5**: 50 pg/μl; **Lane/Tube 6**: 5 pg/μl; **Lane/Tube 7**: 500 fg/μl; **Lane/Tube 8**: 50 fg/μl; **Lane/Tube 9**: 5 fg/μl; **Lane/Tube 10**: Negative control

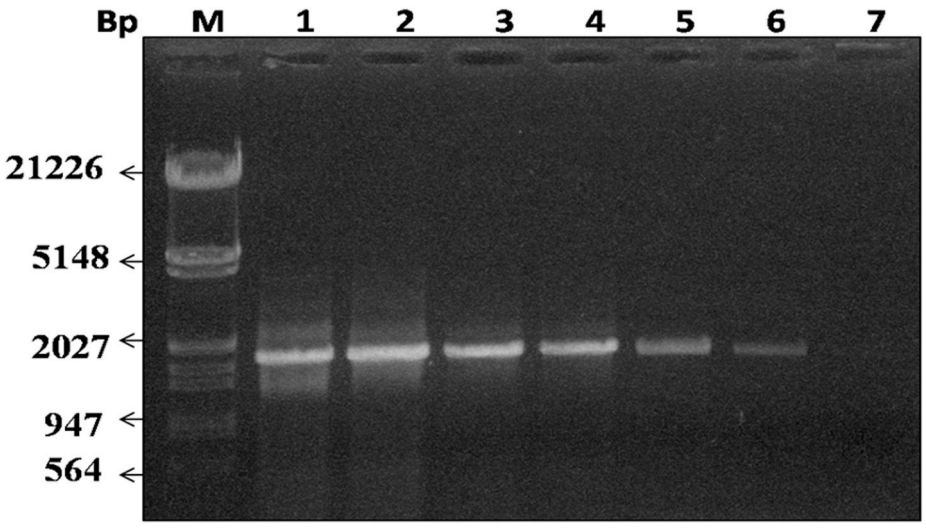

Fig: 6. Sensitivity evaluation of conventional PCR: **Lane M**: λ DNA/*Eco*RI + *Hind*III digested; **Lane 1**: 500 ng/μl; **Lane 2**: 50 ng/μl; **Lane 3**: 5 ng/μl; **Lane 4**: 500 pg/μl; **Lane 5**: 50 pg/μl;**Lane 6**: 5 pg/μl; **Lane 7**: 500 fg/μl.

amplification, it eliminated the possibility of laboratory contamination caused by the opening of tubes post-amplification. The optimized LAMP-PCR (both with and without loop primers) successfully produced positive reaction from both isolates of *B. anthracis*.

We used a simple method for the total DNA extraction from *B. anthracis* [11] and used as template in the optimized LAMP-PCR in addition to the plasmid isolated by commercial kit. As there is no chance of exposure to the active culture during this method of DNA extraction, excluding during initial inoculation of suspected sample, this method will contribute significantly in reducing the risk of laboratory acquired infection during the identification of suspected cultures. The present study demonstrated that this simple method of DNA extraction did not

adversely affect the optimized LAMP-PCR assay results.

The optimized LAMP-PCR was found to be specific for anthrax causing bacteria as there was no false positive reaction with any other tested bacteria (Five *Bacillus* spp. and 12 non- *Bacillus* bacteria) (Fig. 3). At this point, it is important to note that some *B. cereus* strains carrying pXO1 and pXO2 plasmids and can produce anthrax like disease [8,25] which cannot be differentiated from anthrax caused by *B. anthracis* by the present LAMP-PCR. However, recently it was revealed [26] that chromosomes of *B. anthracis* and the other *B. cereus* causing anthrax-like disease are genetically similar and there is no sub-group within *B. cereus* group which are genetically predisposed to anthrax pathogenesis, instead, any member of *B. cereus* may produce anthrax if they acquire the virulence plasmids. Thus clinically it is not important to differentiate such *B. cereus* strains capable of causing anthrax from toxigenic *B. anthracis* during anthrax diagnosis. Thus, the optimized LAMP-PCR can specifically detect all organisms that are capable of producing anthrax.

Sensitivity of the assay was 50 pg of total DNA without loop primers (Fig. 4) and 500 fg of total DNA with loop primers (Fig. 5) per reaction. Sensitivity of conventional PCR was 50 pg of total DNA (Fig. 6). Moser et al. [27] estimated that 1 pg of total DNA of *B. anthracis* contained about 100 copies of *pagA* targets. Therefore, sensitivity of the optimized assay when translated into a copy number limit of detection, can be expressed as 5000 copies of anthrax toxin-specific plasmids for conventional PCR and LAMP assay without loop primers. In the similar manner, sensitivity of the optimized LAMP assay with loop primers was calculated as 50 copies of anthrax toxin-specific plasmids. Therefore, sensitivity of the optimized LAMP assay with loop primers was 100 times more than the conventional PCR. The results also showed that use of loop primers increased the sensitivity and reduced the reaction time of LAMP-PCR without altering the specificity as previously described [22].

In conclusion, the present paper reports optimization of a sensitive LAMP-PCR based assay for specific identification of cultures of bacteria that can cause anthrax. The assay can also be used for determining the virulence of suspected cultures. The assay was about 100 times more sensitive than the conventional PCR. Thus, this can be a promising alternative diagnostic tool for the specific, sensitive, time and cost-effective and easy to perform identification method for anthrax causing bacteria.

Acknowledgement

The authors acknowledge the Director ICAR-IVRI for providing necessary facilities to carry out the experiment. LU is thankful to Indian Council of Agricultural Research for Junior Research Fellowship grant. The authors declare no conflict of interest.

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