DEVELOPMENT AND APPLICATION OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION METHOD FOR RAPID DETECTION OF *PASTEURELLA MULTOCIDA*

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ABSTRACT

Pasteurella multocida is an important pathogen affecting livestock and poultry causing significant economic losses. A loop-mediated isothermal amplification (LAMP) assay using four primers targeting a conserved region of the *kmt1* gene was standardised to diagnose *Pasteurella multocida*. The test was carried out at 64°C for 45 minutes, the LAMP products could be visually confirmed using fluorescent dye SYBR Green I as detection reagent both with naked eye as well as under UV-illumination. The sensitivity of the developed LAMP assay was 10^4 fold higher than PCR. Furthermore, no cross-reactivity was found with the other tested bacteria. The developed LAMP assay allows easy, rapid, accurate and sensitive detection of *Pasteurella multocida*.

Keywords: *Bubalus bubalis*, buffaloes, haemorrhagic septicemia, *Pasteurella multocida*, *kmt1* gene, LAMP

INTRODUCTION

Pasteurella multocida is a gram negative bacterial pathogen known to affect a wide range of domestic as well as wild animals and avian species (Hunt *et al*., 2000). It is associated with Haemorrhagic septicaemia (HS) in bovines, pneumonic pasteurellosis in sheep and goats, fowl cholera in poultry, atrophic rhinitis in pigs and snuffles in rabbits. The organism is divided into five capsular (A, B, D, E, and F) and 16 somatic (1-16) types. HS is an acute primary pasteurellosis affecting buffaloes and cattle and caused by specific serotypes of *Pasteurella multocida* (B:2/ B:2,5/ B:5) in Asian countries (Ataei *et al*., 2009). The buffaloes are highly susceptible to the infections, affected by the acute form and untreated cases invariably succumb to the infection. Diagnosis of the HS is mainly based on the clinical signs and symptom, post mortem findings. Confirmatory diagnosis is done by isolation and identification of causative agent. Polymerase chain reaction (PCR) based techniques have also been developed which not only gives diagnosis but also provide information regarding capsular type of *Pasteurella multocida*. Although PCR targeting different genes have been widely used for detection of *Pasteurella* sp., they are not appropriate for resource limited laboratories because the tests are time consuming, require skilled technicians, costly equipment and are not straight forward. Therefore, development of rapid, accurate, cost effective and more

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practical diagnostic method for diagnosis of HS and identification of *Pasteurella* sp. is of high priority. A promising candidate that could fulfill these requirements is Loop Mediated isothermal Amplification (LAMP) (Nagamine *et al*., 2002). It is an outstanding nucleic acid amplification method which relies on autocycling strand displacement DNA synthesis in a single tube under isothermal conditions and can amplify DNA with high specificity, efficiency and speed under simple laboratory conditions (Notomi *et al*., 2002). In this study, a LAMP assay was developed and compared with conventional PCR for the detection of *P. multocida* from haemorrhagic septicemia affected animals.

MATERIALS AND METHODS

Bacterial strains and DNA preparation

Standard vaccine strain of *Pasteurella multocida* (P52) was used for standardization of the LAMP assay. The extraction of genomic DNA from *P. multocida* isolates was performed as per the method described by Wilson (1987).

Primers for LAMP and PCR

In order to devise effective LAMP primers, nucleotide sequence of the *kmt1* gene of *Pasteurella multocida* serovar B2 was analyzed according to different reference sequence published in Gene bank (Genebank accession no AY225341) (Jabbari *et al*., 2006). The conserved region of the *kmt1* gene from 1 nt to 447 nt was used for primer design. A total of four specific primers for LAMP reaction were designed using the Primer Explorer V4 software (Eiken Chemical Co., Ltd). The primers Past-F3 and Past-B3 are an outer pair whereas primers Past-FIP and Past-BIP are inner pair. These primers specifically recognized 6 distinct regions on target DNA. FIP is comprised of the F1c sequence complementary to F1, a TTTT linker and the F2 sequence; BIP is comprised of the B1c sequence complementary to B1, a TTTT linker and the B2 sequence. Nucleotide sequence used for LAMP primer designing and the sequence primers designed are shown in the Figure 1 and Table 1, respectively.

Optimization of LAMP reaction

All the conditions like the relative concentration of outer and inner primers (1:1 to 1:10), concentration of $MgSO_4$ (2 to 8 mM), dNTPs (0 to 1.2 mM), Betaine (0 to 1M), incubation temperature (58°C to 65°C), reaction length (15 to 60 minutes) were optimized and final reaction conditions were determined.

The finally standardised LAMP reaction was carried out in a 25 µl mixtures containing 0.2 µM of outer primers (the primers Past-F3 and Past-B3), 1.6 µM of inner primers (the primers Past-FIP and Past-BIP), 4 mM of $MgSO_4$ (MBI Fermentas), 0.4 mM of dNTPs (MBI Fermentas), 0.6 M of Betaine (Sigma-Aldrich), 2.5 µl of 10×Thermopol buffer, 8U of *Bst*DNA polymerase (New England Biolabs, USA), approximately 200 ng of genomic DNA of *P. multocida* (P52) and nuclease free water to make reaction volume 25 µl. The reaction was carried out at 64°C in a water bath for 45 minutes and inactivated at 80°C for 10 minutes (Notomi *et al*., 2000). A reaction containing nuclease free water was used as the negative control.

LAMP products were visualized by addition of 1 µl of 1:10 diluted SYBR Green I dye (Life technologies) to the reaction tubes. The change in colour from orange to green was observed with naked eye as well as using UV

transilluminator. 5 µl of LAMP products were subjected to electrophoresis on a 2.0% agarose gel in 1X TBE and stained with 0.5 µg/ml ethidium bromide. The gel was visualized under UV transilluminator.

Specificity and sensitivity of the LAMP reaction

The specificity of the developed Pm-LAMP was assayed by performing the reaction with seven other bacterial genera (*Salmonella, Brucella, Escherichia coli, Staphylococcus, Pseudomonas, Proteus, Klebsiella* along with *P. multocida isolates* (including standard vaccine strain and field isolates n=8).

Conventional PCR was performed with primers targeting *kmt1* gene described by Townsend *et al*., 1998. Sensitivity of the standardized LAMP reaction and Species specific polymerase chain reaction was determined and compared. Serial 10-fold dilutions (400 ng to 0.4 fg) of the DNA were made in nuclease free water and the concentration of DNA was measure with UV spectrophotometer by calculating A260/A280 ratio. LAMP and conventional PCR were performed as per the optimized conditions by taking 2 µl of template DNA from each dilution in both reactions.

PCR was performed in 25 µl reaction volume by adding 2.5 µl 10x Taq buffer, 0.5 mM of each dNTP, 1.5 mM of $MgCl_2$, 0.4 µM of each primer (forward and Reverse), 1U T*aq* DNApolymerase (Qiagen), 2 µl of genomic DNA template and nuclease free water to make final volume of 25 µl. Amplification conditions consisted of an initial denaturation at 94°C for 5min followed by 30 cycles of amplification for 1 minutes at 94°C, 1 minute at 55°C, 1 minute at 72°C and a final extension for 6 minutes at 72°C. PCR products were analysed by agarose gel electrophoresis.

Applicability of the developed LAMP assay on clinical samples

A total of 40 samples including nasal swabs, blood and tissue samples were collected from cattle and buffalo suspected for haemorrhagic septicemia as well as normal animals to evaluate feasibility of the developed LAMP method in clinical detection. All the samples were tested simultaneously both by developed LAMP assay and PCR in parallel.

RESULTS AND DISCUSSIONS

Optimized LAMP assay condition for detection

All of the possible variables were tested to optimize the LAMP reaction. The results indicated that LAMP reaction generated the typical ladder-like pattern of amplification products at all temperatures (58 to 65°C) but 64°C gave the optimal reaction product. Amplification at wide range of temperatures (58 to 65°C) indicating its applicability in the fields where poor temperature control may be a factor (Tsai *et al*., 2009). While optimizing reaction time, not much variation was observed in the results between 45 and 60 minutes.

So the reaction length 45 minutes was selected as the optimal time period. Several other studies with other organisms have also indicated the detection of amplification below 60 minutes (Iwamoto *et al*., 2003; Savan *et al*., 2004).The concentration of betaine at 0.6 M gave the maximal amplification. The dNTPs concentrations ranging from 0.4 to 1.2 mM amplified the target DNA. So the minimum 0.4 mM concentration was selected as the optimal level for further reactions. Betaine is known to help in separation of DNA strands and to increase the specificity. The effectiveness of 0.4M of betaine in *Pasteurella* in contrast to

higher concentration reported by other workers (Inacio *et al*., 2008) may be due to fact that DNA in *Pasteurella multocida* is not GC rich. Out of several ratios of outer to inner primers studied DNA target was amplified in the primer ratio of 1:8. Out of different $MgSO_4$ concentration a minimum of 4 mM concentration gave a positive reaction and no significant change in the yield of amplicon was observed by increasing the concentration beyond 4 mM.

Taken together, the optimal conditions for the LAMP assay were determined as 64°C for 45 minutes with 4 mM $MgSO_4$, 0.6 M betaine, 0.4 mM dNTPs, 0.2 µM each of outer primer, 1.6 µM each of inner primer and 8U *Bst* polymerase. The results of optimised LAMP reaction are depicted in Figure 2.

The dNTPs concentrations ranging from 0.4 to 1.2 mM amplified the target DNA. So the minimum 0.4 mM concentration was selected as the optimal level for further reactions which is also reported by Notomi *et al*., 2000, and much lower than those reported for bacteria by others (Iwamoto *et al*., 2003; Maruyama *et al*., 2003).

Since the ratio of outer and inner primers may affect the sensitivity of the LAMP reaction (Notomi *et al*., 2000; Parida *et al*., 2004) optimum results were obtained when we used an outer and inner primer ratio of 1:8. Out of different $MgSO_4$ concentration a minimum of 4 mM concentration need give a positive reaction however there is no significant change in the yield of Amplicon was observed beyond 4 mM. Naked-eye identification of the result by adding nucleic acid stain like SYBR Green facilitates rapid screening of samples without need of electrophoresis (Yamazaki *et al*., 2008; Inacio *et al*., 2008).

Specificity and sensitivity of the LAMP reaction

The specificity of the designed LAMP primers of the *P. multocida Kmt 1* gene, was indicated by the fact that amplification was observed only in case of *Pasteurella multocida* while all other non-*Pasteurella* organisms gave a negative result. No cross-reactivity was observed with other bacterial genera. Also the reaction gave a positive result when a mixture of DNA from all the bacterial genera including *P. multocida* was used (Figure 3). The involvement of four primers targeting six different regions on the gene are known to impart specificity to amplification (Tsai *et al*., 2009; Maruyama *et al*., 2003, Enosawa *et al*., 2003). No amplification of the DNA from other bacteria included in the study indicated specificity of LAMP reaction.

All the 8 field isolates of *Pasteurella multocida* tested positive with the developed assay (Figure 4). As far as the detection level of LAMP is concerned, LAMP gave a positive reaction on DNA concentration as low as 40 fg whereas PCR was positive only up to 400 pg concentration, thereby indicating that the developed LAMP assay is approximately 10^4 times more sensitive than species specific PCR. Application of LAMP technique on clinical samples indicated that the technique may be appropriately applied for field use however further improvements are possible by incorporating colorimetric detection.

Applicability of the developed LAMP assay in clinical samples

The application of developed LAMP test on the clinical samples indicated that DNA isolated from all the culturally positive clinical samples including nasal swabs, tissues and blood tested positive for both LAMP as well as PCR whereas none of the negative sample showed

Table 1. Primers designed for LAMP reaction.

Primer Name	Sequence(5’-3’)
Past-F3	CGCGAAATTGAGTTTTATGC
Past-B3	CAAGGAAATATAAACCGGCAA
Past-FIP (F1C+tttt+F2)	GCCACAAGCTAAATAAAAGACTACCttttAAATGGCATTATTTTATGGCTCG
Past-BIP (B1C+tttt+B2)	CACAGTTTTGTTGGGCGGAGttttAAATAACGTCCAATCAGTTGC

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1 CCCAGTGGGG CGGTGCGAAT GAACCGATTG CCGCGAAATT GAGTTTTATG CCACTTGAAA
                                  F3
61 TGGGAAATGG CATTATTTTA TGGCTCGTTG TGAGTGGGCT TGTCGGTAGT CTTTTATTTA
           F2
121 GCTTGTGGCA AAGAAAAGCA CAGTTTTGTT GGGCGGAGTT TGGTGTGTTG AGCCAATCTG
                                                F1
181 CTTCCTTGAC AACGGCGCAA CTGATTGGAC GTTATTTATT ACTCAGCTTA TTGTTATTTG
                          B2
241 CCGGTTTATA TTTCCTTGTC AGTCTGATTT ATCAATATTT CCATGTTGAG TTACGTTTCT
       B3
301 TATGGCCATT ATTGAAGCCA TTAACGACAG AGCGGTTTAA TTTATTTATC GTGTATTGGT
361 TACCTATTTT GGTCTTTTTC TTCGTGTTCA ACGGTTTAAT CGTGTCAGTC CAAATGAAAC
421 AAAAAGTGGC GAAGTCGTTT ACAGCAA
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Figure 1. Nucleotide sequence of *Kmt1* gene used for primer designing and position of primers.

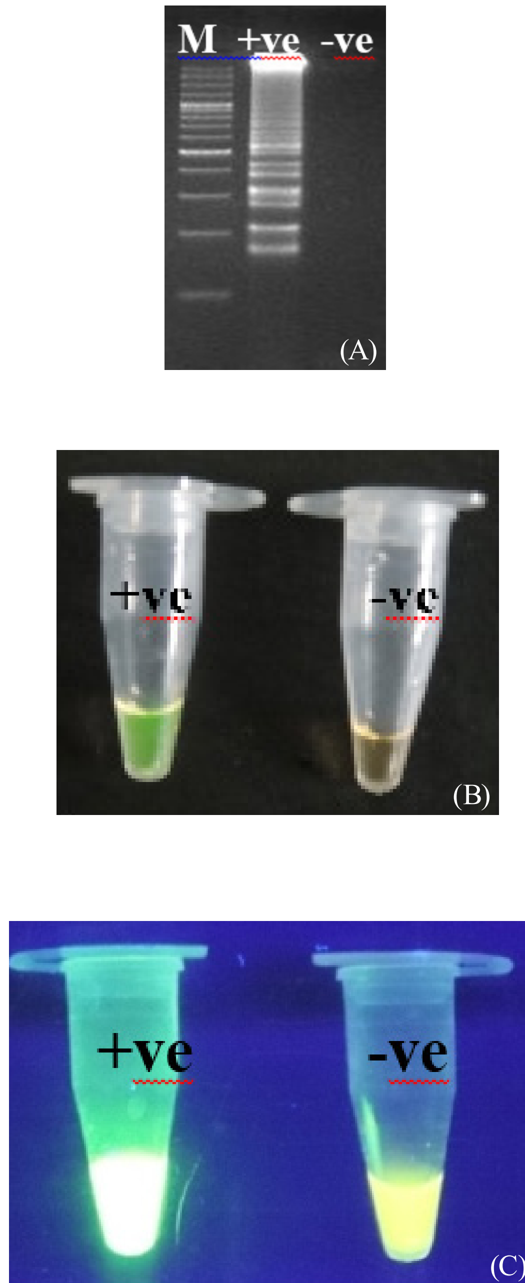
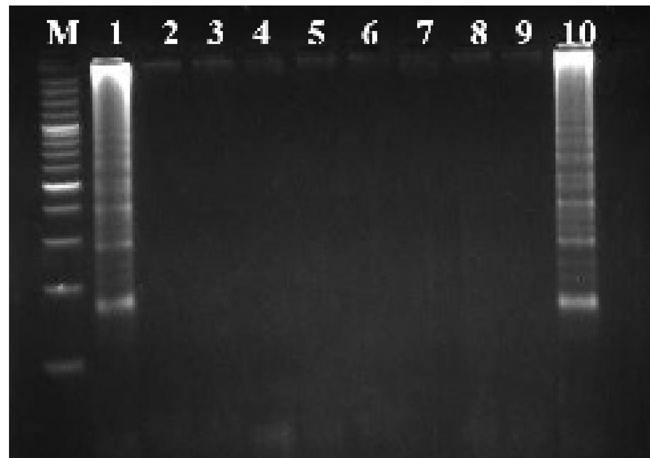
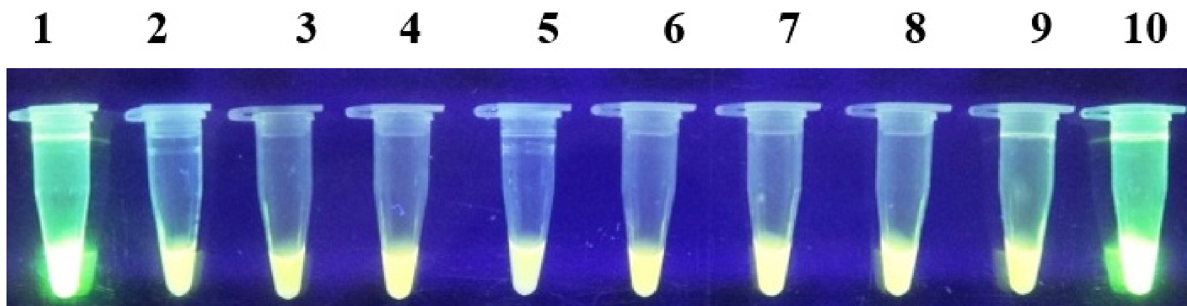


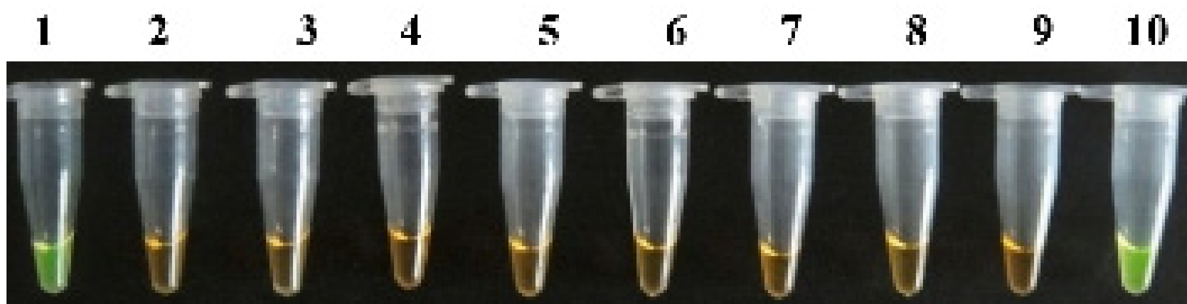
Figure 2. Analysis of optimized LAMP reaction by (A) agarose gel electrophoresis (B) in natural light (C) under UV transilluminator, (M: 100 bp Plus molecular DNA ladder ; +ve: denotes Positive control (with target DNA); -ve: Negative control).



(A)



(B)



(C)

Figure 3. Specificity of LAMP reaction as observed by (A) gel electrophoresis, (Lane M: 100 bp plus DNA ladder. Lane 1: *Pasteurella* sp.; Lane 2: *Brucella* sp.; Lane 3: *Salmonella* sp.; Lane 4: *Escherichia coli*; Lane 5: *Proteus* sp.; Lane 6: *Pseudomonas* sp.; Lane 7: *Staphylococcus* sp.; Lane 8: *Klebsiella* sp.; Lane 9: Negative control; Lane 10: Mixture of DNA of all the tested organisms including *Pasteurella multocida*; (B) Visual detection in natural light and (C) Visual detection on UV transilluminator.

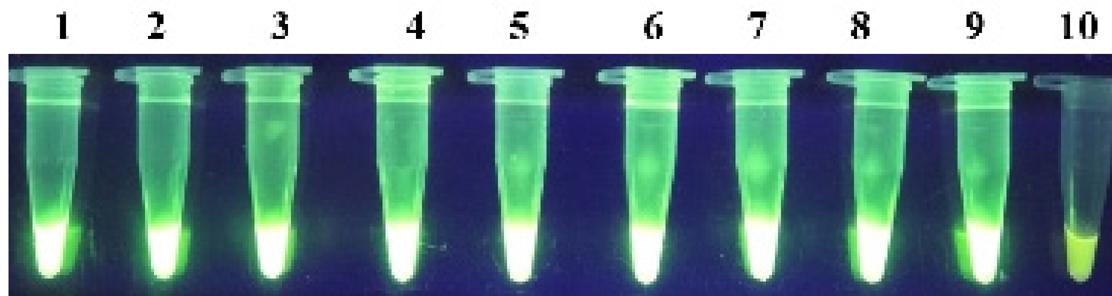


Figure 4. Testing of field isolates with developed LAMP reaction: Tube 1, Positive control; Tubes 2 to 9, field isolates; Tube 10: Negative control.

any amplification. Although all the PCR positive samples also tested positive by LAMP, the ability of the latter to detect very small quantities of DNA makes it to more sensitive than PCR.

CONCLUSION

The LAMP assay developed for detection of *Pasteurella multocida* is specific, sensitive, rapid compared to conventional PCR. The optimal utilisation of the technique holds promise to be of great help for quick diagnosis of haemorrhagic septicaemia in resource limited laboratories.

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