

**DEVELOPMENT OF HIGHLY SPECIFIC, RAPID AND  
SENSITIVE LOOP MEDIATED ISOTHERMAL AMPLIFICATION  
ASSAY (LAMP) FOR IDENTIFICATION OF MUTTON**

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P.V. NARSIMHA RAO TELANGANA VETERINARY  
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FOR THE AWARD OF THE DEGREE OF  
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IN  
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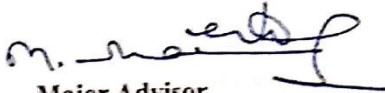
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## CERTIFICATE

Ms. THOKALA MOUNIKA has satisfactorily prosecuted the course of research and the thesis entitled "DEVELOPMENT OF HIGHLY SPECIFIC, RAPID AND SENSITIVE LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) FOR IDENTIFICATION OF MUTTON" submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any university.

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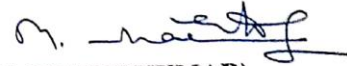


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## CERTIFICATE

This is to certify that the thesis entitled "DEVELOPMENT OF HIGHLY SPECIFIC, RAPID AND SENSITIVE LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) FOR IDENTIFICATION OF MUTTON submitted in partial fulfillment of the requirements for the degree of MASTER OF VETERINARY SCIENCE of the P. V. Narsimha Rao Telangana Veterinary University, Hyderabad is a record of the bonafide original research work carried out by Dr. THOKALA MOUNIKA under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee. No part of the thesis has been submitted for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.



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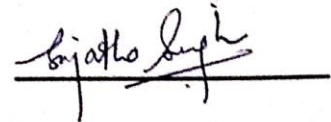
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I, Ms. **THOKALA MOUNIKA** hereby declare that the thesis entitled "**DEVELOPMENT OF HIGHLY SPECIFIC, RAPID AND SENSITIVE LOOP MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) FOR IDENTIFICATION OF MUTTON**" submitted to P. V. Narsimha Rao Telangana Veterinary University for the Degree of **MASTER OF VETERINARY SCIENCE** is a result of original research work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

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Place: Hyderabad

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(THOKALA MOUNIKA)

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THOKALA MOUNIKA

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## LIST OF ABBREVIATIONS

%	Percentage
<sup>0</sup> C	Degree Celsius
ATP	Adenosine triphosphate
AluI	<i>Arthrobacter luteus</i>
ApoI	<i>Arthrobacter protophormiae</i>
A	Adenine
AL	Alkaline lysis
ATPase	Adenosine triphosphatase
BglIII	<i>Bacillus globigii</i>
BM	Binary mixture
Bp	Basepairs
BspTI	<i>Arthrobacter protophormiae</i>
B3	Backward outer
BIP	Backward inner
Bst	<i>Bacillus stearothermophilus</i>
Bsm	<i>Bacillus smithi</i>
C	Cytosine
CO1,2,3	Cytochrome oxidase 1, 2, 3
CFU	Colony forming unit
Cytb	Cytochrome b
CuSO <sub>4</sub>	Copper sulphate
D-loop	Displacement loop
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ELISA	Enzyme linked immunosorbent assay
FAO	Food and Agricultural Organization

Fg	Femtogram
FINS	Forensically Informative Nucleotide Sequencing
Fig	Figure
F3	Forward Outer
FIP	Forward Inner
G	Guanine
Gm	Gram
HhaI	<i>Haemophilus haemolyticus</i>
HincII	<i>Haemophilus influenzae Rc</i>
HindII	<i>Haemophilus influenzae Rd</i>
HinfII	<i>Haemophilus influenzae Rf</i>
HpaII	<i>Haemophilus parainfluenzae</i>
HPLC	High Performance Liquid Chromatography
HNB	Hydroxy Naphthol Blue
Hrs	Hours
ICAR	Indian Council Of Agricultural Research
IgE, G	Immunoglobulin E, G
Kcl	Potassium chloride
LOD	Limit of detection
LF	Loop forward
LAMP	Loop Mediated Isothermal Amplification
LB	Loop backward
M	Molar
Mab	Monoclonal antibody
MBM	Meat and Bone Meal
Mg	Milligram
Mins	Minutes
Mt	Mitochondrial
mM	Milli molar

μL	Microliter
N	Number of observations
ND1-6, 4L	NADH dehydrogenase1-6, 4L
NaOH	Sodium Hydroxide
NCT	Negative control
Ng	Nano gram
NIN	National Institute Of Nutrition
OD	Optical Density
pH	Hydrogen ion concentration
Ppm	Parts per million
PCR	Polymerase Chain Reaction
Pg	picogram
Pvt	Private
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SE	Standard Error
Sec	Second
Spp	Species
T	Thymine
TAE	Tris acetate EDTA
tRNA	Transfer ribonucleic acid
U	Units
UV	Ultraviolet
V	Voltage
U	Units
USDA	United States Department of Agriculture
Viz	Namely

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## **ABSTRACT**

Meat adulteration is a growing concern in market place in recent years because of the increasing incidences of fraudulent substitution of superior quality meat with that of inferior quality to reap monetary benefits. Taboo attached to consumption of meat from a particular species among certain communities and allergy exhibited by few individuals to different meats makes it necessary to develop highly precise identification techniques for species identification. Precise detection of sheep meat is of paramount importance as mutton is the costliest meat in India. Techniques involved in meat speciation take the advantage of variation in composition of biomolecules including protein and DNA in different species. However, DNA is preferred over protein for species identification owing to its high stability under extreme conditions and homogeneity in all tissue types.

The present study aims at development of Loop-mediated isothermal amplification (LAMP) assay for detecting and quantifying adulteration of mutton both in raw and cooked forms. DNA was extracted by using alkaline lysis method. Extracted DNA samples were subjected to spectrophotometric analysis, where most of the samples showed an OD ratio 1.4-1.8 between a wavelength of 260 and 280 nm. For checking the quality, DNA samples were electrophoresed in 0.85 per cent agarose gel. For LAMP assay four primers were designed by targeting Mitochondrial D loop region and amplification was carried out under constant temperature 60 °C using dry bath. All results were visually detected and confirmed by electrophoresis and flurometric assay.

Specificity of the designed primers for sheep was assessed by performing LAMP assay with the DNA templates isolated from domestic species including cattle, buffalo, pork, goat and chicken and no cross amplification was detected. The experiment was repeated for six times and consistent results were obtained.

Amplification was possible even in heat treated mutton samples subjected to cooking at 60, 80, 100, 121 °C for 30 min. Sensitivity evaluation showed that these assays can detect 0.5 ng of sheep DNA per reaction. Detection of 0.1 % of mutton spiked with 99.9 % of beef in binary mixtures is indicative of the sensitivity and robustness of these assay. In this study LAMP amplicons were electrophoresed in QIAxcel capillary gel electrophoresis for testing the specificity of designed primers, were in high degree of amplification was visible only in sheep meat indicated by multiple bands. Results obtained in the present study are reliable, hence the developed LAMP assay combined with Alkaline lysis method of DNA extraction can be further used as a prototype for meat species identification at field level.

# INTRODUCTION

# CHAPTER I

## INTRODUCTION

Meat is considered highly nutritious among animal foods (Bhat *et al.*, 2015). It is an excellent source of protein, vitamins and minerals. There is a marked increase in demand for meat and meat products, consequent to the rise in the human population and their disposable income and increasing urbanization. In India, chicken is the highest produced meat (47.32 %) followed by bovines (24.45 %), goat (14.22 %), sheep (7.60 %) and pork (6.41 %) (Basic Animal Husbandry & Fisheries Statistics 2018). Among these, mutton and chevon are costliest as their demand often far exceeds supply. Generally, mutton is preferred meat in south India especially in states of Telangana, Andhra Pradesh and Karnataka while in north India chevon is preferred. The total mutton production in India was 602 thousand tones in the year 2017-18 which contributes 7.94 % of total meat production (Basic Animal Husbandry & Fisheries Statistics 2018). As per survey undertaken by Registrar General of India (2014), 71.6 % of the Indians are non-vegetarians. Among states, Telangana ranks number one with 98.7 % while meat consumption in Andhra Pradesh was 98.25 %.

Consumers across the world are increasingly concerned about the meat they eat and also demand accurate labeling. But there is increasing evidence suggestive of economic fraudulence of misrepresentation and adulteration of meat which are mainly driven by variation in prices of meat and restrictions on slaughter of certain meat animal species. As per Subbaiah *et al.* (2018), in the samples analyzed at meat speciation laboratory of Indian Council of Agriculture Research– National Research Centre on Meat, Hyderabad, out of 112 samples received and analyzed to identify the presence of either cattle (male/female) or buffalo, 8 were found to be female cattle (7.14 %) and 63

were male cattle (56.25 %). 22 samples were found to be buffalo only, 11 samples were admixture of cattle and buffalo, 2 sheep, 1 goat and 2 chicken respectively. Interestingly, 3 samples suspected as female cattle were found to be camel meat and the sample suspected as dog were found to be sheep, on DNA analysis. Meat adulteration has got social, religious, economic and public health concerns. It is important to respect consumer's choice, their lifestyle, religion, and diet and health concerns. Further, accurate labelling is essential to support a fair-trade in meat sector and to comply with regulations put forth by Food Safety & Standards Authority of India (FSSAI), 2011. Identification of sheep meat (mutton) has immense importance, especially in southern India, because it is highly preferred meat, costlier and vulnerable for adulteration with cheaper alternatives like beef. Also, some retailers adulterate mutton with chevon to meet the consumer demand and achieve monetary gain. To prevent such practices, to protect consumer sentiments and to implement statutory regulations, development of techniques for species identification of meat are essential. Further, in view of global concerns regarding threat of prion diseases and their linkage to small ruminants, it has become imperative to develop a specific and sensitive diagnostic assay for the rapid detection of sheep meat.

Vast array of techniques have been developed for identification of species of meat. The traditional methods such as anatomical, histological, organoleptic, chemical, electrophoretic, chromatographic and immunologic techniques are either cumbersome or lack repeatability, reproducibility, less sensitive and are reliable only in unprocessed raw meats (Plowman and Close, 1988). Several proteins based methods have also been used for speciation such as electrophoretic methods viz., SDS-PAGE (Bhilegaonkar *et al.*, 1990), Isoelectric focusing (King, 1984) and immunological methods viz. counter immunoelectrophoresis (Sherikar *et al.*, 1993), peroxidase antiperoxidase (PAP) technique (Karkare *et al.*, 1989) and Enzyme Linked Immunosorbent Assay (ELISA) (Martin *et al.*, 1991).

Recently, nucleic acid based techniques involving DNA analysis have become popular. These techniques have been proved to be promising in providing satisfactory solutions to the problem of identification of meats of animal species. Analysis of DNA rather than protein has been exploited for species identification due to its stability at high temperature and its conserved structure within all tissues of an individual animal. Both mitochondrial as well as nuclear DNA are being used for meat differentiation, but mitochondrial DNA was the most commonly used DNA, because of its highly conserved sequences in different species of animals (Antoinette *et al.*, 1995 and Fajardo *et al.*, 2010).

Several molecular techniques such as polymerase chain reaction (PCR) (Ilhak and Arslan, 2007), Restriction Fragment Length Polymorphism (RFLP) (Girish *et al.*, 2005), Random Amplified Polymorphic DNA (RAPD), Finger printing (Ganai *et al.*, 2000), DNA sequencing (FINS) (Girish *et al.*, 2004) and Loop-mediated isothermal amplification assay (LAMP) (Deb *et al.*, 2016) were used for identification of species in meat and meat products.

PCR is a rapid and specific nucleic acid amplification method for the identification of meat and number of PCR assays have been described for its identification. In spite of this, the PCR-based method continues to have some disadvantages, such as complicated operation and requirement of special heating cycle equipments.

Loop-mediated Isothermal Amplification (LAMP) is a recent innovative nucleotide amplification technique developed by Notomi *et al.* (2000) that amplifies target DNA with high specificity and sensitivity at isothermal temperatures using a DNA polymerase with high strand displacement activity with specially designed 4 to 6 lamp primers and obviating need for the expensive thermal cyclers or post-amplification procedures of signal detection (Erwanto *et al.*, 2011). As in PCR, LAMP assay could

amplify target DNA several folds ( $10^9$  copies in a span of an hour) under isothermal conditions without compromising specificity and having capability of the visual detection of amplified targets using specific dyes (Yang *et al.* 2014). Consequently, LAMP assay has emerged as an alternative tool to PCR technique for the purpose of testing food safety hazards including detection of meat adulteration (Abdulmawjood *et al.*, 2014; Abdullahi *et al.*, 2017).

In the era of globalization and increased concern for animal food quality and safety, there is a great need to develop rapid, sensitive, specific and reproducible and cost effective methods for authentication of meat.

Since the reports on species identification of mutton by LAMP assay are scarce, this study is proposed to develop simple, rapid & reliable speciation techniques for authentication of mutton with the following objectives.

- 1. Designing of species specific LAMP primers for identification of sheep meat.**
- 2. Development of LAMP assay for species authentication of Mutton.**

# **REVIEW OF LITERATURE**

## CHAPTER II

### REVIEW OF LITERATURE

#### 2.1 MEAT

Meat is a rich source of proteins, containing all the essential amino acids and a good source of minerals like iron, phosphorus, zinc, selenium and vitamins like riboflavin, niacin, vitamin B6, vitamin B12, choline etc. Red meat serves as one of the best sources for iron and zinc which is well absorbed by the body (Biesalski, 2005; Pereira and Vicente 2013; Bhat *et al.*, 2015). Meat is also able to supply omega 3 fatty acids and conjugated linoleic acids to a limited extent (Devi *et al.*, 2014).

##### 2.1.1 Fraudulent substitution of meat

Act of adding inferior quality meat with superior one is known as fraudulent substitution. There is a variation in the taste, nutritive and aesthetic value of meat from different food animal species. The demand and cost of the meat therefore varies according to the species of food animal. Fraudulent substitution of more expensive meat with cheaper meat is one of the major problems in the meat industry. Identification of meat substitution is gaining importance in the recent times for quality control management, conservation of laws, veto-legal cases, and safeguarding health and religious sentiments etc., (Mahajan *et al.*, 2011; Singh *et al.*, 2014). Poaching i.e., the illegal killing of the wild animals for its products, also needs the identification of species to safeguard the law and conservation of the endangered species (Guha and Kashyap 2005; Bhat *et al.*, 2015).

In the recent past, reports of fraudulent substitutions of dearer meats by the cheap priced ones were frequent in the meat industry (Fajardo *et al.*, 2010 and Mane *et al.*, 2011). The prevalence of such malpractices in the trading circles call for food authenticity to curb them besides preventing the negative consequences. Meat authenticity is

important to protect the consumers besides the implementation of national regulations (Singh and Sachan, 2011).

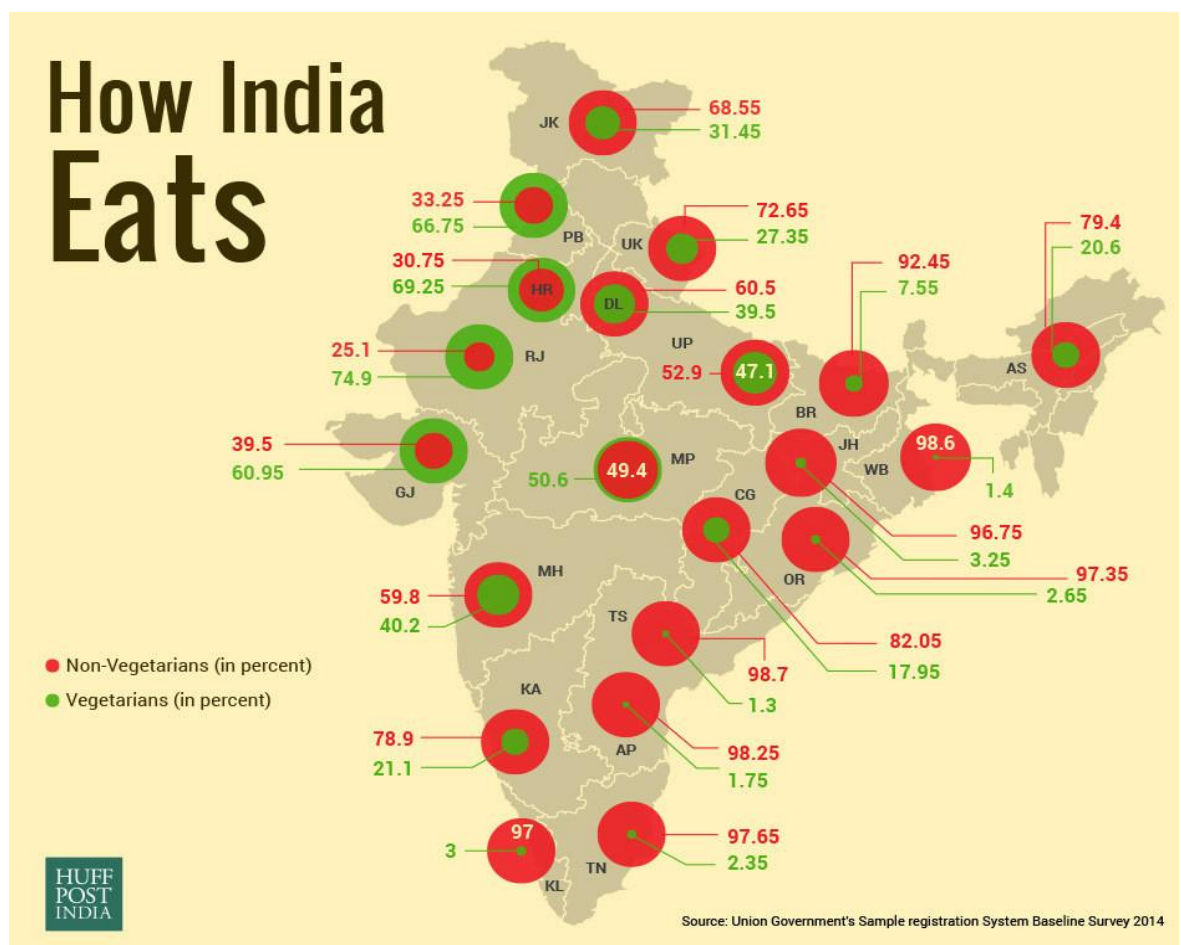
Emergence and spread of potentially zoonotic diseases such as Bovine spongiform encephalopathy (BSE), Creutzfeldt-Jakob disease (CJD) and variants of CJD (Adam, 2001), and their possible link to the scrapie in sheep led to the ban on incorporation of animal derived material in ruminant feed (LMG, 1992). Therefore, such a ban on raw materials derived from ruminant raw materials in animal feed necessitated sound speciation tools for authentication of animal products or by products for incorporation into those products that had even undergone thermal processing (LMV, 1995). The risk associated with infectious transmissible spongiform encephalopathy in humans discouraged many individuals around the globe from consuming beef (Mohamed *et al.*, 2007). Hence, authentication of meat species and reliable labelling of foods originating from them are thus important in terms of providing quality assurance and food safety to the consumer.

### **2.1.2 Meat allergies**

Allergies to meat are seen when the individuals consume meat, which is adulterated with the meat for which they are allergic. Allergy to meat is generally seen at young age and is rare in adults. Allergy to beef is most commonly encountered meat allergy. Meat allergies are majorly due to serum albumin and immunoglobulins. Allergies to muscle proteins like actin, myosin and tropomyosin have been reported (Restani *et al.*, 2009). Allergies to carbohydrate moieties like an oligosaccharide galactosyl 1, 3-galactose have also been reported (Mullins *et al.*, 2012). In addition, few individuals were also reported to show sensitivity to gelatin (Saleh *et al.*, 2012).

### 2.1.3 Meat production and consumption pattern in India

According to FAO 2016, India is one of the developing countries that contribute to a part of 80 % of the global ovine meat production. The total mutton production in India was 602 thousand tons, which contributes to about 7.94 % of total meat production (Basic Animal Husbandry and Fishery statistics 2018). Survey conducted by the Registrar General of India (2014) revealed that more than 71 % of Indians were meat consumers.



**Figure-1: Prevalence of Vegetarianism and Non-vegetarianism among the Indian population (Sample Registration System) 2014)**

## 2.1.4 Nutrient composition of mutton

According to Gopalan *et al.*, (1989) the nutritive values of mutton (100 gm) are as given below (Nutritive Value of Indian Foods, NIN, 1989)

**Table-1: Nutritive values of mutton**

NUTRIENT	UNIT	MUTTON
Moisture	gm	71.5
Protein	gm	18.5
Fat	gm	13.3
Minerals	gm	1.3
Energy	Kcal	194
Calcium	mg	150
Phosphorous	mg	150
Iron	mg	2.5
Vitamin B12	µg	2.6

## 2.2 CHOICE OF MATERIAL OF ANALYSIS: DNA Vs Protein

Analytical methods for detection of species in meat and meat products employ the measurements of protein and DNA (Ballin *et al.*, 2009). Today, however, DNA is considered to be the most appropriate molecule for species detection and identification in foods (Singh and Neelam, 2011). While the methods involving protein identification work well and are acceptable for the analysis of raw tissue, they are inadequate for thermally treated material due to the degradation of protein epitopes (Hird *et al.*, 2003; Rodriguez *et al.*, 2005). In contrast, DNA can be analyzed not only in fresh and frozen food products, but also in processed, degraded and mixed commodities (Lenstra, 2003).

Degeneracy, high thermal stability and presence of DNA in almost all the cells being identical renders DNA a better choice over protein for use in species determination (Lockley and Bardsley, 2000). The high diversity of DNA allows it for the differentiation even between the closely related species (Ballin, 2010).

### **2.2.1 Genomic DNA Vs Mitochondrial DNA**

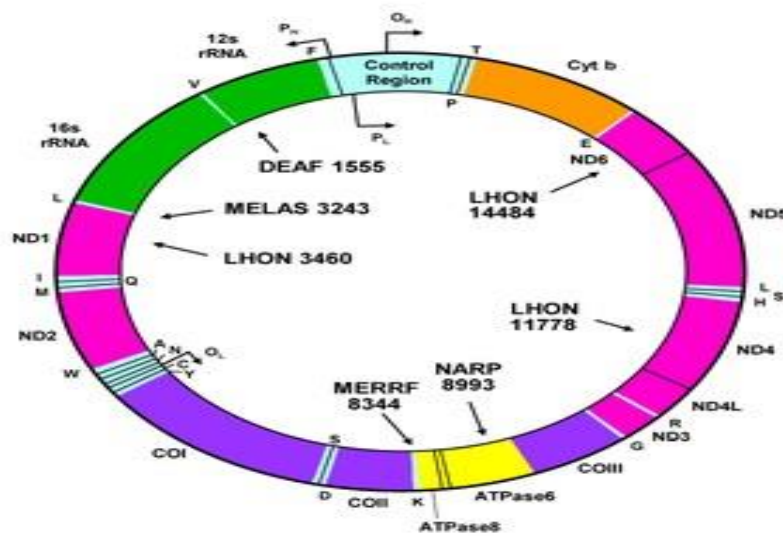
Nuclear DNA (genomic DNA) is present in a single copy, making it possible for the better quantitative analysis of meat in correlation with the DNA equivalents, but has higher LOD. It is much easier to design species specific primers for the nuclear DNA (Ballin *et al.*, 2009).

Mitochondrial DNA is a small, extra-chromosomal genome typically with 16 kb size (Boore, 1999). Its genome in most of the mammals contains 13 protein encoding genes including CO1, CO2, CO3, Cytb, ND1-ND6, ND4L and ATPase subunits 6 and 8 (ATP6 and ATP8) (Luo *et al.*, 2011). Mitochondrial DNA has a considerably high rate of mutation and evolution and has rarely any non-coding segments. As a result, there is a high sequence variation even between the closely related species that helps in effective species discrimination. It is thought to be strictly of uniparental (maternal) inheritance, whereas nuclear DNA is of biparental inheritance (Schwartz and Vissing, 2002).

Mitochondrial DNA is present in more number of copies per cell, in contrast to the nuclear DNA which is present only in a single copy (Pereira *et al.*, 2008, Kolesar *et al.*, 2013). Adequate research has been done on mitochondrial DNA and all its Genbank sequences are also available which makes it a choice of material for species identification (Dalmaso *et al.*, 2004).

Higher copies of mitochondrial DNA are present in a cell which can be extracted in intact form from heat treated processed meat products (Dooley *et al.*, 2004).

All the above factors make mitochondrial DNA a better choice over nuclear DNA for species identification.



**Figure-2: showing mitochondrial genome of mammals. Genes: ND1-ND6 and ND4I (complex subunits); COI-COIII (Cytochrome c oxidase subunits); ATPase 6 and 8 (ATP synthase subunit) cytochrome b (complex III); 12S rRNA and 16S rRNA (ribosomal RNA); D-loop (noncoding region) (jones *et al.*, 2008)**

The commonly targeted mitochondrial genes for species identification included cytochrome-b (Meyer *et al.*, 1995; Wilson and Walker, 1995; Zehner *et al.*, 1998; Matsunaga *et al.*, 1999; Wolf *et al.*, 1999; Halder, 2007; Mohammed *et al.*, 2007; Murugaiah *et al.*, 2009 and Rohita *et al.*, 2011), mt rRNA (5s, 12s, 16s, 18s etc.) (Rodriguez *et al.*, 2001; Girish *et al.*, 2005 and Gurudeep *et al.*, 2007) and the D-loop genes (Lopez *et al.*, 1996; Foran *et al.*, 1997; Fajardo *et al.*, 2007; Nagappa *et al.*, 2011 a & b; Kumar *et al.*, 2011 a & b and Mane *et al.*, 2011).

### 2.3 DNA EXTRACTION METHODS

DNA extraction is the vital step in undertaking molecular techniques. It should be emphasized that DNA quality, purity and quantity has considerable effect in the species

identification and because of that, methods for extracting DNA should be carefully selected. Various methods of isolation procedures have been analyzed and identified and used for isolation of DNA from meat which are further used for the LAMP and PCR assay.

### **2.3.1 Phenol chloroform isoamyl alcohol method (PCI)**

It is the most common and convenient method of extraction and purification of DNA used for isolation of DNA from meat and fat tissue (Matsunga *et al.*, 1999; Girish *et al.*, 2005). In this method, DNA is extracted using 25:24:1 phenol: chloroform: isoamyl alcohol into the aqueous phase with simultaneous removal of protein contaminants. DNA is then precipitated with isopropanol and the precipitated pellet is finally washed with 70 % alcohol to remove any salts or organic matter (Sambrook *et al.*, 1989).

### **2.3.2 Spin column method**

This method utilizes the bind-wash-elute approach where the DNA binds to membrane like that of silica in the presence of chaotropic salts, while the contaminants are washed away using two types of wash buffers. The membrane is then dried and DNA is eluted from it (Ivanova *et al.*, 2012). DNA was extracted from horse meat using DNeasy tissue kit method employing spin column composed of a silica gel membrane. Concentration of DNA obtained by this method was in the range of 67.3-56.7 ng/ $\mu$ l, with OD<sub>260/280</sub> ranging from 1.76-1.78. When the DNA extracted through this procedure was subjected to PCR the LOD was found to be 2.5 pg/ $\mu$ l (Pinto *et al.*, 2007).

### **2.3.3 Magnetic beads**

This method comprises binding of polynucleotides non-specifically to a solid surface such as magnetic micro-particles followed by washing with high ionic strength buffer and then eluting the polynucleotide with low ionic strength buffer.

### **2.3.4 Kit procedures**

Oster *et al.* (2001) successfully isolated DNA from raw and ground beef and chicken, raw and smoked fish and processed meats using Bugs'n beads kit in which polyvinyl alcohol based magnetic beads are used for isolation of DNA.

DNA was successfully isolated from cattle and buffalo fat using QIAMP DNA stool mini kit. The isolated DNA was subjected to amplification using universal primers for 12S rRNA and 16S rRNA and species specific primers for mitochondrial D-loop region. The LOD was established to be 0.47 ng for cattle and 0.23 ng for buffalo DNA in simplex assay, while 0.47 ng for cattle and 0.12 ng for buffalo in the duplex assay (Vaithiyanathan and Kulkarni, 2016).

When extracting DNA from these commercially standardized kits, high quality DNA with very good quality yield can be obtained. Price per sample is however higher than in phenol chloroform extraction (Girish *et al.*, 2005)

Salt precipitation, silica column based extraction and chloroform-phenol methods were compared for their efficiency of genomic DNA extraction, of which salt precipitation and column based extraction were proven to be more efficient (Muhammed *et al.*, 2015).

### **2.3.5 Alkaline lysis method**

Girish *et al.* (2013) reported that the rapid extraction of DNA from meat can be achieved by alkaline lysis method as it takes 30 mins for extraction and the method is economical involves only simple chemicals like NaOH and Tris HCL, they also conclude that by combining alkaline lysis method of DNA extraction and species specific PCR the time for identification of meat has been reduced.

## **2.4 DIFFERENT TECHNIQUES FOR SPECIES IDENTIFICATION**

Various methods have been developed over a period of time to identify the meat of various food animal species but with certain associated limitations. These included: anatomical, organoleptic, chemical, (Plowman and Close.,1988; Meyer *et al.*, 1994; Matsunaga *et al.*,1999a and Ilhak and Arslan, 2007), immunological (Hofmann.,1997 and Hsieh *et al.*,1998), electrophoretic (Skarpeid *et al.*,1998; Ozgen and Ugur., 2000; Renon *et al.*, 2003 and Belinda *et al.*, 2010), chromatographic ( Wissiack *et al.*, 2003) and DNA based molecular techniques (Girish, 2003; Girish *et al.*, 2004, 2005; Rohita *et al.*, 2011; Nagappa *et al.*, 2011a & b and kumar *et al.*, 2011a & b).

The traditional techniques such as anatomical, organoleptic, chemical, immunological, electrophoretic and chromatographic, spectroscopic methods described previously for the animal speciation had limitations of repeatability, reproducibility and sensitivity. In addition, they were observed as cumbersome besides being unsuitable for processed samples (Plowman and Close, 1988).

### **2.4.1 Immunological tests**

#### **2.4.1.1 Agar Gel Immunodiffusion Test (AGID)**

It is a qualitative immunological test that has an ability to demonstrate the similarities and resolve the differences in related protein based on the formation of the specific immunoprecipitin lines that form after they have reached their optimum proportions due the diffusion of antigens and their specific antibodies. Extracts from beef, chicken, pork and donkey meats were prepared and injected subcutaneously into rabbits for the preparation of species specific antisera. Agar Gel ImmunoDiffusion was then performed which showed the formation of specific immunoprecipitin lines that occurred

due to the diffusion and reaction between the antigens extracted from the meats and their respective antibodies raised in the rabbits (El-Nasser *et al.*, 2010).

#### **2.4.1.2 Enzyme Linked Immunosorbent Assay (ELISA)**

ELISA is an immunological technique that involves an enzyme to detect the presence of an antibody or an antigen in a sample. There are two main types of ELISA, Indirect and Sandwich ELISA. The indirect ELISA utilizes two antibodies, one of which is specific to the antigen and the other is coupled to an enzyme. This second antibody will cause a chromogenic or fluorogenic substrate to produce a signal. In the sandwich ELISA the antigen is bound between two antibodies: the capture antibody and the detection antibody.

The detection antibody can be coupled to an enzyme or can bind the conjugate (enzyme-linked antibody) that will produce the biochemical reaction (Asensio *et al.*, 2008). Monoclonal antibodies (MAb 5H9) were developed against a heat stable skeletal muscle protein of swine and conjugated to biotin which was used in pork adulteration detection. The detection limit was determined to be 0.8 % of pork in beef mixtures (Chen and Hsieh, 2000).

#### **2.4.2 Electrophoretic methods**

##### **2.4.2.1 Isoelectric focusing**

It is an electrophoretic technique which is based on the migration of the proteins in pH gradient, in which charge on the surface of the protein is utilized to drive it through gradient gel. The process stops when the surface charges of the proteins become neutral, i.e., at their respective isoelectric point (IP). Protein extracts from the meat samples obtained from swine, cattle, calves, chicken, goat and turkeys were collected and separated on 1.5 % agarose gel using direct contact electrophoresis. After the proteins

were absorbed by the gel, the samples were removed and the proteins absorbed by the gel were electrophoresed. The proteins were then fixated and the gel was stained and immunoblotted. The blotted membrane was then soaked in solution containing the primary antibody, anti-titin monoclonal antibody IgM9D10. Secondary antibody employed was horse radish peroxidase conjugated with rabbit immunoglobulins to mouse immunoglobulins. The IP of the extracts was then compared against a mixture of nine protein markers having different IPs (Pospiech *et al.*, 2005).

Polyacrylamide gel isoelectric focusing of sarcoplasmic protein, tetrazorium oxidase, esterase, heme protein, creatine kinase, adenylate cyclase, and phospho-glutamase was performed to identify raw and cooked meat samples of cattle, horse, sheep, goat and pig. LOD was determined to be ranging from 1 to 5 % (Matsuoka *et al.*, 1992).

Enzymes in sarcoplasmic protein fraction of eight gadoid fish species, including lactate dehydrogenase, glycerol 3-phosphatase dehydrogenase allowed their differentiation by isoelectric focusing in the pH range of 3.5-9.5 (Pineiro *et al.*, 2000).

### **2.4.3 Chromatographic techniques**

#### **2.4.3.1 Immunochromatographic strip test**

It is a technique similar to a typical test strip design that utilizes analytic specific antibodies coated onto colloidal particles as well as analyte-specific antibodies immobilized onto a nitrocellulose membrane. Anti- MBM (Meat and Bone Meal) MAb and goat anti-mouse IgG were blotted onto nitrocellulose membranes and immunochromatographic strips were prepared. The strips were placed in the liquid sample extract for 10 min and results were interpreted to be positive if two lines were seen and negative if only one line was noticed. This technique could detect 0.1 % MBM in animal feed (Muldoon *et al.*, 2004).

### **2.4.3.2 Ultra Performance Liquid Chromatography (UPLC)**

Ultra Performance Liquid Chromatography is a technique which employs particles less than 2 µm in diameter for separation of proteins and peptides with a better resolution, and sensitivity compared to HPLC (Wu and Engen 2006). Myoglobin extracted from the meat samples of pork and beef, after its conversion to metmyoglobin was subjected to Anionic Ultra Performance Liquid Chromatography comprising of a quaternary ammonium column. The retention times of these myoglobins were observed to be 23.12 min and 24.29 min respectively for pork and beef. An adulteration of 5 % of pork or beef in the mixed meat samples could be detected through this technique (Giaretta *et al.*, 2013).

## **2.5 IDENTIFICATION TECHNIQUES EMPLOYING DNA**

Molecular methods employing DNA include DNA hybridization and Polymerase chain reaction. There are also different PCR techniques including PCR-RAPD, PCR-RFLP, PCR with species specific primers, real time PCR and PCR- nucleotide sequencing (Farg *et al.*, 2015). Higher sensitivity of PCR techniques allows the detection of even 0.1 % of adulteration (Cawthraw *et al.*, 2009).

### **2.5.1 DNA hybridization**

It is a qualitative technique in which DNA probes hybridize to the complementary target DNA and aid in the sample detection, the result of which is interpreted on the basis of colour development or autoradiography. DNA isolated from heated pork samples was subjected to gel electrophoresis then immobilized on to nylon membranes. These fragments were then hybridized to P32 labelled probes made from porcine genome. The signal intensity was then determined by laser densitometry of autoradiographs. The LOD was determined to be 0.1 % pork in beef in raw samples, while in heat treated samples it was 0.5 % pork in beef (Ebbehoj and Thomsen, 1991).

DNA probes were generated by amplification with the primers targeting species specific satellite DNA and labelled. These probes were blotted and fixed onto a nylon membrane and could recognize and bind complementary sequences thereby detecting species origin of the meat. LOD by this method was found to be 0.1-0.5 % and 0.5-5 % respectively in raw and autoclaved meat mixtures (Janssen *et al.*, 1998).

### **2.5.2 PCR RAPD (PCR- Randomly Amplified Polymorphic DNA)**

RAPD analysis consists of the analysis of amplification of DNA fragments using short arbitrary primers that tie multiple locations on the genomic DNA, followed by separation of amplified fragments based on their sizes using gel electrophoresis. RAPD was a powerful technique in instances where little or no information on the DNA sequence is available (Ballin, 2010). RAPD was a simple and rapid method that can be used for halal authentication of meat without complex analytical steps like DNA restriction, sequencing or hybridization. However, its disadvantage is the difficulty of obtaining reproducible data as PCR amplifications have to be developed under strictly controlled and standardized conditions such as temperature, number of cycles and reagent concentration. RAPD also required high quality starting DNA in order to achieve reproducible RAPD profiles, which limits its application in highly processed meats with excessively degraded DNA. Additionally, RAPD analysis was not suitable for identification of a target species in admixed meats consisting of more than one species due to the non-specific nature of the PCR reaction (Fajardo *et al.*, 2010).

Gurdeep *et al.* (2007) tested PCR fingerprinting methods like RAPD and actin bar-coding to generate species-specific 'fingerprints'. Results suggested that among PCR fingerprinting approaches, RAPD was more discriminatory, accurate and efficient than actin fingerprinting, but the major disadvantage of RAPD was its inability to detect mixed species (meat) besides lacking the quantification of the extent of adulteration.

### 2.5.3 PCR using species specific primers

Species specific PCR has plenty of advantages; it offers simple, rapid, specific and highly sensitive authentication of species (Ilhak and Arslan, 2007). The species specific PCR has been developed for different species viz., cattle (Mane *et al.*, 2012; buffalo (Mane *et al.*, 2011); sheep and goat (Deepak *et al.*, 2011); chicken and cat (Ilhak and Arslan, 2007); pig (Aida *et al.*, 2005). Primers specific for buffalo targeting the mitochondrial D-loop region were designed and DNA was amplified producing an amplicon of 358 bp. The primers showed no cross reactivity with other species (Kumar *et al.*, 2011).

Deepak *et al.* (2011b) developed a highly specific single step polymerase chain reaction (PCR) for the detection of goat (*Capra hircus*) meat by using goat-specific primers designed to target mitochondrial D-loop gene that yielded a 294 bp PCR product. Specificity of the primers was confirmed by testing them with DNA from other commonly used meat animal species, i.e., cattle, buffalo, sheep, swine and poultry. PCR amplification of target DNA with goat-specific primers was repeated 15 times and consistent results were received. The specificity of goat-specific PCR provided a valuable tool for identification of goat meat and to avoid its fraudulent substitution and adulteration.

Nagappa *et al.* (2011 b), developed a highly species-specific polymerase chain reaction (PCR) for identification of sheep meat (mutton) using mitochondrial D-loop.

Rohita *et al.* (2011) developed species specific primers for cattle and buffalo using mitochondrial Cyt-b gene.

#### **2.5.4 Restriction Fragment Length Polymorphism (RFLP)**

PCR-RFLP was another convenient, rapid and sensitive molecular technique, which could be used for the meat species identification (Verkaar *et al.*, 2002). It was difficult to design species-specific primers particularly when species were closely related. In these cases, it was being suggested to apply a technique known as PCR-RFLP which is based on the analysis of the single species-specific mutations (sites of diagnosis). In PCR-RFLP, a conserved region of a marker gene was amplified and subsequently it was digested with the specific restriction enzymes yielding specific product pattern for a particular species.

Girish *et al.* (2005) utilized PCR-RFLP technique for identification of beef, buffalo meat, mutton and chevon which involved PCR amplification of mt 12S rRNA gene (amplicon size 456 bp) followed by restriction digestion with Alu I, Hha I, Apo I and BspT I restriction enzyme targeting species specific restriction site, which yielded products of size 359 & 97 bp, 246 & 210 bp, 329 & 127 bp and 323 & 133 bp, respectively after digestion. This technique could identify and differentiate each of the above species but did not yield satisfactory results in case of meat mixtures/meats. However, consistent results were obtained with both fresh and processed meat samples.

The analysis of PCR-RFLP of Cyt-b fragments was successfully applied for species differentiation in heated and processed meat products e.g. sausages (Meyer *et al.*, 1995; Satyabrata, 2007). However, the possible individual mutations make the selection of endonucleases in conserved restriction sites difficult. Several enzymes were often needed to discriminate between phylogenetically related species if several species were to be differentiated simultaneously (Wolf *et al.*, 1999). In addition, the use of many restriction enzymes and analysis of more than one diagnosis site made PCR-RFLP difficult to be automated. The RFLP of PCR products constituted alternative to

sequencing for the identification of genetic variation between and within species (Girish *et al.*, 2005). Chen *et al.* (2010) studied PCR-RFLP for species authentication.

### **2.5.5 Real time PCR**

Real Time PCR detects PCR products using fluorescent probes or DNA binding dyes, such as SYBR Green. It can quantify PCR products with greater reproducibility while eliminating the need for post-PCR processing.

A common forward primer and reverse primers specific for cattle and buffalo were used to amplify *cytb* gene in these species. When real time PCR was performed using these primers, melting peaks at 76.2 °C and 78.2 °C for cattle and buffalo respectively were observed. When these qPCR products were analyzed on 2 % agarose bands of 113 bp amplicon in case of cattle and a 152 bp amplicon in case of buffalo were obtained (Kumar *et al.*, 2015).

Real time PCR was performed on meat mixtures of beef and lamb using universal primers targeting mitochondrial 16S rRNA gene for total meat content measurement and beef specific primers targeting the D-loop for individual (beef) meat content estimation. A comparison of the first detection point was made between the amplicons of universal and species specific primers. The amplification with the beef specific primers resulted in earlier first detection point than that with the universal primers (Sawyer *et al.*, 2003).

### **2.5.6 Multiplex PCR assay**

Multiplex PCR assay was performed and *cytb* gene variability was determined in different meat samples including those obtained from cattle, buffalo, sheep, goat, swine, horse and chicken. A common forward primer for all the species but a different reverse primer for each species was used to obtain species specific DNA fragments. DNA

fragments of 157, 227, 274, 274, 331, 398 and 439 bp in goat, chicken, cattle, buffalo, sheep, swine and horse meats respectively were obtained (Jain *et al.*, 2007).

Matsunaga *et al.*, (1999) performed a multiplex PCR assay on cooked meats (heated to 100–120 °C except in horse where it was cooked to 120 °C) using the primers targeting the *cytb* gene. A common forward primer for all the species and reverse primers specific for each species were designed and used. The amplicons when analyzed using 4 % agarose gel showed 157, 227, 274, 331, 398 and 439bp amplicons for goat, chicken, cattle, sheep, swine and horse meats. Detection limit observed was 0.25 ng of DNA for all meat samples.

## **2.6 LAMP (LOOP MEDIATED ISOTHERMAL AMPLIFICATION)**

LAMP is a technique where nucleic acid amplification occurs with high specificity, efficiency and rapidity under isothermal conditions (Notomi *et al.*, 2000).

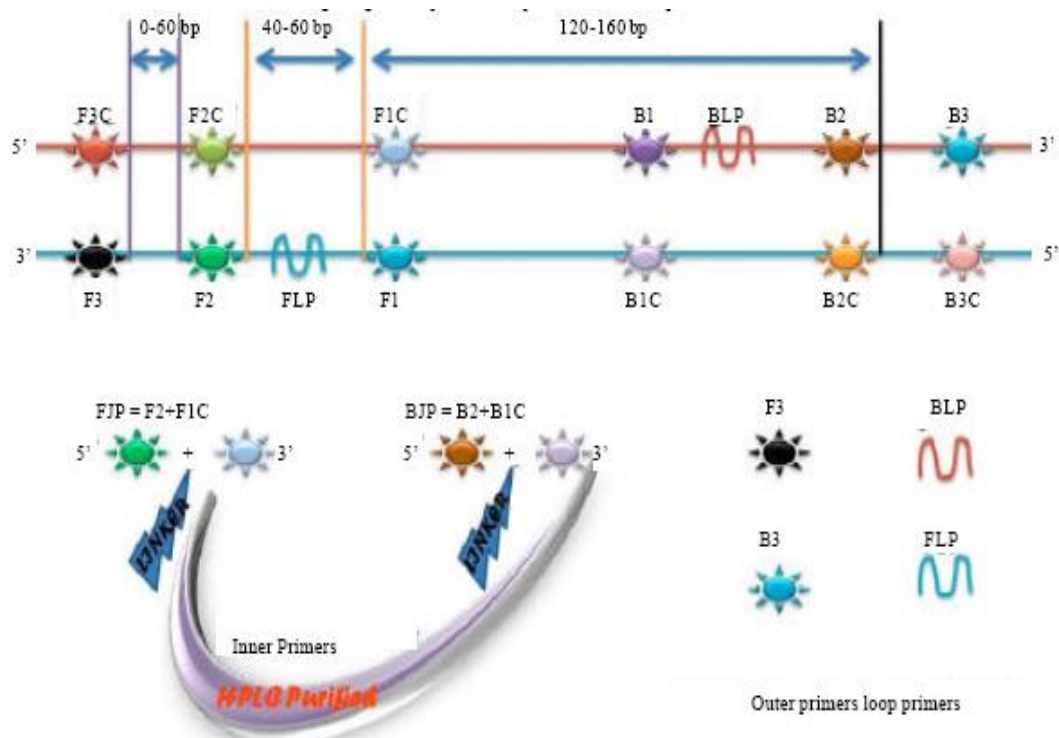
**LAMP:** Loop-mediated isothermal amplification as the name implies the reaction takes place at an isothermal temperature which is the greatest credit to the test. LAMP uses a DNA polymerase which has unique property of strand displacement along with the usual polymerization property. LAMP is a simple screening assay and when compared to PCR is more sensitive and specific, So that it can amplify a negligible amount of DNA to more than  $10^9$  copies which happens within an hour's time (Notomi *et al.*, 2000).

LAMP amplifies DNA at greater concentration when compared to PCR based amplification and allows easier visualization due to release of pyrophosphate that causes turbidity due to precipitation. The amplification technique can also be quantitative (Mori *et al.*, 2001) and depends on DNA synthesis by auto cycling strand displacement, performed by a DNA polymerase with high strand displacement activity (Whiting and Champoux, 1998; Ayyadevara *et al.*, 2000).

## 2.6.1 Requirements of LAMP

**Primer:** Performance of LAMP mainly relies on crafting of Primers which should be very specific. Unlike PCR, LAMP requires minimum 4 primers which are named as F3 (Forward outer), B3 (Backward outer), FIP (Forward inner) and BIP (Backward inner) primers. Two more primers namely LF (Loop forward) and LB (Loop backward) can also be incorporated which accelerates the reaction hence completes the reaction still faster (Nagamine *et al.*, 2002). The F3 and B3 have their major role during strand displacement and called as strand displacing primers. FIP and BIP have their function in loop formation (Parida *et al.*, 2008). The FIP and BIP should be of High Performance Liquid Chromatography (HPLC) purified primers. The primers are designed based on the eight target regions present on the gene: F3c, F2c, F1c and FLP which are in 3' side and B1, B2, B3 and BLP in the 5' side. The inner and loop primers act via different mechanisms. Moreover, the loop primers facilitate the LAMP reaction specifically within a period of half an hour in comparison to when original LAMP method is used. Loop primer inclusion yields a large amount of DNA in short time (Nagamine *et al.*, 2002).

**Enzyme:** Enzyme can be called as the heart of the LAMP. An enzyme which has DNA polymerizing capacity along with the crucial ability to displace the strand is selected for LAMP. The enzymes with recommended ability are *Bst* polymerase isolated from *Bacillus stearothermophilus* and *Bsm* polymerase isolated from *Bacillus smithii*. Both enzymes have unique property of strand displacement and can catalyse 5'-3' DNA polymerization but they don't have 5'-3' exonuclease activity (Nagamine *et al.*, 2001; Wozniakowski *et al.*, 2012). The *Bst* polymerase has its enzyme activity till 66 °C and *Bsm* polymerase has its activity till 63 °C and best at 60 °C.



**Figure-3: showing the target region of primers**

Other components which are required for LAMP are dNTPs for providing required nucleotides, Magnesium sulphate which forms magnesium pyrophosphate during the course of the reaction which enables to visualize the result based on the turbidity formed (Tomita *et al.*, 2008). Betaine is a chemical used to stabilize the AT and GC content and finally buffer which contains, Tris-HCl with a pH 8.8, (NH<sub>4</sub>)<sub>2</sub> SO<sub>4</sub>, MgSO<sub>4</sub> and KCl are commonly used.

### **2.6.2 Methods for detection of amplified LAMP products and results interpretation:**

LAMP results can be interpreted with naked eye which is the phenomenal thing about LAMP. Because of this feature it can be effortlessly applied in the field as a diagnostic technique and a semiskilled person can interpret the results. Magnesium pyrophosphate is produced as a by-product of amplification turbidity

due to which indicates the formation and quantity of targeted genomic region. Gene amplification products can be detected by agarose gel electrophoresis as well as by real-time monitoring in a relatively inexpensive turbid meter which in addition to visualization, aid in quantification of the gene copy number. Varying sizes of bands (i.e., ladder pattern) are observed from the amplified LAMP products in agarose gel electrophoresis. New LAMP protocols must be verified by restriction enzyme analysis alone or followed by nucleotide sequencing when tested for the first time (Notomi *et al.*, 2000).

Results can be visualized by adding up SYBR green which is a DNA binding dye (Monis *et al.*, 2005; Parida *et al.*, 2005, 2008). The DNA binding dyes like ethidium bromide, Picogreen (Dukes *et al.*, 2006; Curtis *et al.*, 2008), or propidium iodide (Hill *et al.*, 2008) can also be used.

Metal ion indicator like calcein can be used to visualize the result (Tomita *et al.*, 2008). Hydroxy naphthol blue a colouring dye can also be used (Goto *et al.*, 2009). The benefit about calcein and Hydroxyl Naphthol Blue (HNB) is that both can be added during the start of the reaction unlike SYBR green which has to be added once the reaction is completed. Calcein and HNB are added in the start of the reaction; hence chance of carry over contamination is very less. Turbidity can also be seen once the amplified product is spun for a short burst of time, white precipitate settles down in the bottom of the tube (Mori *et al.*, 2001).

### **2.6.3 Salient advantages of LAMP**

- LAMP can be called as an equipment free technique because it doesn't require costly equipment's and only water bath which is commonly available can be used. Hence, under field condition, the technique is suitable Notomi *et al.* (2000).
- Diagnosis by LAMP is rapid; gets completed within an hour or 30 min (when loop primers are used) (Nagamine *et al.*, 2002a).

- Unprocessed or partly processed samples can be used as template and hence it a robust technique.
- LAMP works at a constant temperature.
- No need of post amplification processing. Results can be seen directly by adding SYBR green, HNB or Calcein. So electrophoresis is not needed which also reduces the time (Njiru, 2012).

#### **2.6.4 LAMP for meat species identification**

(Sul *et al.*,2019) applied direct LAMP assay to detect chicken in processed meat products by targeting mitochondrial 16S rRNA gene, the limit of detection of chicken specific LAMP assay was 10 fg and the LOD s of the direct LAMP assay for chicken /pork and chicken/beef mixtures were 0.1 % in raw and in heat treated meat. This assay successfully amplified the target sequence and identified the presence of chicken meat in processed meat products approximately within 30 mins.

Kumari *et al.* (2019) developed a species specific LAMP assay for identification of tissue of cattle by targeting mitochondrial DNA, they revealed that the lowest level of detection was 0.01 ng of absolute DNA from target species and they reported that LAMP assay can detect 1 % cattle tissue in binary meat mixtures.

Ya Shi *et al.* (2017) developed a nucleotide detection method by combining LAMP and LFD for detection of duck genes in meat products targeting mitochondrial DNA region at 65 °C for 30 mins , the method has a sensitivity of 3 pg duck DNA /reaction which is 10 times higher than real time PCR .The method was able detect 0.1 % duck meat within 35 mins in meat mixtures sample containing duck and beef meat, they developed a simple on site DNA extraction process conducted within 8 mins by eliminating the complicated DNA extraction process ,using the simple DNA extraction step. The LAMP-LFD was able to detect 1 mg duck meat within 30 mins.

Abdullah *et al.* (2017) developed a rapid and sensitive Loop-mediated isothermal amplification assay for detection of swine DNA in adulterated meat and meat products. Six sets of LAMP primers designed based on porcine tRNA lysine gene and ATPase subunit 8 genes were used for the assay. Detection limit of the assay was 0.03 femtogram (fg) much higher than the PCR assay, and detection probability of the assay was 100 %. Detection of 0.5 % of pork spiked with 99.5 % of cattle beef is indicative of the sensitivity and robustness of the assay.

(Deb *et al.*, 2016) applied the LAMP for rapid detection of cow components adulteration in buffalo meat/milk samples. The cow DNA in buffalo samples were visualized by SYBR Green I/HNB dyes and the level of detection was up to 5 % and the entire test completed within 1 hr 40 mins starting from DNA extraction.

Ran *et al.* (2016) developed a LAMP assay targeting mitochondrial DN1 gene sequence for visual detection of pork DNA in meat products, the method was capable of detecting 0.5 ng pork DNA /reaction and 0.01 % pork in the meat mixture sample containing beef or mutton. They also reported that sensitivity of the LAMP method was not affected by high temperature treatments.

Li *et al.* (2016) developed rapid visual identification method for identification of meat species by combining LAMP with immunochromatographic strips, the limit of detection of beef in meat mixtures was 0.1 %.

(Lixia *et al.*, 2014) developed a one-step real time LAMP assay targeting mitochondrial cytochrome b gene sequence for visual detection of pork DNA, the method was capable of detecting 1 pg pork DNA /reaction and 0.01 % pork in the meat mixture sample containing beef.

The LAMP method for visual detection of bovine and ovine gene in animal food stuff (raw and processed meat products) was developed using primers targeting col and

cytb gene sequence of mitochondrial DNA region. The amplification occurred only in bovine and ovine species and not in horse, pork the sensitivity for bovine and ovine DNA was  $5 \times 10^{-3}$  ng whereas for bovine and ovine the sensitivity was 0.01 % and 0.001 % respectively in meat mixtures, they concluded that the LAMP method had lower sensitivity than real time PCR for the detection of genomic DNA from mixed DNA samples (Ma *et al.*, 2016).

A LAMP method was developed for the specific detection of horse meat in processed food products (Zahradnik *et al.*, 2015) the primers targeting mitochondrial NADH dehydrogenase showed no cross reactivity with the beef, pork, chicken samples the method showed 100 % specificity for horse and donkey meat and the sensitivity was 0.1 ng of horse DNA. In processed meat (sausage) the method detected 0.1 % horse meat content within 26 mins.

Abdulmawjood *et al.* (2014) conducted a LAMP assay integrated with real- time fluorometry for identification of ostrich meat, the method showed higher sensitivity 0.01 % when mitochondrial cytochrome b gene was targeted. In parallel a direct swab method without nucleic acid extraction using the HYPLEX LPTV buffer was also evaluated.

Cho *et al.* (2014) designed LAMP assays targeting species-specific mitochondrial DNA to identify and discriminate eight meat species; cattle, pig, horse, goat, sheep, chicken, duck, and turkey and unique annealing temperature were found for each species. The limits of detection (LODs) of the LAMP assays in raw and cooked meat were determined from 10 pg/ $\mu$  L to 100 fg/ $\mu$  L levels, and LODs in raw and cooked meat admixtures were determined from 0.01 % to 0.0001 % levels.

The D loop region of mitochondrial DNA from wild boar was targeted for LAMP identification at 45 min, 65 °C, in these instead of costly dyes  $\text{CuSO}_4$  was added to the amplified LAMP products for visual detection of animal species, the beef and negative

control samples showed a ring shaped deposit of white precipitate of  $\text{Cu}(\text{OH})_2$ , while no such precipitate was shown by pork samples (kanchanaphum *et al.*, 2014).

Yang *et al.* (2014) reported that the sensitivity of pork meat was 1 pg/ml by conducting LAMP assay targeting the mitochondrial DNA which was 100 times higher than conventional PCR, the method detected 0.1 % pork meat in binary meat mixtures.

LAMP proceeded by electrochemical detection of amplicons was employed for quick detection of meat adulteration. This technique using species specific primers for pork could identify up to 20 % pork in binary mixtures made of pork and chicken (Ahmed *et al.*, 2010).

### **2.6.5 LAMP for microbial detection in meat**

LAMP technique coupled with ELISA was developed to detect salmonella serogroup D strains in meat sample (Ravan *et al.*, 2012), in this method the detection of salmonella serovars enteritidis and typhi was done using a serogroup D/A specific primers targeting the prt gene, the products were labeled with digoxigenin using labelled nucleotides and then hybridized with a serogroup -specific oligonucleotide probe for specific detection, the method was highly specific with sensitivity of 4 cfu/tube which is 12 fold higher than PCR-ELISA (50 cfu/tube) targeting the same gene.)

(Yamazaki *et al.*, 2008) applied Loop mediated isothermal amplification assay (LAMP) for detection of campylobacter jejuni and campylobacter coli. The sensitivity of the LAMP assay for c.jejuni and c.coli in spiked human stool specimens was 1.4 cfu per test tube and 1.2 cfu per test tube respectively. They also reported that the entire process for detection of c.jejuni and c.coli in stool specimens was less than 2 hours.

Yang *et al.* (2011) developed a rapid LAMP method for detection of staphylococcus aureus in meat products. This amplification was based on heat stable nuc

gene. The sensitivity of this method was 1.25 cfu/reaction in pure culture and 10.3 cfu /reaction in artificially contaminated meat products. They also reported that this sensitive LAMP has 97.93 % specificity.

# **MATERIALS AND METHODS**

## CHAPTER III

### MATERIALS AND METHODS

A study on development of Loop-mediated isothermal amplification assay (LAMP) for identification of Mutton was carried out at ICAR-National Research Centre on Meat (NRCM), Chengicherla, Uppal, Hyderabad with the aim to identify mutton and differentiate from other meat animal species.

#### 3.1 COLLECTION OF SAMPLES

Samples of beef, buffalo, mutton and chevon were obtained from Municipal slaughterhouse (Chengicherla, Hyderabad, and Telangana). Samples of pork and chicken were obtained from the authentic retail shops located at Hyderabad city. Meat samples were collected individually into sterile containers and stored at -20 °C until further analysis.

#### 3.2 EXTRACTION OF DNA

DNA from different species of meat were extracted under sterile condition by Alkaline Lysis (AL) method as described by Girish *et al.*, 2013.

##### 3.2.1 Reagents

- 0.2 N NaOH
- 0.04 M Tris Hcl

##### 3.2.2 Alkaline lysis method for DNA extraction

500 mg of meat sample was taken and transferred on to glass slide and cut into small pieces with sterile scalpel blade, transferred on to petri plate and triturated with eight volumes (4 mL) of sodium hydroxide solution (0.2 N). 5 µL of this extract was mixed with another 40 µL of 0.2 N sodium hydroxide solution and heated at 75 °C in dry

bath for 20 minutes. After thermal lysis, reaction mix was neutralized using 360  $\mu\text{L}$  of Tris-HCl (0.04 M, pH 7). 2  $\mu\text{l}$  of final mix was used for assessing DNA concentration and purity. DNA samples were stored in  $-20\text{ }^{\circ}\text{C}$  till further analysis.

### 3.2.3 Qualitative and quantitative analysis of extracted DNA

The concentration of DNA was assessed with the help of Nano spectrophotometer at 260 nm and analyzed for purity by determining the optical density at 260/280 (Make: Denovix, Model: DS-11FX). (The recommended optimum values for purity lies between 1.7 to 1.9). The DNA which exhibited the ratio within the limit were taken for further study. All the extracted samples were kept at  $-20\text{ }^{\circ}\text{C}$  for further use.

The concentration of DNA was estimated by using following formula

$$\text{DNA concentration } (\mu\text{g/ml}) = \frac{\text{OD}_{260} \times \text{Dilution Factor} \times 50}{1000}$$

(1 OD value at 260 nm is equivalent to 50 ng dsDNA /  $\mu\text{l}$ )

### 3.3 DESIGNING OF PRIMERS FOR THE LAMP ASSAY

Two sets of primers (F3/B3, FIP/BIP) were designed for the LAMP assay by targeting mitochondrial D loop region using Primer Explorer V5 software; the 5'→3' primer sequences of the designed primers is as given below:

**showing sequences of primers designed for LAMP assay targeting mitochondrial D loop region of sheep**

S.NO	Primer	Sequence	Length
1	F3	5' -GAAGTCAAATCCGTCCTA GT-3'	20 bp
2	B3	5' -GATGAGATGGCCCTGAAG-3'	18 bp
3	FIP	5' -AAGCGGGTTGTTGGTTTCACACATGCATATCCCGTCCA-3'	38 bp
4	BIP	5' -GCAAGGATC CCTCTTCTCGCAAGAACCAGATGCCTGTT-3'	38 bp

### **3.4 DETERMINATION OF LADDER PATTERN**

Ladder pattern of LAMP reaction to identify right proportion of ingredients and temperature for optimum results was determined as per Deb *et al.* (2016). The ladder pattern in LAMP assay was optimized by varying different reaction components like Magnesium Sulphate, betaine and temperature.

#### **3.4.1 Standardization of betaine**

Betaine is one of the major component in LAMP assay. For standardization of LAMP ladder pattern betaine at different concentration *viz.*, 1.2 M, 1.4 M, 1.6 M, 1.8 M and 2 M were used.

#### **3.4.2 Standardization of temperature**

Temperature is also playing one of the major role in LAMP reaction. We used five amplification temperatures *viz.* 58, 59, 60, 61 and 62 °C for one-hour incubation. Among all temperature gradient 60 °C temperature gave good amplification which was confirmed by fluorometer reading and agarose gel electrophoresis.

#### **3.4.3 Standardization of magnesium sulphate**

MgSO<sub>4</sub> is also significant factor for all successful LAMP assay. The experiment was also carried out at different concentration of Magnesium sulphate 1 mM, 2 mM, 4 mM, 6 mM, 8 mM and 10 mM. The 6 mM showed best absorbance result in flurometer reading as well as in gel electrophoresis image.

Based on the results of the experiment, 1.4 M betaine, 60 °C temperature and 6 mM Magnesium sulphate, were standardized for getting good ladder pattern in LAMP Assay.

### **3.5 PREPARATION OF BINARY MEAT MIXTURES**

Binary mixtures of meat were made with each mixture made up to 500 mg by mixing the mutton and beef in different proportions and labelled from BM 1 to BM 13

for the detection of mutton in beef using AL-LAMP assay. Different proportions of mutton in beef were made in various percentages as shown in Table 3. After thorough mixing, meat mixtures were subjected for DNA extraction using AL method.

**Table-3: Composition of Binary meat mixtures**

<b>Sample</b>	<b>Mutton %</b>	<b>Beef %</b>
<b>BM1</b>	50	50
<b>BM2</b>	40	60
<b>BM3</b>	30	70
<b>BM4</b>	20	80
<b>BM5</b>	15	85
<b>BM6</b>	10	90
<b>BM7</b>	5	95
<b>BM8</b>	1	99
<b>BM9</b>	0.8	99.2
<b>BM10</b>	0.6	99.4
<b>BM11</b>	0.4	99.6
<b>BM12</b>	0.2	99.8
<b>BM13</b>	0.1	99.9

All the samples were stored at -20 °C until further processing. Prior to processing the samples were thawed for 3-4 h and DNA is extracted from the samples by using alkaline lysis method.

### **3.6 PREPARATION OF HEAT TREATED SAMPLE**

Sheep meat samples of about 500 mg were heated at 60, 80, 100 and 121 °C for 30 minutes in a dry bath (Model: Ultimate Dry Bath Incubator). Thereafter, DNA was extracted from thermally processed samples using alkaline lysis method as described by Girish *et al.* (2013), followed by LAMP assay.

### **3.7 CHECKING SPECIFICITY OF THE DEVELOPED LAMP ASSAY**

For checking specificity of the sheep primers, DNA is extracted from closely related species viz., cattle, buffalo, pork, goat, pig and chicken and LAMP reaction was carried out for all the species.

### **3.8 CHECKING SENSITIVITY OF THE DEVELOPED LAMP ASSAY**

Sensitivity of developed LAMP assay was assessed by diluting the template DNA so as to get concentrations viz., 100, 75, 50, 40, 30, 25, 20, 10, 5, 2.5, 1, 0.5 ng/ $\mu$ L, followed by the LAMP assay using the novel set of primers designed.

### **3.9 LAMP REACTION**

Loop mediated isothermal amplification (LAMP) reaction mix consists of 10X Thermopol buffer (3  $\mu$ L), 1.4 M Betaine (3  $\mu$ L), 6 mM MgSO<sub>4</sub> (3  $\mu$ L), 10 mM dNTP mix (4  $\mu$ L), 10 pmol F3 primer (1  $\mu$ L), 10 pmol B3 primer (1  $\mu$ L), 40 pmol FIP primer (4  $\mu$ L), 40 pmol BIP primer (4  $\mu$ L) and template DNA (5  $\mu$ L). The reaction mix was heated at 95 °C for 5 minutes and 8 units (1  $\mu$ L) of *Bsm* enzyme was added and incubated at amplification temperature of 60 °C for 1 hour followed by heating at 80 °C for 2 min for the inactivation of *Bsm* enzyme. Heating of the LAMP reaction mix was done in dry bath. Then finally 1 $\mu$ L of SYBR green dye was added for visualization of amplification.

**Table-4: Composition of LAMP Reaction mixture.**

<b>COMPONENTS</b>	<b>VOLUME (<math>\mu\text{L}</math>)</b>
10X thermopol buffer	3
6 mM MgSO <sub>4</sub>	3
1.4 M betaine	3
10 mM dNTPmix	4
Template DNA	5
Bsm enzyme(8 units/ $\mu\text{L}$ )	1
F3 10 pico molar	1
B3 10 pico molar	1
FIP 40 pico molar	4
BIP 40 pico molar	4

### **3.10 VISUALIZATION OF LAMP RESULTS**

After completion of required LAMP reaction, 1  $\mu\text{L}$  of SYBR green I dye (1:10, 10,000X, Sigma-Merck) was added for the visual detection of amplification products. Positive reaction was indicated by green color whereas, orange color indicated a negative reaction. For confirmation, all LAMP products were subjected to agarose gel electrophoresis and fluorometry.

#### **3.10.1 Fluorometric assay**

Intensity of colour developed after the addition of SYBR green I dye to the final reaction mix of LAMP product was analyzed using a fluorometer (Denovix, Model: DS-11FX) at 565–650 nm fluorescence. The final reaction mix was diluted with

nuclease free water to 200  $\mu$ l volume and the resultant diluted mix was used for recording fluorescence.

### **3.10.2 Agarose gel electrophoresis**

Finally, the LAMP amplification was confirmed by agarose gel electrophoresis.

#### **3.10.2.1 Reagents:**

- TAE buffer (50X)
- Agarose
- Ethidium bromide
- 6X Gel loading buffer
- 100 bp ladder

#### **3.10.2.2 Procedure**

- For preparation of 2 % agarose 2 gm of agarose was dissolved in 100 ml 1X TAE buffer by heating and after cooling 10  $\mu$ l of ethidium bromide was added.
- The gel was then poured into a casting tray and a comb was inserted and the gel was allowed to set. After setting, the gel was immersed in the 1X TAE buffer contained in the electrophoresis tank.
- The LAMP products were then loaded into the wells after mixing them with the 6x gel loading buffer (2  $\mu$ l gel loading buffer/5 $\mu$ l LAMP product).
- A 100 bp ladder was also loaded in the first well.
- The electrophoresis was carried out at 80 V for 1 hr.
- The gel was then visualized under UV light in gel documentation system and bands viewed were interpreted in comparison with the 100 bp ladder

### **3.11 QIAXCEL CAPILLARY ELECTROPHORESIS**

DNA analysis was performed on the QIAxcel system by using QIAxcel DNA high resolution kit.

#### **3.11.1 Materials**

- Wash buffer
- Separation buffer
- Alignment marker
- Calibration marker
- DNA ladder
- Mineral oil

#### **3.11.2 Procedure**

First calibration of the instrument was done by adding 8 ml of wash buffer and 3 ml of the mineral oil in first small channel of the tray, then we added 16 ml separation buffer and 3 ml mineral oil in the bigger channel of the tray ,then load 15 ml calibration marker in 12 strip tube followed by 2 ml mineral oil and allow for calibration of the instrument After successful calibration replace the calibration marker with alignment marker and then load 10  $\mu$ L of LAMP samples in 12 well strip with 10  $\mu$ L of molecular weight marker 15 to 600 bp in one tube.

Then allow to run the equipment for 45 mins. The separation of DNA fragments can be achieved within 45 mins by applying high voltage. The results were displayed as gel image and electropherogram was obtained from QIAxcel advanced system software.

## **3.12 MATERIALS**

### **3.12.1 Chemicals**

- NaOH and Tris hydrochloride were procured from SRL pvt ltd Mumbai.
- Primers were designed and procured from Eurofins pvt ltd .
- LAMP reagents (Betaine, Magnesium sulphate, dNTP, SYBR green dye ) were procured from SIGMA MERCK Bangalore.
- Bsm enzyme and Buffer from GENETIX.

### **3.12.2 Equipments:**

- Dry Bath (Make: Model: Ultimate Dry Bath Incubator).
- Spectrophotometer (Make: Denovix, Model: DS-11FX)
- Fluorometer (Make: Denovix, Model: DS-11FX)
- Gel documentatiom system

# RESULTS

## CHAPTER –IV

### RESULTS

Present work was undertaken with an objective to develop simple, rapid and economical assay for species identification of mutton and to differentiate it from other closely related meat animal species. Technique developed was based on Loop-mediated Isothermal Amplification (LAMP) assay which enables amplification of targeted gene at constant temperature, in quick time with minimum instrumentation.

#### 4.1 DNA QUANTIFICATION AND PURITY

##### 4.1.1 DNA Quantification of different meat animals

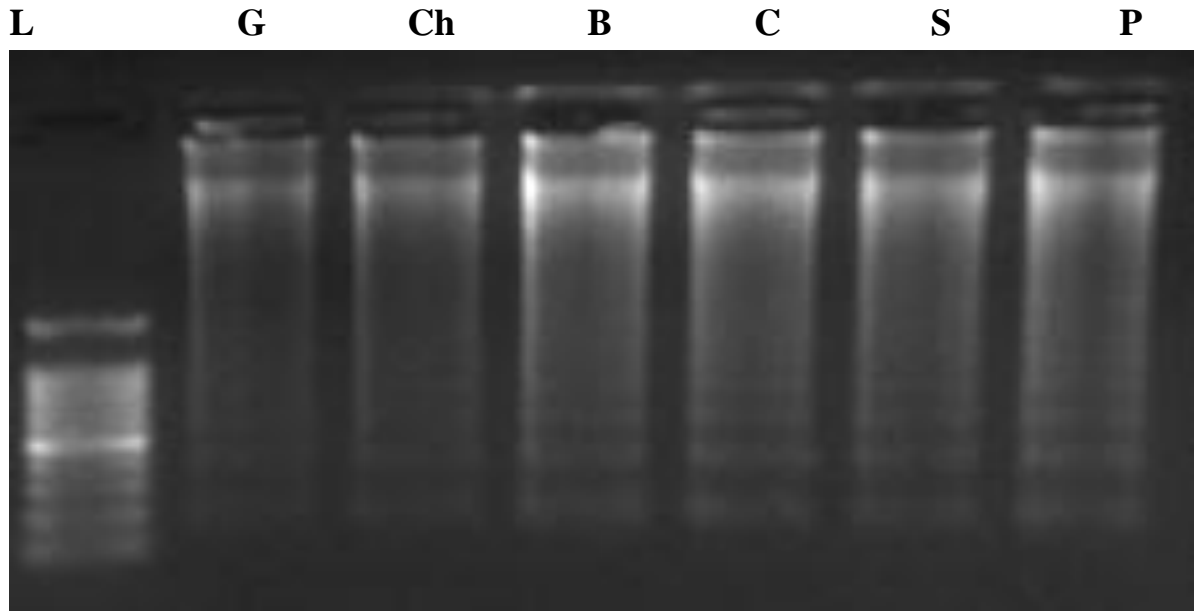
In the present study, the meat samples collected from different food animal's viz., cattle, buffalo, goat, sheep, chicken and pork were subjected to DNA extraction using alkaline lysis method as described by Girish *et al.* (2013). The mean DNA concentrations of the different meat samples with their standard errors are shown in the table 5. The OD 260/280 values obtained were in the range of 1.4 to 1.8 and it was considered as good quality and used for LAMP.

**Table-5: Mean values of concentrations of the DNA (ng/μl) extracted from different species**

Species	DNA Concentration (ng/μl)
Cattle	136.08±8.09
Buffalo	169.77±11.19
Sheep	157.23±11.26
Goat	110.73±14.40
Pork	148.86±12.45
Chicken	118.48±9.20

Values are Mean ± SE, N=6

Gel image of the DNA run on agarose gel is given in figure 4. Result indicated minimum shearing which was indicated by lower intensity of smear in each well.



**Figure-4: DNA extracted by alkaline lysis method from fresh meat samples run on 0.85 % agarose gel**

**L: DNA; G: Goat; Ch: Chicken; B: Buffalo; C: Cattle; S: Sheep; P: Pork**

#### **4.1.2 Binary mixtures of meat**

Binary mixtures of meat were made with each mixture made up to 500 mg by mixing the mutton and beef in different proportions and labelled from BM 1 to BM 13 and subjected to alkaline lysis method of DNA extraction. The mean DNA concentrations of the 13 binary meat mixtures with their standard errors are shown in the table 6. The OD 260/280 values obtained were in the range of 1.4 to 1.8.

**Table-6: Mean values of the concentrations (ng/μl) of the DNA extracted from binary meat mixtures**

<b>Binary Mixture</b>	<b>DNA Concentration (ng/μl)</b>
BM1	172.86±16.41
BM2	136.63±13.38
BM3	141.35±6.01
BM4	151.83±16.66
BM5	85.98±9.29
BM6	169.05±10.66
BM7	149.74±14.57
BM8	148.68±15.34
BM9	151.05±13.03
BM10	99.44±11.72
BM11	107.78±10.98
BM12	141.66±16.61
BM13	106.56±8.45

Values are Mean ± SE, N=6

#### **4.1.3 DNA quantification in heat treated samples**

The mean DNA concentrations of the heat treated mutton samples, subjected to alkaline lysis method with their standard errors are shown in the table 7. The OD<sub>260/280</sub> values obtained were in the range of 1.4 to 1.7.

**Table-7: Mean values of the concentrations (ng/μl) of the DNA extracted from heat treated mutton samples**

<b>Temperature (°C)</b>	<b>DNA Concentration (ng/μl)</b>
60	112.33±17.26
80	170.66±15.01
100	205.50±13.18
121	90.00±4.94

Values are Mean ± SE, N=6

## **4.2 OPTIMIZATION OF LADDER PATTERN IN LAMP ASSAY**

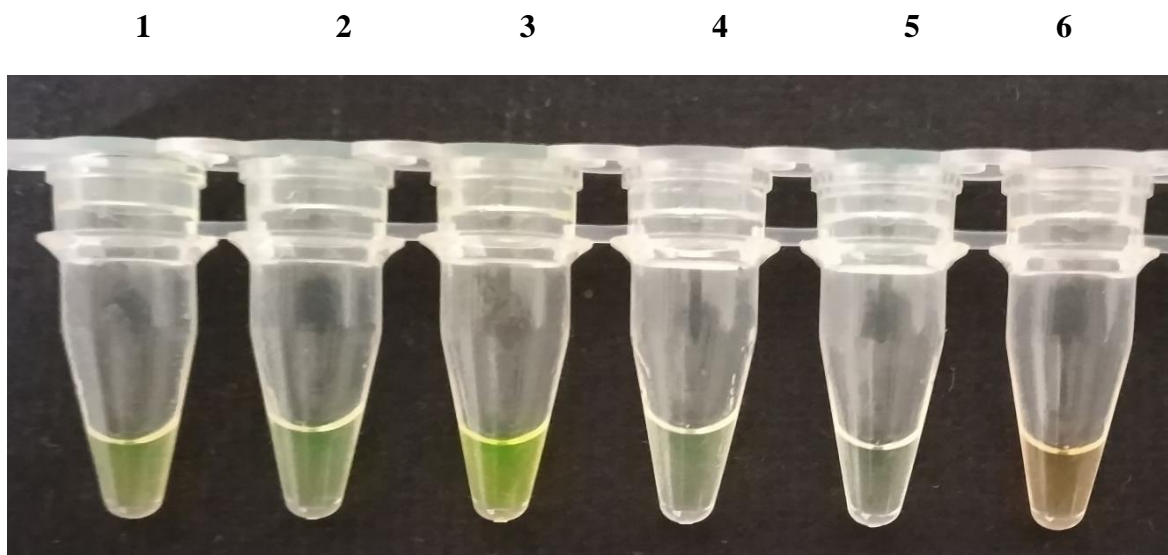
MgSO<sub>4</sub>, betaine and temperature of reaction are critical parameters considered for developing typical ladder pattern during LAMP assay. In the present study, we optimized the concentration of these components to get a better ladder effect by keeping the other parameters standard. Betaine (buffering action) concentration was standardized in the range of 1.2-2 M; amplification temperature at 58- 62 °C for one hour and MgSO<sub>4</sub> at 1-10 mM concentrations.

Characteristic green colour of the of amplified DNA produced after adding SYBR green dye after loop displacement action was checked by flurometer reading while ladder pattern was checked by agarose gel electrophoresis using 2% agarose gel in 1X TAE (Tris- Acetic Acid – Ethylene Diamine Tetra acetate) buffer run for 40 min at 100 V.

### **4.2.1 Standardization of temperature**

Reaction temperature is a significant factor for any successful LAMP assay. The assay was carried out at different temperatures ranging from 58-62 °C The optimal temperature for the present assay was found as 60 °C, at this temperature, peacock green color was obtained (fig 5) and intense banding of ladder pattern was observed in agarose

gel electrophoresis stained with ethidium bromide (fig 9). Fluorometric reading of the reaction mixtures is given in table 8. Highest fluorimeter reading of 25,583 was recorded at 60 °C. Each experiment was repeated 3 times for confirmation.



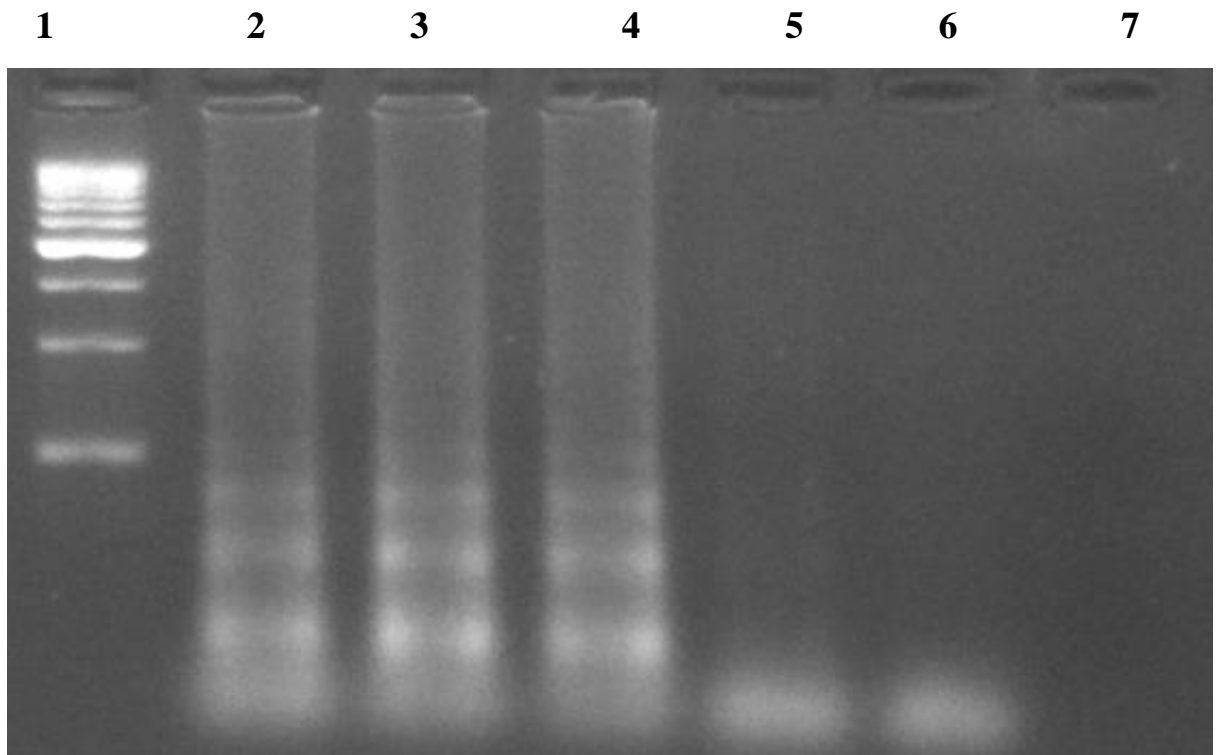
**Figure-5: Visualization of LAMP results in reaction mixture with different reaction temperatures**

**1: Reaction mix with 58 °C; 2: Reaction mix with 59 °C; 3: Reaction mix with 60 °C**

**4: Reaction mix with 61 °C; 5: Reaction mix with 62 °C 6: Negative control**

**Table-8: Fluorimeter reading in LAMP with different reaction temperature**

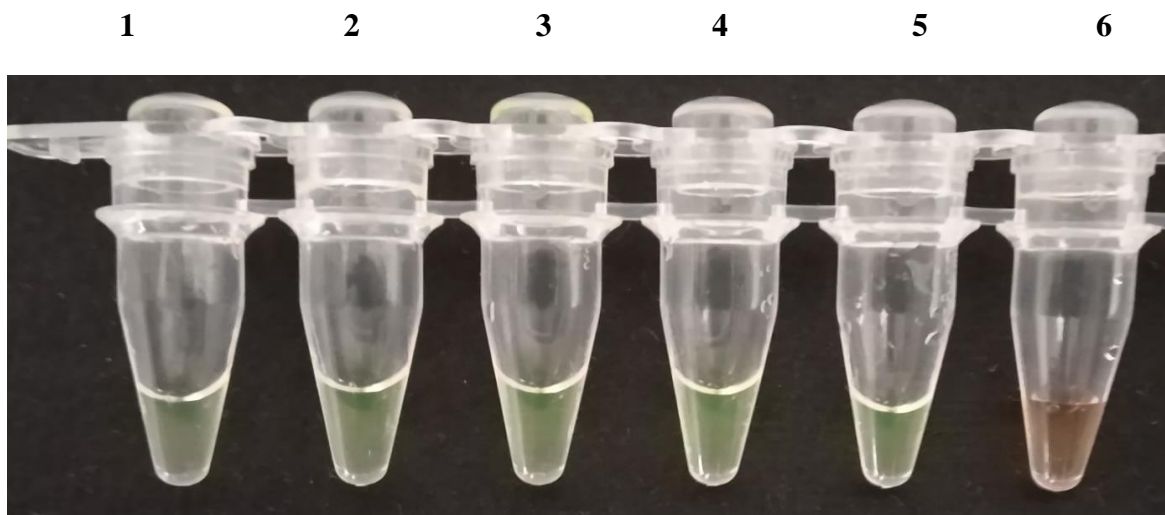
S No	Reaction Temperature (°C)	Mean average of fluorimeter reading (N=3)
1	58	19,713
2	59	20,768
3	60	25,583
4	61	15,826
5	62	13,235
6	Negative control	4,321



**Electrophoretic analysis showing the effect of temperature on LAMP assay: Lane 1: 100 base pair ladder; Lane 2: 58 °C; Lane 3: 59 °C; Lane 4: 60 °C; Lane 5: 61 °C; Lane 6: 62 °C; Lane 7: Negative control**

#### **4.2.2 Optimization of betaine concentration for LAMP reaction**

Betaine is an essential component of the LAMP reaction mix. Chemically, betaine is an amino acid analogue *i.e.* N, N, N-trimethyl glycine that has both positive and negative charges at neutral pH. Betaine equilibrates melting temperatures ( $T_m$ ) of base pairings (Jensen *et al.*, 2010) and increases specificity of the LAMP amplification (Rees *et al.*, 1993). Based on intense banding of ladder pattern achieved using agarose gel stained with ethidium bromide (fig 8) coupled with higher fluorometric readings (28,287) (table 9). Optimum concentration of betaine was fixed at 1.4 M for the sheep specific LAMP assay.



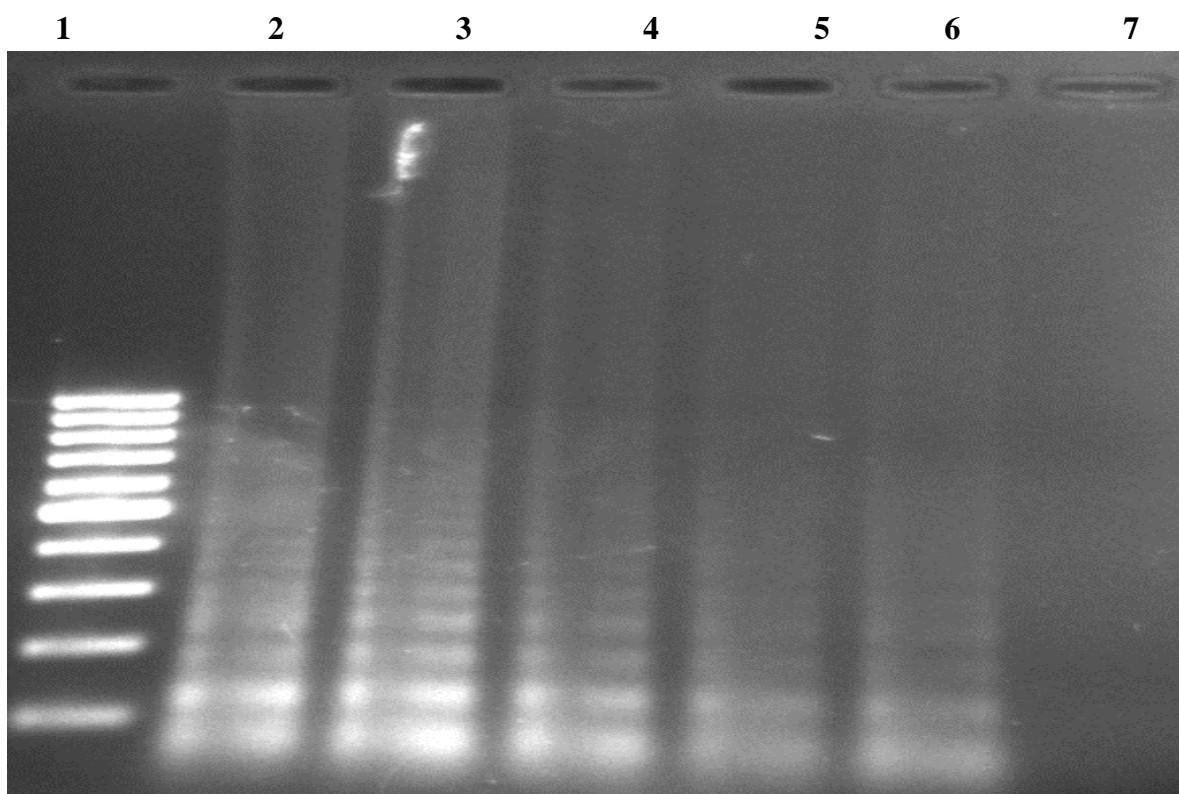
**Visualization of LAMP results in reaction mixture with different betaine concentrations**

**1: Reaction mix with 1.2 M; 2: Reaction mix with 1.4 M; 3: Reaction mix with 1.6M**

**4: Reaction mix with 1.8 M; 5: Reaction mix with 2 M 6: Negative control**

**Flurometer reading in LAMP with different betaine concentration**

<b>S No</b>	<b>Concentration of Betaine (M)</b>	<b>Mean average of flurometer reading (N=3)</b>
1	1.2	21,891
2	1.4	28,287
3	1.6	23,825
4	1.8	23,238
5	2.0	24,350
6	Negative control	3,219

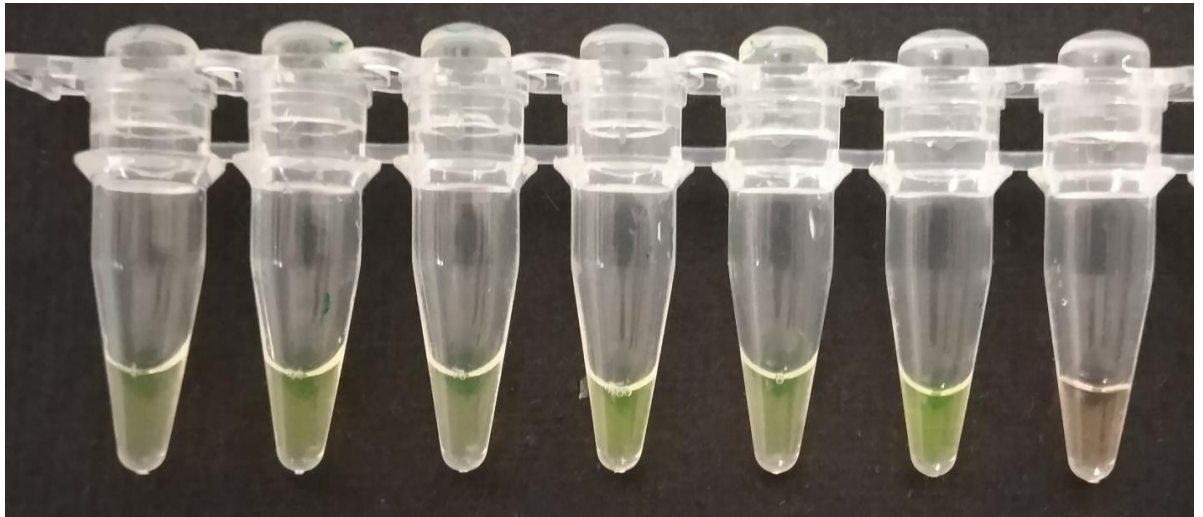


**Electrophoretic analysis showing the effect of betaine concentration on LAMP assay: Lane 1: 100 base pair ladder; Lane 2: 1.2 M; Lane 3: 1.4 M; Lane 4: 1.6 M; Lane 5: 1.8 M; Lane 6: 2 M; Lane 7: Negative control**

#### **4.2.3 Optimization of magnesium ions for the LAMP reaction**

$Mg^{++}$  ions greatly influence LAMP amplification reaction and dimer formation; this requires optimization of  $Mg^{++}$  ions concentration in the LAMP assay. At 6 mM concentration, fluorometer reading of 37,311 was recorded, which was higher as compared to the readings recorded at other concentrations *viz.*, 1, 2, 4, 8 and 10 mM. Therefore, concentration of 6 mM was finalized after comparing the intensity of green color developed (fig 9, intensity of ladder observed in agarose gel electrophoresis (fig 10) and fluorometer reading at different concentrations (table 10).

1 2 3 4 5 6 7



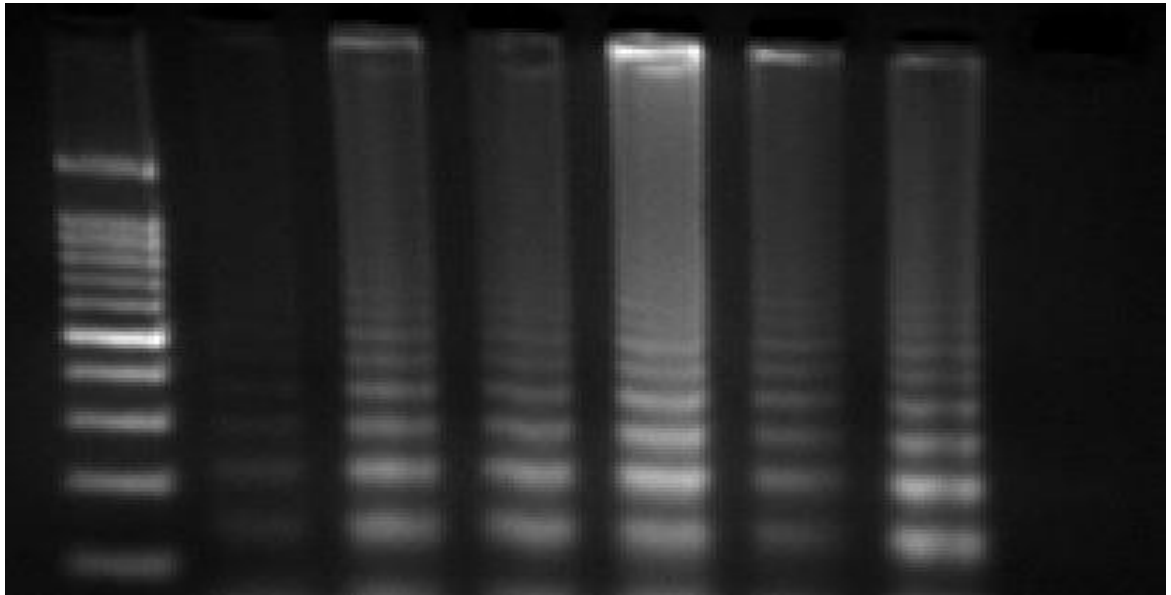
**Visualization of LAMP result in reaction mix containing different concentration of MgSO<sub>4</sub>**

**1: Reaction mix with 1 mM; 2: Reaction mix with 2 mM; 3: Reaction mix with 4 mM;  
 4: Reaction mix with 6 mM; 5: Reaction mix with 8 mM 6: Reaction mix with 10 mM  
 7: Negative control**

**Table: Flurometer reading in LAMP with different MgSO<sub>4</sub> concentration**

<b>S No</b>	<b>Concentration of MgSO<sub>4</sub> (mM)</b>	<b>Mean average of flurometer reading (N=3)</b>
1	1	13,229
2	2	26,448
3	4	32,190
4	6	37,311
5	8	28,771
6	10	30,216
7	Negative control	2,545

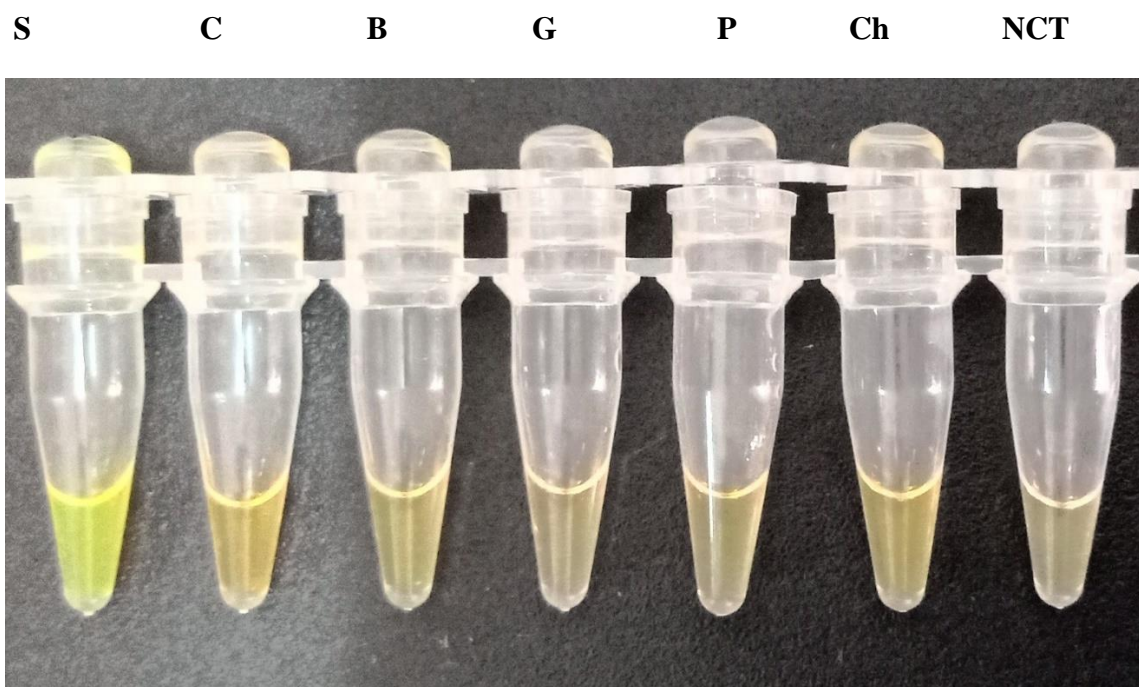
**1                      2                      3                      4                      5                      6                      7                      8**



**Electrophoretic analysis showing the effect of  $\text{MgSO}_4$  concentration on LAMP assay: Lane 1: 100 bp ladder; Lane 2: 1 mM; Lane 3: 2 mM; Lane 4: 4 mM; Lane 5: 6 mM; Lane 6: 8 mM; Lane 7: 10 mM; Lane 8: NCT**

### **4.3 SPECIFICITY OF THE PRIMERS**

Possibility of cross amplification of sheep specific primers was eliminated by testing sheep specific primers with DNA of cattle, buffalo goat, sheep, pork and chicken. The sheep specific primer pair was able to produce the green colour in sheep DNA only. No amplification was observed in the DNA of other species tested including negative control and thus the specificity of designed sheep specific primers was confirmed further by ladder pattern (fig 11) and flurometry (table 11). The experiment was repeated for 6 times with 6 different samples.

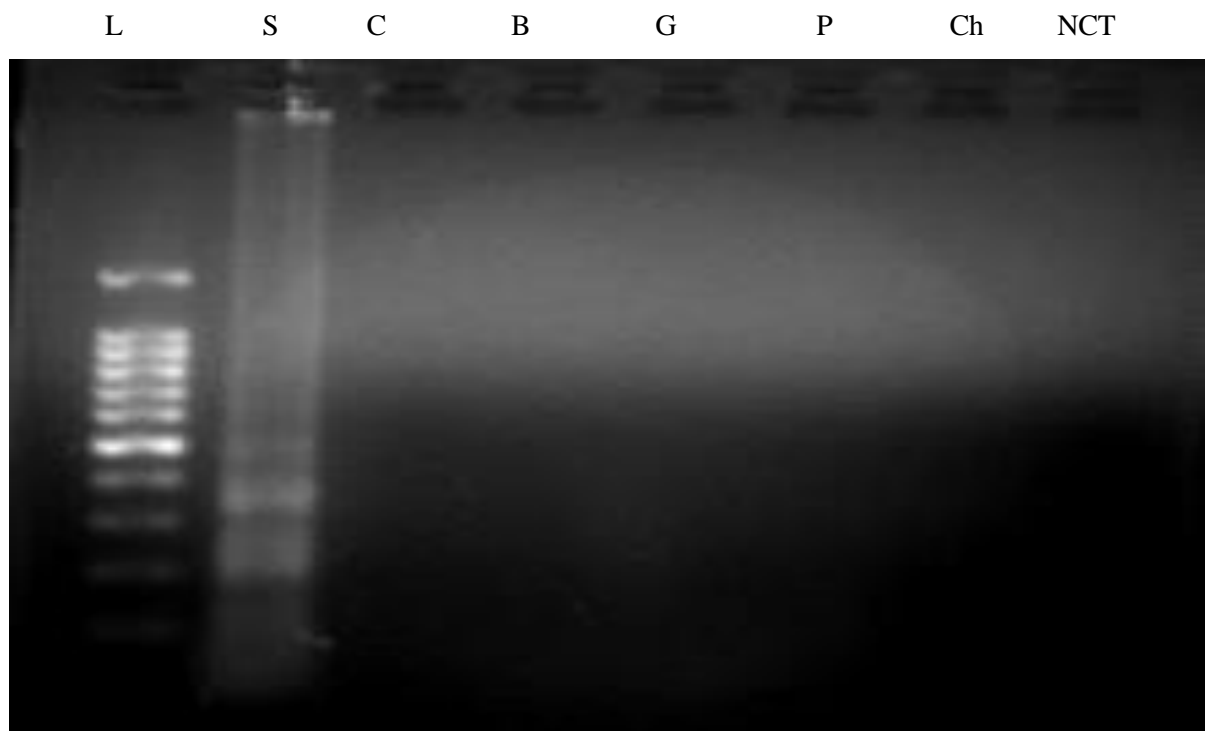


**Checking cross reactivity of sheep specific LAMP assay with different meat animal species**

**S: Sheep meat; C: Cattle meat; B: Buffalo meat; G: Goat meat; P: Pork meat Ch: Chicken; NCT: Negative control**

**Table-Fluorometer reading of LAMP reaction mixture containing DNA of different species**

<b>S No</b>	<b>Species</b>	<b>Mean average of fluorometer reading (N=3)</b>
1	Sheep	31,801
2	Buffalo	6,357
3	Chicken	6,225
4	Pig	8,374
5	Cattle	8,012
6	Goat	4,983
7	Negative control	2,984

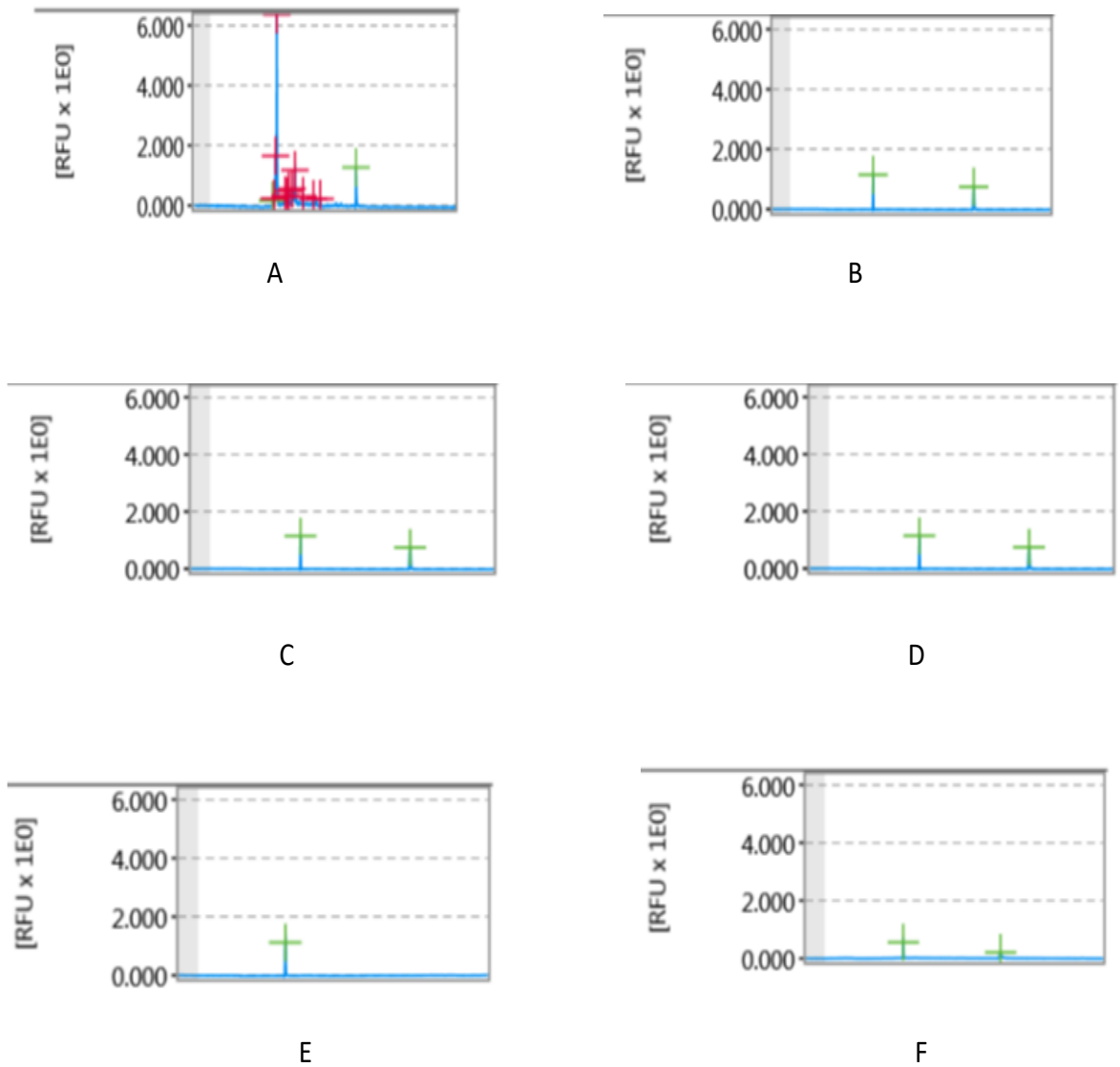


**Electrophoretic analysis of cross-species specificity test of the designed primers with non-target species.**

**Lane L: 100 bp ladder; Lane S: Sheep; Lane C: Cattle; Lane B: Buffalo; Lane G: Goat; Lane P: Pork; Lane Ch: Chicken; Lane 7: Negative control**

**Checking specificity of the designed primers by QIAxcel capillary electrophoresis system**

QIAxcel capillary electrophoresis was done for checking the specificity of primers. Various multiple bands are observed only in sheep meat as shown in fig 13 by red colour bands. No amplification was possible in other meat species. This technique was used to quantify the amplified DNA fragments. Various amplicons of size 15, 19, 24, 70, 89, 106, 126, 174, 234, 251, 273, 600 were recorded in mutton. The quantitative data of all LAMP products, as integrated by QIAxcel software, is displayed in table 12



**Specificity of the designed primers by capillary gel electrophoresis**

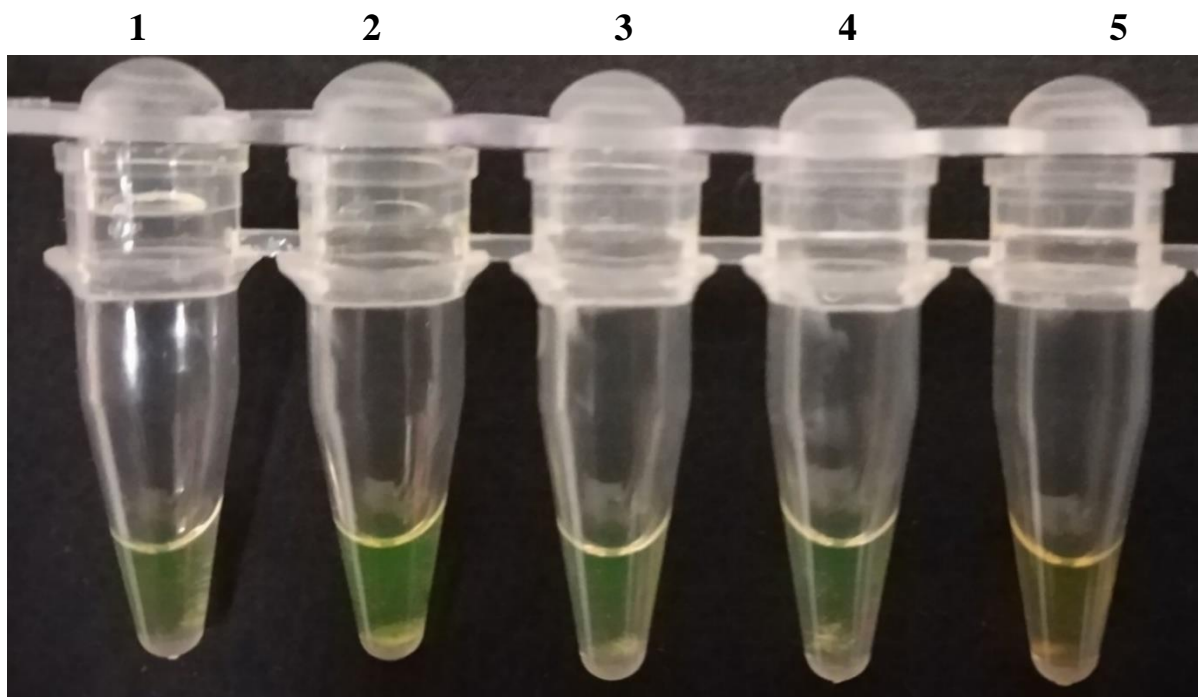
**The electropherograms of LAMP products in different species A) Sheep B) Cattle  
C) Goat D) Pork E) Chicken F) Buffalo**

**Table-12: Amplicons of sheep specific LAMP run on capillary electrophoresis**

<b>S NO</b>	<b>species</b>	<b>Amplicon size</b>
1	Sheep	15, 19, 24, 70, 89, 106, 126, 174, 234, 251, 273, 600
2	Cattle	No amplification
3	Buffalo	No amplification
4	Goat	No amplification
5	Pork	No amplification
6	Chicken	No amplification

### **LAMP IN THERMALLY PROCESSED MEAT SAMPLES**

LAMP assay was carried out in heat treated mutton samples cooked under 60, 80, 100, 121 °C for 30 mins in dry bath. Amplification was observed in all processing temperatures. High degree of amplification was noticed at 60 °C indicated by high flurometer reading (31,482) as shown in table 13. These results indicated that LAMP amplification was also possible even at high cooking temperatures but flurometer reading was decreased with decreasing temperature, lowest value 16,497 was recorded at 121 °C. Although DNA gets degraded in to small fragments at higher temperatures, amplification of target region was still satisfactory.

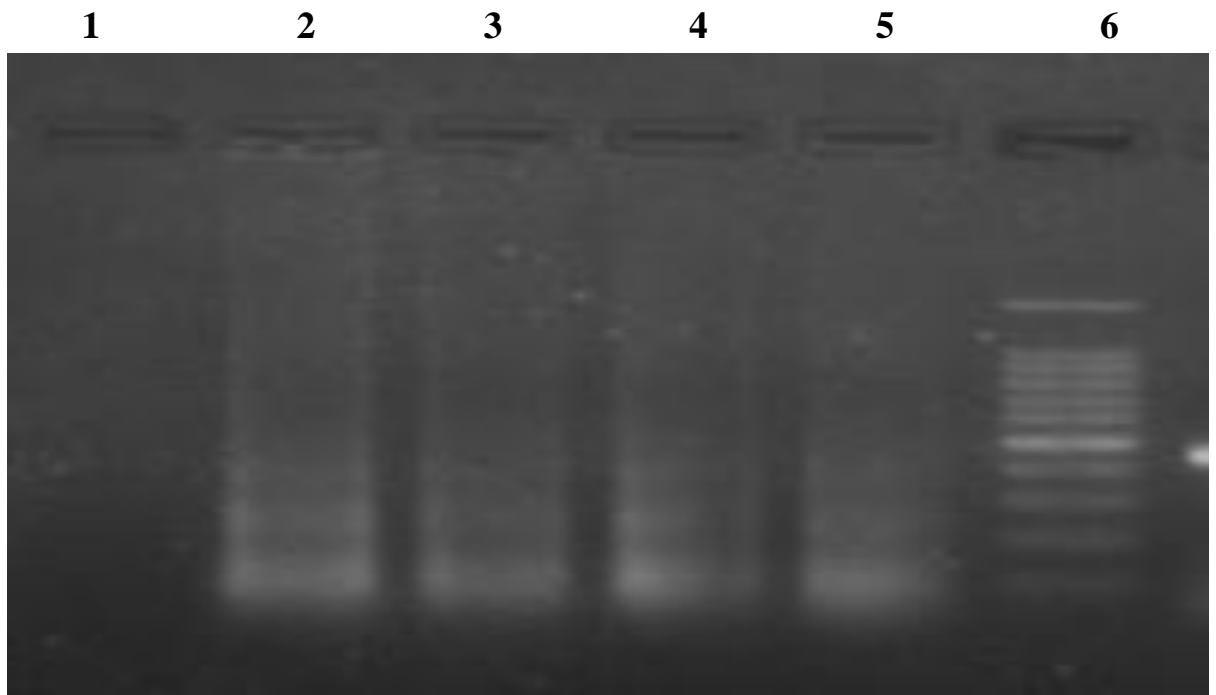


**Visualization of LAMP result in reaction mix containing DNA extracted from sheep meat processed to different temperatures**

**1: 60 °C;    2: 80 °C;    3: 100 °C    4: 121 °C;    5: Negative control**

**Table- Flurometer reading of LAMP reaction mixture in thermally processed meat samples**

<b>S No</b>	<b>Temperature (°C)</b>	<b>Mean average of flurometer reading (N=3)</b>
1	60	31,482
2	80	22,930
3	100	18,229
4	121	16,497
5	Negative control	1,650

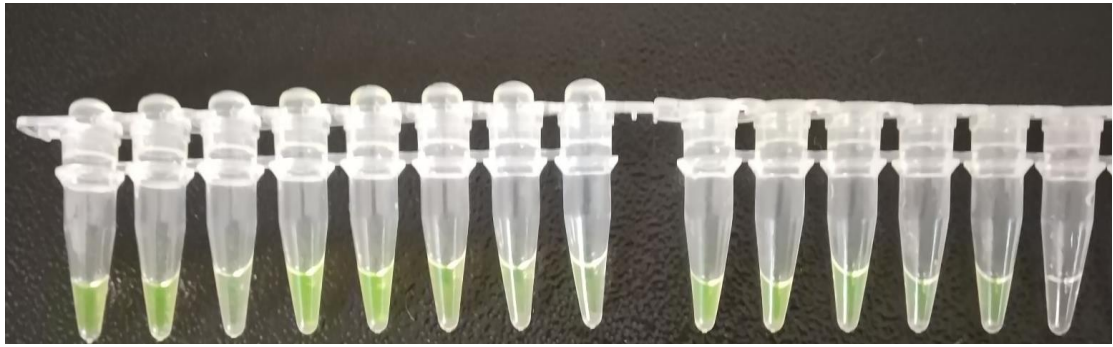


**Figure- Electrophoretic analysis of heat treated samples of LAMP Lane 1: Negative control; Lane 2: 60 °C; Lane 3: 80 °C; Lane 4: 100 °C; Lane 5: 121 °C; Lane 6: DNA Ladder**

#### **4.5 LAMP IN BINARY MIXTURES**

Binary mixtures of each mixture up to 500 mg were made in different proportions. After thorough mixing, meat mixtures were subjected for DNA extraction using AL method, then followed by LAMP assay. It was found that sheep-specific LAMP assay developed in the present study was highly sensitive to identify meat adulteration up to extent of 0.1 %, which is confirmed by change in colour after addition of dye as shown in fig: 16 and also confirmed by flurometer reading, highest flurometer reading value (38,343) was noticed in binary mixture with 50 % mutton in 50 % beef and the values were decreasing with decreasing percentage of mutton in beef.

1 2 3 4 5 6 7 8 9 10 11 12 13 14



**Figure-16: Visualization of LAMP result in binary meat mixtures made by mixing mutton with beef in different proportions**

- 1) 50 % 2) 40 % 3) 30 % 4) 20 % 5) 15 % 6) 10 % 7) 5 % 8) 1%  
 9) 0.8 % 10) 0.6 % 11) 0.4 % 12) 0.2 % 13) 0.1 % 14) Negative control

**Table -14: Flurometer reading in LAMP reaction for detection of sheep meat in beef**

S. No	% sheep meat in beef	Mean average of flurometer reading (N=3)
1	50	38,343
2	40	35,918
3	30	32,476
4	20	31,363
5	15	31,130
6	10	29,300
7	05	27,261
8	01	25,777
9	0.8	25,333
10	0.6	25,308
11	0.4	23,380
12	0.2	22,973
13	0.1	20,803
14	Negative control	2,307

#### 4.6 CHECKING SENSITIVITY OF LAMP DNA

The sensitivity of sheep-specific AL-LAMP assay was done using different concentrations of sheep DNA made by diluting the template DNA so as to get concentrations *viz.*, 100, 75, 50, 40, 30, 25, 20, 10, 5, 2.5, 1, 0.5 ng/ $\mu$ L, followed by LAMP assay wherein, limit of detection (LOD) of sheep DNA was established as 0.5 ng as shown in fig 17. Highest flurometer value of 30,502 was recorded in sample having 100 ng of template DNA. Lower the amount of template DNA lower is the LAMP amplification indicated by decreasing flurometer reading as shown in table 15.

1 2 3 4 5 6 7 8 9 10 11 12 13



**Figure-17: Visualization of LAMP result in reaction mix containing different concentration of DNA extracted from sheep meat**

**1:100 ng; 2:75 ng; 3: 50 ng; 4: 40 ng; 5: 30 ng; 6: 25 ng; 7: 20 ng;  
8: 10 ng; 9: 5 ng; 10: 2.5 ng; 11: 1 ng ; 12: 0.5 ng; 13: Negative control**

**Table-15: Flurometer reading in LAMP reaction mixture with different concentration of sheep meat DNA**

<b>S No</b>	<b>Concentration of DNA (ng/<math>\mu</math>L)</b>	<b>Mean average of flurometer reading (N=3)</b>
1	100	30,502
2	75	29,835
3	50	24,316
4	40	22,710
5	30	20,818
6	25	20,488
7	20	18,271
8	10	18,710
9	5	16,375
10	2.5	16,515
11	1	13,167
12	0.5	12,366
13	Negative control	2,132

# DISCUSSION

## CHAPTER V

### DISCUSSION

Globally, DNA based molecular techniques have become the techniques of choice for species identification of meat due to stability, versatility and unambiguous nature of DNA molecule. Array of DNA based techniques have been developed and employed for speciation of meat of which Polymerase Chain Reaction (PCR) based methods are commonly used. In recent years, Loop-mediated isothermal amplification technique which amplifies targeted gene at isothermal temperature is gaining importance. LAMP overcomes certain drawbacks of PCR based methods in terms of time, instrumentation, consumables required and the overall cost.

In the present study, an attempt was made to develop and standardize Loop-mediated isothermal amplification assay for species identification of mutton by utilizing four primers designed targeting mitochondrial D loop region. In addition, the technique was combined with simple DNA extraction method *viz.*, 'Alkaline lysis method' to achieve rapid and authentic speciation of mutton.

#### 5.1 DNA EXTRACTION

Alkaline lysis protocol described by Girish *et al.* (2013) was used in this study. DNA was extracted from meat samples of different meat animal's *viz.*, cattle, buffalo, sheep, goat, chicken and pig. The DNA extracted by following this method was of good quality and gave the successful LAMP amplification of mitochondrial D loop gene. Alkali *i.e.* sodium hydroxide at 0.2 N strength lyse cells at higher temperature leading to the release of DNA, Tris-HCl neutralize the alkali and the resultant DNA containing solution was used for LAMP reaction. Strong alkaline solution used in the AL method denatures proteins and nucleases, disrupt nucleus and release DNA. Denatured DNA gets

dissolved in solution; nevertheless, since primary structure DNA is relatively stable it sustains such treatment (Felicello & Chinali., 1993).

Although, OD<sub>260/280</sub> ratio was found below the recommended ratio of 1.8 to 2.2, DNA quality was sufficient enough for undertaking LAMP reaction. Moreover, this method is cost effective and it takes only 20 to 30 minutes. Similar opinion was expressed by Girish *et al.* (2013). He observed that alkaline lysis method was more economical and less laborious when compared to Phenol: Chloroform: Isoamyl alcohol (PCI) and commercial kit methods.

Further, Alkaline lysis method was able to successfully extract the genomic DNA from cooked meat samples (sheep) which were heat treated at 60, 80, 100 and 121 °C for 30 min in dry bath. Although DNA got degraded into small fragments at high temperatures and shearing of DNA was also observed, shearing did not make any difference in the amplification of targeted mt D loop region, which was satisfactory. According to findings of Girish *et al.* (2005) and Nagappa *et al.* (2011) PCR amplification is possible even in thermally processed meat samples. The presence of high copy number of mitochondrial DNA in a cell may contribute to the survival of few copies, when the tissue is subjected to cooking.

## **5.2 GENE OF CHOICE**

Mitochondrial genes especially cytochrome b, 12S rRNA, 16S rRNA and D loop regions are commonly used for meat species identification (Kumar *et al.*, 2015). In this study we designed the sheep-specific LAMP primers targeting the mitochondrial D loop region. In the previous studies, LAMP primers targeting D loop region of mitochondrial DNA for identification of buffalo meat (Deb *et al.*, 2016) and for wild boar (Kanchanaphum *et al.*, 2014) were designed, but to our knowledge the D loop region of Sheep meat has not

been targeted so far for the LAMP assay, Hence, we designed primers for identification of mutton targeting D loop region.

### **5.3 OPTIMIZATION OF LADDER PATTERN**

In this study, four primers were designed targeting D loop region of sheep for LAMP. For better ladder pattern various critical factors were standardized *viz.* MgSO<sub>4</sub>, betaine and temperature. Various temperatures ranging from 58 to 62 °C were tested in order to select a suitable temperature that could amplify target region in sheep. After several experiments it was found that amplification was possible with temperature ranging from 58 to 62 °C, but at 60 °C amplification was higher among all the temperatures selected, which was confirmed based on the instant colour change of LAMP product after addition of SYBR green I dye which was visualized by naked eye under day light, fluorometer reading and gel electrophoresis. Hence, 60 °C was considered as ideal for LAMP. Previous studies revealed that the optimum temperature with Bst enzyme was 64 °C (Kumari *et al.*, 2019) and 63 °C (Deb *et al.*, 2016, Li *et al.*, 2017) but the enzyme used in this study is Bsm which has its activity till 63 °C and best at 60 °C (Dhama *et al.*, 2014).

Various concentration of betaine from 1.2 to 2 M and MgSO<sub>4</sub> from 1 to 10 mM were also standardized. The inclusion of MgSO<sub>4</sub> at 6.0 mM level yielded better pattern as compared to the 4.00 mM level. Similarly, the inclusion level of 1.4 M betaine gave desirable ladder pattern as compared to 1.6 M level. Many researchers have also standardized MgSO<sub>4</sub>, betaine, temperature for authentication of meat species using LAMP (Deb *et al.*, 2016, Kumari *et al.*, 2019). All these LAMP assays took about 60 min time to accomplish detection of diagnostic signals at temperatures between 61 and 65 °C. Results of our newly developed sheep specific LAMP assay developed in the present study was in accordance with the LAMP assays developed by other investigators for the

detection of meat animal species. However, the present LAMP reaction was done in combination with DNA extraction by alkaline lysis method which brings down the overall time required for species identification of meat.

#### **5.4 SPECIFICITY OF THE PRIMERS**

Previously many studies on meat speciation were based on the main molecular biology technique PCR, such as conventional PCR, PCR-RFLP (Murugaiah *et al.*, 2009, Girish *et al.*, 2005), species specific PCR (Girish *et al.*, 2011) and Forensically Important Nucleotide Sequencing (FINS) targeting mitochondrial genes (Girish *et al.*, 2004; Chandre Gowda *et al.*, 2015). Although, PCR is accurate and authentic method, it is costly, time consuming, require sophisticated equipments. Therefore, in this direction a novel, LAMP technique has been developed to authenticate meat as it is proved to be practical, simple, and quicker approach (Notomi *et al.*, 2000 Nagamine *et al.*, 2002). In the present study, a partial sequence of mitochondrial D loop gene was targeted for LAMP study, so as to identify sheep and differentiate with other five animals.

The possibility of cross amplification was precluded by analyzing sheep specific primers with DNA extracted from cattle, buffalo, pork, goat and chicken. The amplification was evident in sheep DNA only and no amplification was observed in the DNA of other species including negative control (fig 11). Repeatability of the LAMP assay was confirmed by testing in six different samples. Since the designed primers exhibited stringent specificity further assays were carried on using these primers.

Similar studies for the specificity of primers in other species were also done *i.e.*, cattle specific LAMP developed targeting mitochondrial COI (cytochrome oxidase subunit 1) and D loop region was done by (Ma *et al.*, 2016; Deb *et al.*, 2016), pork specific by (Abdullah *et al.*, 2016; Yang *et al.*, 2014; Ran *et al.*, 2016), (Abdulmawjood *et al.*, 2014) for ostrich and Duck specific by (Ya shi *et al.*, 2017).

As an alternative method for confirming the LAMP amplicons, the present study used the QIAxcel capillary electrophoresis system, a computer controlled system that provides electronic documentation which was innovatively used in different aspects (Barakat *et al.*, 2014). Results of capillary electrophoresis showed multiple banding pattern indicative of ladder pattern observed in agarose gel electrophoresis. Electrophorogram of capillary electrophoresis is shown in fig 13 and size of the amplicons in capillary electrophoresis is given in table: 12. No amplification was observed in other meat animal species which indicated specificity of the assay.

## **5.5 SENSITIVITY OF LAMP ASSAY**

Quality of any molecular diagnostic test is determined by its sensitivity and specificity. We, therefore performed an analytical sensitivity of the LAMP assay using series of serially diluted mutton DNA samples starting from 100 ng to 0.5 ng for each template. The LOD for the assay was established as 0.5 ng for sheep (fig 17, table 14). Similar studies were also done by using mitochondrial cytochrome b gene by Abdulmawjood *et al.* (2014) who reported that the analytical sensitivity was 0.35 pg DNA in beef. Pork specific LAMP tests against mitochondrial D loop, cytochrome b and ND1 targets showed sensitivity of 0.5-1 pg DNA (Lee *et al.*, 2016; Yang *et al.*, 2014; Ran *et al.*, 2016). Kumari *et al.* (2019) reported that the sensitivity of LAMP assay was 0.01 ng in cattle tissue. Abdullah *et al.* (2016) confirmed that the sensitivity was 0.03 fg when pork DNA was targeted.

## **5.6 LAMP IN BINARY MIXTURES**

To test the sensitivity of LAMP in binary mixtures, various proportion of meat mixtures containing mutton and beef were made (table 3) and DNA was isolated from these mixtures. LAMP assay was conducted using these DNA templates to identify the sensitivity of the assay in binary meat mixtures. The assay was performed in triplicates to analyze the reproducibility. Interestingly, the optimized LAMP assay was able to detect 0.1 % mutton

spiked with 99.9 % of beef which was confirmed by visual change in color after addition of dye and fluorometer reading (fig16, table 13). However, the intensity of fluorometer was decreasing with decreasing percentage of mutton in beef as shown in table 13 but still clearly detectable with color intensity of SYBR green dye. These findings are in substantiation with Li *et al.* 2016 and Yang *et al.* 2014 who also reported that detection limit in meat mixtures was 0.1 %.

## **5.7 SUITABILITY OF AL-LAMP FOR THE DETECTION OF MUTTON IN HEAT TREATED MEAT**

Any meat authentication test in addition to raw meats must also detect processed samples such as heat treated. In order to evaluate suitability of the AL-LAMP assay for thermally processed meat, mutton samples were cooked at 60, 80, 100, 121 °C for 30 mins in dry bath. Amplification was possible with all the temperatures as shown in table 12, fig 14. The obtained result clearly indicated that cooking treatment of mutton do not affect the LAMP amplification of sheep D-loop, these findings are in agreement with Sul *et al.* (2019) who developed chicken specific LAMP assay targeting mitochondrial 16S rRNA gene reported the detection levels were 0.1 % and 1 % chicken in raw and autoclaved meats, respectively. Under routine conditions, cooking temperatures will not exceed 121 °C and the LAMP assay developed in this study could be conveniently used for detection of mutton even in cooked meat samples.

In recent years several PCR based methods have been reported for identification of sheep meat. The PCR detection of sheep meat species has been previously studied by Panwar *et al.* (2015) by targeting mitochondrial cytochrome b gene and producing 337 bp amplicons by employing a universal common forward primer and designed species specific reverse primers. In this Duplex PCR assay the sensitivity in sheep and goat binary mixtures was detected as 10 % for both species with an analytical sensitivity of 5 ng DNA. Nagappa *et al.* (2011) developed a highly species specific PCR assay for identification of raw and

heat treated mutton by targeting mitochondrial D loop region and sensitivity of these assay was 0.1 %. Nischala *et al.* (2016) conducted a touchdown duplex PCR assay for detection of mutton and chevon using a common forward primer and species specific reverse primers for sheep and goat targeting the *cytb* gene. Amplicons of 254 bp and 453 bp for sheep and goat respectively were produced with an LOD of 5 ng of mutton DNA. Bhatt *et al.* (2016) developed a multiplex PCR for the simultaneous detection of beef, buffalo meat and mutton. Girish *et al.* (2005) utilized PCR-RFLP technique for identification of beef, buffalo meat, mutton and chevon which involved PCR amplification of mt 12S rRNA gene followed by restriction digestion. This technique could identify and differentiate each of the above species but did not yield satisfactory results in case of meat mixtures/meats.

However, these PCR techniques requires a thermocycler and post-PCR electrophoresis for the interpretation of results. Also, limit of detection of adulteration was less whereas, AL-LAMP technique could detect meat adulteration up to 0.1 %. Specificity and sensitivity of LAMP assay is higher than conventional PCR, this could be attributed to LAMP primers that can detect even a single nucleotide difference and formation of loop-mediated secondary DNA structure. Of the several current alternatives to the PCR for the purpose of species authentication, the LAMP assay proved to be the best option due to its rapid and sensitive detection of the even fewer diagnostic target signal under isothermal conditions (Nagamine *et al.*, 2002; Erwanto *et al.*, 2011; Dhama *et al.*, 2014; Abdullahi *et al.*, 2015) and is well suited for even field conditions lacking sophisticated infrastructure.

Results indicate that combination of alkaline lysis method of DNA extraction and Loop-mediated Isothermal Amplification (LAMP) reaction targeting mitochondrial D loop region can help in rapid authentication of sheep meat (mutton) in both fresh and processed forma. AL method requires only dry bath and simple chemicals for DNA extraction. LAMP amplification reaction is undertaken at constant temperature which precludes the requirement of expensive thermocycler for amplification of genes. Results of the reaction

can be interpreted by colour change with addition of SYBR green dye which obviates the requirement of electrophoresis unit and gel documentation system. Overall, AL – LAMP can be a quick and economical technique for species identification of mutton which can be undertaken with minimum instrumentation which makes it a technique suitable at both laboratory and field level.

# SUMMARY

## **CHAPTER VI**

### **SUMMARY**

The meat industry in recent times has come across incidences of misrepresentation of higher commercial valued meats by low-priced ones and the fraudulent labelling of meat species. Food authenticity is an issue of major concern for food authorities, since incorrect labelling of animal foods may have remarkable negative consequences. The consumers require higher protection from falsely labelled meat products for a variety of economic, religious, social and health concerns. Identification of sheep meat has immense importance because, being costly meat it is often mixed with low value meat for economic gain. For instance, mutton may be adulterated with buffalo meat thereby jeopardizing sentiments of the consumers.

In view of the above, an experimental study was undertaken with the objective to develop LAMP assay by designing two pairs of primers targeting mitochondrial D loop region for authentication of mutton (sheep meat) and to differentiate with other closely related meat species.

Meat samples were collected from the experimental abattoir of ICAR- NRC on Meat, Hyderabad and local retail outlets for validation of LAMP assay. Two sets of primers comprising of forward outer, forward inner, backward inner and backward outer targeting the mitochondrial D loop gene of sheep were designed using Primer Explorer V5 software. DNA was extracted from the authenticated samples using alkaline lysis method. Extracted samples were subjected to spectrophotometric analysis where most of the samples showed an OD ratio 1.4-1.8 between a wavelength of 260 and 280 nm.

Samples exhibiting an OD ratio in this range only were selected for LAMP assay. For checking the quality, DNA samples were electrophoresed in 0.85 per cent agarose gel.

Cross species specificity of the primers was assessed by performing a LAMP assay with DNA templates of cattle, buffalo, pork, goat and chicken, where no cross amplification was detected. Capillary electrophoresis was also performed for testing the specificity of the primers which indicated high degree of amplification in sheep only.

Similar studies were conducted for mixed meats also. For this purpose, various proportions of mutton in beef meat were prepared, and DNA was extracted from these mixtures by alkaline lysis method. It was observed that this assay could detect 0.1 % of mutton when mixed with beef which was visualized by addition of SYBR green dye.

The amplification was also obtained in thermally processed mutton. For this study the mutton samples were cooked at varying temperatures like 60, 80, 100, 121 °C for 30 min. Amplification was observed in all temperatures including 121 °C. Presence of high number of mitochondrial DNA in a cell may contribute to the survival of at least few copies, when the sequences were subjected to extreme processing condition.

LAMP assay was performed with the designed primers for detecting the analytical sensitivity of the assay by using series of diluted DNA samples. The limit of detection (LOD) of these assay was 0.5 ng of DNA confirmed by visual detection and flurometer reading.

To conclude, the combined approach of Alkaline lysis method of DNA extraction and the Loop mediated isothermal amplification assay (AL-LAMP) has been proved for its rapidity, specificity and sensitivity, thereby obviating the use of costly instrumentation such as thermal cyclers. AL-LAMP can be recommended for identification of mutton even in cooked sample at field level with minimal facilities.

**LITERATURE  
CITED**

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