# PCR BASED IDENTIFICATION OF PROCESSED CATTLE AND HORSE MEAT[#]

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**ABSTRACT**

A simple and efficient PCR reaction used to identify and differentiate processed cattle and horse muscle meat. To simulate the various processing technology, meat samples were putrefied by keeping unpreserved at room temperature for 48-72 h and heated at various temperatures and conditions. To amplify partial cyt b gene of mtDNA, a common forward and specific reverse oligonucleotide primers were used. The PCR amplified 274bp and 439 bp fragments from cattle and horse mtDNA. The test is a valuable tool for meat authentication and screening of cooked, putrefied and mixed samples of cattle and horse.

***Key words**:* Meat speciation; polymerase chain reaction; cattle; horse, mtDNA, *cyt b*

## Introduction

The authenticity of food is currently a major issue for researchers, consumers, industries and policy markers at all levels of the production process. Animal based protein has been part of staple diet from ancient times and it is a major concern that comes under food security. This is also important issue rooted to socio-religious approach. Some people, depending on their religious beliefs, do not eat certain meats (Gash, 2013).

In Hindu religion cows are perceived as holy deity and slaughter of cows is prohibited in most states of India and export of beef (cattle meat) is totally prohibited (Girish *et al*., 2013). Judaism and Islam do not permit the consumption of pork or horse meat (Taylor and Willett, 2013; Sistani, 2013). Early Christianity bans the consumption of horsemeat, and even early Greek and Roman laws had bans on consuming horse flesh (Taylor and Willett, 2013). Traditional methods used for species identification in meat samples generally based on the protein and DNA analysis. Protein based tests proved useful in meat detection are electrophoresis (SDS-PAGE), isoelectric focusing (IEF) and HPLC, immune based method such as ELISA (Ghovvati *et al*., 2009), immune diffusion tests, sensory analysis, anatomical differences, histological differentiation of the hair that may possibly exist in the meat, properties of tissue fat, level of and glycogen in muscle tissue (Ilhak and Arslan, 2007). The major disadvantages of using these methods in processed meat is that these methods are tissue dependant (Pirondini *et al*., 2010) and they can't differentiate between closely related species (Stamoulis *et al*., 2010). High temperature and processing of sample damages the original structure of proteins, in this way test usually is not successful in species determination in meat sample. Among DNA-based methods, polymerase chain reaction (PCR) is the most well developed molecular technique up to now and provides a simple, rapid, highly sensitive and specific tool for detecting constituents of animal origin in foods (Tobe and Linacre, 2008). Most of the PCR based meat identification based on the use of mitochondrial DNA (mtDNA) rather than nuclear DNA for the identification, the origin of meat products, because processed meats are likely to contain degraded DNA. (mtDNA) is more suitable than nuclear DNA due to the high copy number of (mtDNA) per cell, which thereby increases the chance of getting good DNA from samples (Hsieh *et al*., 2005).

## Materials and Methods

Eight fresh muscle meat samples from both species were collected from a local slaughter house and stored at -20°C until use. Meat samples (1-3 g) from each species were divided into four replicates and subjected to various experimental procedures of fresh, heating (cooking) and putrefaction. Fresh meat samples were kept frozen at -20°C until they were processed to DNA isolation. Meat samples were cooked at 100°C in dry (hot air oven) and moist heat (water bath and autoclave) for 30-45 min to simulate cooking. Autolysis of meat samples were also stimulated by allowing them to putrefy for a period of 48-72 hours at room temperature in unpreserved conditions. Pre-treatment of tissue samples was done by freezing them in liquid nitrogen and then subsequently grounded into powder for easy isolation of DNA. DNA was extracted by using the method described by as described by Sambrook *et al*. (1989). The DNA samples were evaluated both quantitatively and qualitatively using spectrophotometer and agarose gel electrophoresis, respectively. The quantity and quality (A260/A280 ratio, i.e absorbance at wavelengths of 260 and 280) of the DNA samples were assessed by using a Spectrophotometer, Bio Drop cuvette (Isogen Life Science, Netherlands).

## Polymerase chain reaction (PCR)

Species specific fragments of cytochrome b gene of mitochondrial DNA were amplified from fresh cooked and putrefied meat samples of both cattle and horse. PCR amplification was done in 50 µl of reaction mixture containing 10 µl of 5X PCR assay buffer without $MgCl_2$, 1.5 mM $MgCl_2$, 200 µM dNTPs mix, 20 pmol of each primer forward and reverse, 50 ng target DNA and 1 unit of Taq polymerase enzyme (Promega, USA). Oligonucleotide primers used for this study were described by Matsunaga *et al*. (1999). A common forward primer (5'-GACCTCCCAGCTCCATCAAACAT CTCATCTTGAT GAAA-3') and species specific reverse primers for cattle (5'-CTAGAAA AGT GTA A GACCCGTAATATAAG-3') and horse (5'-CTCAGA TTCA CTCG AC GAGGGTAGTA-3'). Amplification was performed in Veriti Thermal Cycler (Applied biosystems) with the following cycling conditions; after an initial heat denaturation at 94°C for 5 min, thirty-five cycles of amplification were run using PCR as follows: denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 30 sec and final extension at 72°C for 5 min. Following amplification, samples

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were electrophoresed through 1.5% agarose gel containing ethidium bromide (at the rate of 0.5 µg/ml of gel solution) in 1X TBE buffer at 80V for 50 min. The sizes of the bands produced were compared with the 100 bp DNA marker.

**Results and Discussion**

Partial cytochrome b gene fragments of 274 bp and 439 bp of cattle and horse were amplified by PCR, which were confirmed by the products parallel to 100 bp molecular weight marker (Fig.1). Since the forward primer is common to both species, a duplex reaction was also carried out using common forward primer and both reverse primers. Cattle and horse DNA was mixed in equal quantity and used as template for duplex PCR reaction and generated two bands in the agarose gel i.e. 274 bp and 439 bp. This experiment did not shown any false positive/negative results, demonstrating the reliability of the procedure and repetitive test proved the practicality and reproducibility of the method.

Many a time species identification of cooked meat is warranted. The processing technology (salting, drying, smoking, and cooking) applied during the manufacture of meat products are those steps, which mainly affect the integrity of the extractable DNA causing its degradation into small size fragments (Dias *et al*., 1994, Martinez and Man, 1998). For this reason, in the present study meat samples were cooked at 100 °C in dry (hot air oven) and moist heat (water bath and autoclave) for 45 minutes to simulate cooking. Proper cooking was evident from discoloured meat. In many cases samples are brought to the laboratory after one or two days of slaughter under unpreserved conditions. To replicate the reality of this situation, different levels of autolysis were stimulated by allowing the meat samples to putrefy for a variable period (48 hours or more) of time at room temperature in unpreserved conditions to stimulate the autolysis in meat. PCR successfully amplified small fragment of *cyt b* gene from cooked and putrefied meat samples, indicating that partial degradation of DNA because of cooking or putrefaction of meat does not inhibit amplification of *cyt b* gene region. The primer was found to be specific to the target gene as it specifically amplified the designed product of that gene by PCR. Mitochondrial DNA was used as a base in the study as it offers two main advantages: first that mtDNA is present in thousands of copies per cell (as many as 2,500 copies). This increases the probability of achieving a positive result even in the case of samples undergone severe DNA fragmentation due to intense processing conditions (Bellagamba *et al*., 2001) and second that the large variability of mtDNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species (Prado *et al*., 2002).

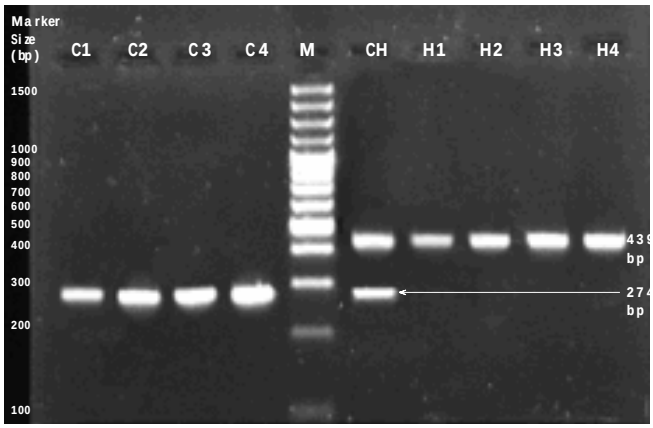


Fig.1: Agarose gel electrophoresis of PCR amplified products from processed cattle and horse meat. Lane C1 to C4 contains amplified cattle specific PCR products and lane H1 to H4 are horse specific PCR products. Sample 1 is dry heat treated meat DNA, sample 2 is wet heat treated meat DNA, sample 3 is putrefied meat DNA and sample 4 is fresh meat DNA as target sequence for PCR. Lane CH contains mixed cattle and horse DNA amplified with common forward primer and species specific reverse primers for cattle and horse. Lane M is molecular weight size marker.

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