Short Paper

LAMP assay for rapid diagnosis of cow DNA in goat milk and meat samples

Deb, R.[1*]; Sengar, G. S.[2]; Singh, U.[1]; Kumar, S.[1]; Raja, T. V.[1]; Alex, R.[1]; Alyethodi, R. R.[1] and Prakash, B.[1]**

[1]Molecular Genetics Laboratory, ICAR-Central Institute for Research on Cattle, Meerut-250 001, Uttar Pradesh, India; [2]MSc in Biotechnology, Molecular Genetics Laboratory, ICAR-Central Institute for Research on Cattle, Meerut-250 001, Uttar Pradesh, India

[*]***Correspondence:*** *R. Deb, Molecular Genetics Laboratory, ICAR-Central Institute for Research on Cattle, Meerut-250 001, Uttar Pradesh, India. E-mail: drrajibdeb@gmail.com*

[*, **]*These authors contributed equally to this work*

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Summary

Animal species detection is one of the crucial steps for consumer's food analysis. In the present study we developed an in-house built loop-mediated isothermal amplification (LAMP) assay for rapid detection of adulterated cow DNA in goat milk/meat samples. The cow milk/tissue DNA in goat milk/meat samples were identified in the developed LAMP assay by either naked eye visualizing with SYBR Green I dyes or by detecting the typical ladder pattern on gel electrophoresis. This test can detect up to minimum 5% level of cow components admixed in goat milk/meat samples and can be completed within 1 h 40 min starting from DNA extraction from milk/meat samples and can be performed in a water bath. Developed LAMP methodology is simple; rapid and sensitive techniques that can detect adulterant like cow components in goat milk/meat are more accurate than other existing DNA based technologies.

Key words: Cow, Goat, LAMP assay, Meat, Milk

Introduction

Species determination in food products has a remarkable importance in food traceability and deception control. For the cause of both ethical issues as well as consumers choice there is an urgent need for developing a suitable sensitive technique for dairy as well as meat factories for detection of such adulteration (Lee *et al*., 2004). Certain efforts were made to differentiate cow and goat milk/meat samples using enzymatic, electrophoretic, chromatographic, mass spectrometric assays and molecular tools (Bitri *et al*., 1993; Lopez-Calleja *et al*., 2004; Abdel Rehman *et al*., 2007; Zhang *et al*., 2007; Calvano *et al*., 2012; Khanzadi *et al*., 2013; Volk *et al*., 2014). However, the tests/assays used have certain disadvantages like they are time consuming, have lengthy protocol requirements, require costly equipment and reagents. Here we developed a loop-mediated isothermal amplification (LAMP) based methodology to identify cow milk/tissue samples adulterated in goat counterparts. This study is the extension of our previous reports which developed for detecting cow milk/meat in buffalo counter parts (Deb *et al*., 2016). The technique requires a set of primers as well as conditions different from those used for normal PCRs. The LAMP reaction typically occurs within 30 to 60 min with temperatures ranging from 60 to 65°C under an isothermal condition with a simple heating block instead of a thermocycler, thus avoiding standard lengthy PCR protocols (Notomi *et al*., 2000). Loop-mediated isothermal amplification assays have now been reported for detection of various pathogens (Pawar *et al*., 2014; Wang, 2015); however, its application for species identification is scanty (Abdulmawjood *et al*., 2014). In the present study, we developed an in-house built rapid, economical and user-friendly LAMP assay protocol to identify the cow milk/meat samples adulterated in goat milk/meat.

Materials and Methods

Cow and goat milk samples were procured from dairy farm and local households. Tissue samples/scrap materials from dead animals of cattle and meat samples of goat from the local slaughter house were collected aseptically. Immediately after collection samples were transferred on ice to the laboratory aseptically. Milk/meat/tissue samples were subjected to genomic DNA extraction using standard phenol-chloroform methods with a few modifications. The mitochondrial D-loop gene sequences of *Bos taurus* (accession No. KJ789953) were selected from the NCBI database. Four primer sets viz. outer forward (F3), outer reverse (B3), inner forward (FIP) and inner reverse were designed using Primer Explorer V4 (http://primerexplorer.jp/e) online software after multiple sequence alignment. Details of the primers designed for the present LAMP assay are presented in Table 1.

The LAMP products were run in 2.5% agarose gel

Table 1: List of primers designed for LAMP assay in the present

Primer code	Primer sequence (5´-3´)	No. of bases	Free energy (kcal/mol) at 5´ and 3´ end	Nucleotide position in the mitochondrial D-loop sequence (KJ789953)
F3	CCTTCATAAAAATTTCCCCCTTA	23	5´: -4.86 3´: -4.53	21-43
B3	GTTGGGAGACTCATCTAGG	19	5´: -5.61 3´: -4.27	205-223
FIP	GCTAAATTGAGTATTGAAGAGCG TG-TCTACCACCACTTTTAACAGA	46		98-122 48-68
BIP	CAAAGTCAATATATAAACGCAGG CC-GTGCCTTGCTTTGGGTTA	43		132-156 172-196
F2	TCTACCACCACTTTTAACAGA	21	5´: -4.43 3´: -4.41	48-68
F1c	GCTAAATTGAGTATTGAAGAGCGTG	25	5´: -4.09 3´: -6.57	98-122
B2	GTGCCTTGCTTTGGGTTA	18	5´: -6.24 3´: -4.69	172-196
B1c	CAAAGTCAATATATAAACGCAGGCC	25	5´: -4.16 3´: -6.69	132-156

electrophoresis followed by visualization after ethidium bromide staining for 5 min. Optimization of the ladder pattern was done by changing different reaction components ($MgSO_4$ and betaine) as well as reaction temperature. The assay was conducted with different concentrations of betaine (0.2 to 1.6 M), $MgSO_4$ (2.0 to 10 mM) and different reaction temperature (61 to 65°C) at water bath. Optimization for the LAMP reaction was conducted both at water bath (Labindia, Mumbai, India) and at thermocycler.

The LAMP reaction was conducted in a PCR tube on a scale of 25.0 µL mixture containing each outer primer (F3 and B3) at the concentration of 5 pmol, each inner primer (FIP and BIP) at the concentration of 40 pmol, 4.0 mM $MgSO_4$ (New England Biolabs, USA), dNTP mix at the concentration of 1.6 mM (Sigma, USA), 8 U Bst DNA polymerase (New England Biolabs, USA), thermo polymerase reaction buffer (20 mM Tris-HCl, 10 mM $(NH4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100) and 2 µL of targeted genomic DNA. The amplification reaction was carried out at 61°C for 60 min and terminated by incubating in water bath at 85°C for 5 min.

Post amplification color change was detected by addition of 1.0 µL SYBR Green l (1:1000) dye (Invitrogen, USA) in each LAMP reaction tube. Since SYBR Green I can only bind to the double-stranded DNA to emit a green color, henceforth it is noted whether the color turned to green which, under natural light is representative of the positive reaction. Cow milk/meat samples were deliberately mixed with pure goat milk/meat samples at different percentages (100 to 0.5% of cow milk). The limit of detection for cow DNA in the admixed samples was determined by the in-house built LAMP assay and visualized by SYBR Green I dye. Further, ladder pattern was also evaluated in all the LAMP products after running at 2.5% agarose gel electrophoresis followed by staining with ethidium bromide.

Results

The LAMP products were subjected to visualization after addition of SYBR Green I dye, cow amplified products led to the formation of green color, while control with no template as well as unadulterated goat samples remain as orange color (Fig. 1). Under UV light, the cow reaction gave a bright fluorescence. The LAMP products were also detected on agarose gel, the positive amplified products of cow milk/meat DNA exhibited a typical laddering pattern, due to formation of stem-loop structures indicative of a successful LAMP assay. No laddering pattern was perceived with the negative control and pure goat DNA samples. We deliberately admixed goat milk/meat samples with different percentile of cow milk/meat. Isolated genomic DNA from each of the admixed samples was subjected for the LAMP assay, which revealed that the admixture of cow milk/meat up to 5% can be detected by the test (Figs. 2A-B and 3A-B). The products were also run in 2.5% agarose gel, to visualize the ladder pattern, which revealed that up to 5% cow milk/meat can depict ladder effect during LAMP assay (Figs. 4 and 5).

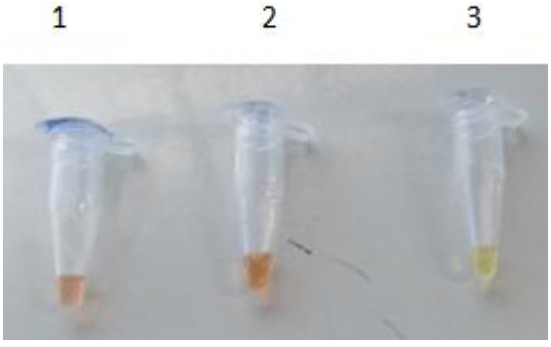

Fig. 1: Visualization of the LAMP assay products under white light after adding SYBR Green I dye. Lane 1: No template control (NTC), Lane 2: Goat DNA, and Lane 3: Cow DNA

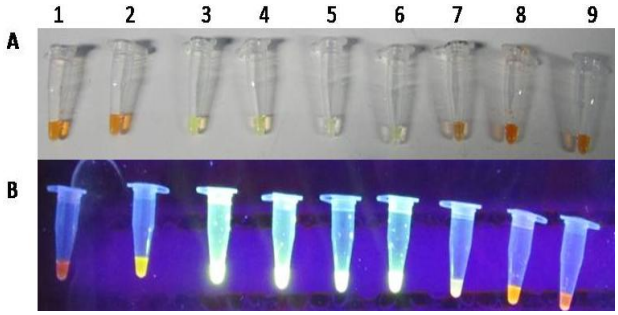

Fig. 2: Sensitivity detection of developed LAMP assay for identifying cow milk components in goat. Goat milk samples were deliberately mixed with cow milk in different ratio and cow components were detected using the LAMP assay with SYBR Green I (A: under normal light and B: under UV light). M: 100 basepair ladder. Lane 1: No template control (NTC), Lane 2: 100% goat milk, Lane 3: 100% cow milk, Lane 4: 50% cow and 50% goat, Lane 5: 10% cow and 90% goat, Lane 6: 5% cow and 95% goat, Lane 7: 3% cow and 97% goat, Lane 8: 2% cow and 98% goat, and Lane 9: 1% cow and 99% goat

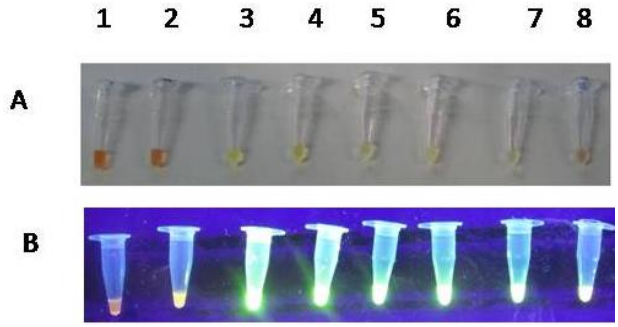

Fig. 3: Sensitivity detection of developed LAMP assay for identifying cow tissue components in goat. Goat meat samples were deliberately mixed with cow tissue in different ratio and cow components were detected using the LAMP assay with SYBR Green I (A: under normal light and B: under UV light). Lane 1: No template control (NTC), Lane 2: 100% goat meat, Lane 3: 100% cow tissue, Lane 4: 50% cow and 50% goat, Lane 5: 10% cow and 90% goat, Lane 6: 8% cow and 92% goat, Lane 7: 5% cow and 95% goat, and Lane 8: 3% cow and 97% goat

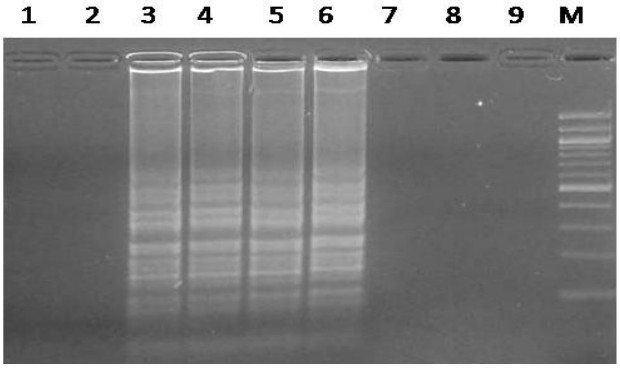

Fig. 4: Detection of ladder pattern at post LAMP assay for identifying cow milk components in goat milk samples. M: 100 basepair ladder. Lane 1: No template control (NTC), Lane 2: 100% goat milk, Lane 3: 100% cow milk, Lane 4: 50% cow and 50% goat, Lane 5: 10% cow and 90% goat, Lane 6: 5% cow and 95% goat, Lane 7: 3% cow and 97% goat, Lane 8: 2% cow and 98% goat, and Lane 9: 1% cow and 99% goat

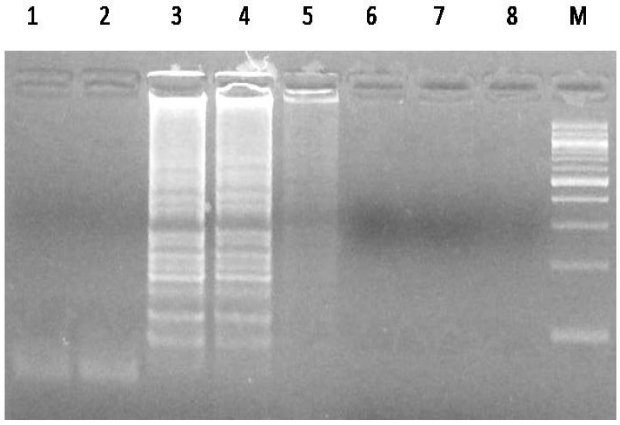

Fig. 5: Detection of ladder pattern at post LAMP assay for identifying cow tissue components in goat tissue samples. M: 100 basepair ladder. Lane 1: No template control (NTC), Lane 2: 100% goat meat, Lane 3: 100% cow tissue, Lane 4: 10% cow and 90% goat, Lane 5: 5% cow and 10% goat, Lane 6: 3% cow and 97% goat, Lane 7: 2% cow and 98% goat, and Lane 8: 1% cow and 99% goat

Discussion

The development of the present LAMP technique will be suitable under field condition which is in agreement with the earlier observation (Notomi *et al*., 2000). $MgSO_4$ and betaine are two important parameters for detecting typical LAMP assay ladder pattern. In the present study, we optimized the concentration of both the components to get a better ladder effect during the developed LAMP assay by keeping the other parameters of LAMP assay as standard; we optimized the $MgSO_4$ and betaine concentration for obtaining better ladder patterns. Our results indicated that, the best amplification was achieved with 4.0 mM $MgSO_4$ and 1.6 M betaine with the optimal temperature at 61°C. Loop-mediated isothermal amplification assay developed here was used to identify cow genomic DNA in goat milk or meat samples through color based visualization and ladder pattern. It can thus be concluded that the novel LAMP tools developed in the present study can help in the detection of fraud like adulteration in goat milk or chevon more accurately and rapidly. This will ultimately benefit both the consumers as well as producers and can aid in reducing/can aid in reduction of economic losses as well.

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