



A novel approach for simultaneous differentiation of closely related meat species by PCR assay using single primer pair

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ABSTRACT

The aim of the present study was to design the primer pair for differentiation of closely related meat species i.e. cattle from buffalo, and sheep from goat employing PCR assay. The primer pair was designed based on the mitochondrial 12S rRNA gene sequences of buffalo and then designed primer pair matched with other species available 12S rRNA gene sequences. The designed primer pair was successfully amplified to the desired DNA fragments of 316 bp from the buffalo and sheep in single PCR reaction assay without any cross reaction with cattle, goat, pig and chicken species. The developed PCR assay was able to differentiate the closely related meat species; however, this assay was not suitable for differentiation of buffalo from sheep species meat. This PCR assay was also effective in heat treated meat and meat emulsion without any adverse effect on amplification of DNA. It can be concluded that the developed PCR assay has potential application for the purpose of differentiation of closely related meat species.

Key words: Cattle, Differentiation, Heat treatment, Meat species, PCR assay, Sheep

Detection of species origin of meat and meat products is a major problem for food regulating agencies due to lack of rapid and sound techniques for differentiation of closely related meat species such as cattle from buffalo and sheep from goat. The task is rather complicated for regulatory agencies, if meat is in the processed form or heat treated (Mane *et al.* 2007). DNA based PCR techniques were recently found to be very useful for meat species detection due to heat stability and conserved structure of DNA within all individuals of the same species (Mane *et al.* 2009). The specific detection of species origin of meat by PCR technique using species-specific targeted primers is relatively quick, precise, sensitive and cost effective as compared to other PCR based assay (Girish *et al.* 2005). The PCR based assay targeted both genomic as well as mitochondrial DNA (Arslan *et al.* 2006) however, mitochondrial DNA has various advantages as compared to the genomic DNA for the identification of meat species even in highly processed and heat treated meat and meat products due to the maternal inheritance of mitochondria gene. The mitochondrial genes are present in thousands of copies per cell as against single copy of genomic DNA and the 10– fold higher rate of point

mutation compared to nuclear genes in vertebrates results in enough point mutations that accumulate quickly to allow the discrimination of even closely related meat species, which ultimately increases the chances of positive result even in highly fragmented DNA due to severe processing conditions (Unsel *et al.* 1995, Greenwood and Paboo, 1999, Bellagamba *et al.* 2001, Mane *et al.* 2012). The targeted approach for the species identification was used for the meat species detection/differentiation, even in the heat treated meat and meat products (Arslan *et al.* 2006, Mane *et al.* 2011, Doosti *et al.* 2011, Yusop *et al.* 2011). Most of studies were not able to differentiate the buffalo from cattle (Gouli *et al.* 1999, Arslan *et al.* 2006, Ilhak and Arslan, 2007) by species-specific targeted approach, however Mane *et al.* (2012) was able to differentiate the buffalo meat from cattle meat (ox) even in admixed meat and meat products processed under different manufacturing conditions. The present study was targeted to differentiate the closely related meat species i.e. buffalo from cattle and sheep from goat using designed primer pair based on 12S rRNA gene by single PCR reaction for the purpose of reliable, rapid, sensitive and cost effective differentiation of meat species.

MATERIALS AND METHODS

Sample collection and extraction of DNA: The fresh meat samples of buffalo, beef, sheep, goat, pig and chicken were collected from local slaughterhouses and experimental

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abattoir of the Institute. After collection, samples were kept at -20°C till further processing. The blood samples were also collected from different species, where availability of meat samples is not possible. The blood was collected in sterile 15 ml polypropylene tube containing 0.5 ml of 0.5 M ethylene diamine tetra acetate (EDTA) solution, which acts as an anticoagulant. The collected blood samples were also preserved at -20°C till DNA isolations. The DNA samples of certain breeds of different species were also collected from different labs of this institute. Blood and tissue kit was used for extraction of DNA from meat samples as per the instructions given by manufacturer. The same kit was also used for extraction of DNA from heat treated meat and meat emulsion.

Design of primer pair: The oligo-nucleotide primer pair based on the mitochondrial 12S rRNA gene sequences of buffalo was designed, which was compared with sheep for positive amplification and other species for negative amplifications. For this, published DNA sequences of the different species were retrieved from the National Center for Biotechnology Information (NCBI) GenBank and buffalo and sheep specific primer pair was designed using primer designing software (DNA-STAR Inc., USA). The primer pairs designed were synthesized from Metabion International, Germany. The details of primer pair used in the present investigation are given below:

Forward: 5' TTG GCG GTG CTT TAT ATC CC 3'

Reverse: 5' GAT CCG GTA CTT CGT GCG TG 3'

PCR amplification and electrophoresis analysis: The reaction mixture was prepared in a 500 μl PCR tube in a total volume of 50 μl containing 5 μl of 10X PCR buffer, 15 mM MgCl_2 , 200 μM each of dNTP, 1–2 Units of Taq DNA polymerase, 20 p mol each of forward and reverse primer, 1 μl of DNA template (20–30 ng) and remaining nuclease free water. The PCR conditions programmed on master cycler gradient thermocycler were: initial denaturation at 94°C for 2 min followed by 30 and 35 cycles for fresh and heat treated meat, respectively, of denaturation at 94°C for 0.5 min, annealing at 55.5°C for 0.5 min and extension at 72°C for 1 min. Final extension was done at 72°C for 5 min. The PCR product was kept at -20°C for further use.

The submarine horizontal agarose gel electrophoresis was used for analysis of PCR products using 2% agarose in gel. For that 0.4 g of agarose was put in 20 ml of 1X TBE solution and heated to completely dissolve the agarose. Then 1 μl (5%) ethidium bromide solution was added as gel visualizing agent and mixed thoroughly. The electrophoresis was done for 90 min at 80V. The PCR product was finally analyzed using UV transilluminator and documented by gel documentation system. The ready to use 100 bp ladders was used in the present work as a known molecular marker.

Specificity and efficiency of PCR assay in meat and meat emulsion: The specificity of PCR assay was cross tested with DNA of other meat species used in this study. DNA was

isolated from raw as well as heat treated meat and meat emulsion for efficiency in various processing conditions. Meat emulsion was prepared as outlined by (Mane *et al.* 2009) while the meat and meat emulsion were heat treated at different temperatures to evaluate applicability of developed PCR assay. The heat treatments given to meat and meat emulsions were: steam cooking at 100°C for 45 min and autoclaving at 121°C (15 psi) for 15–20 min. The experiment is repeated for several times in fresh as well as in processed meat and meat products.

RESULTS AND DISCUSSION

Authentic and rapid testing of species origin of meat is indispensable to avoid unfair market competition and protection of consumer from fraudulent practices of meat adulteration. In the present work, specific primer pair was self-designed based on mitochondrial 12S rRNA gene to amplify the DNA from buffalo and sheep without any non-specific amplification from their closely related meat species i.e. buffalo from cattle and sheep from goat species simultaneously in the single PCR reaction. The PCR was successfully optimized for amplification of desired DNA fragments of 316 bp from DNA extracted from meat of buffalo and sheep using the designed primer pair without any non-specific spurious amplification after repeated testing. Earlier researchers used the species-specific PCR assay for meat species identification (Meyer *et al.* 1994, Calvo *et al.* 2002, Arslan *et al.* 2006, Ilhak and Arslan, 2007, Mane *et al.* 2009, and Mane *et al.* 2011), but only single species was targeted while designing the primer pair. However, in the present study, buffalo and sheep were targeted for simultaneously amplification in the single PCR reaction using single primer pair from their closely related meat species i.e. cattle and sheep respectively.

The optimized PCR assay using self-designed primer pair was cross tested for the specificity with DNA extracted from cattle, buffalo, sheep, goat, pig and chicken meat. After repeated testing, single DNA fragment of 316 bp was amplified only from buffalo and sheep meat DNA without any cross reaction with their closely related meat species i.e. cattle and goat respectively, even from pig and chicken meat DNA (Fig. 1). Most of the researchers failed to differentiate the closely related meat species i.e. in between buffalo and cattle (Gouli *et al.* 1999, Arslan *et al.* 2006, Ilhak and Arslan 2007) and in between sheep and goat (Lahiff *et al.* 2001, Mane *et al.* 2007) except recent report of specific detection of buffalo in meat and meat products, even in heat treated admixed meat products at the level of 1% (Mane *et al.* 2012). Even, in the earlier work using species-specific primer pair by PCR assay identified various meat species and reported their specificity and sensitivity for authentic identification of species origin of meat and meat products (Arslan *et al.* 2006, Ilhak and Arslan 2007, Mane *et al.* 2011, Doosti *et al.* 2011, Yusop *et al.* 2011).

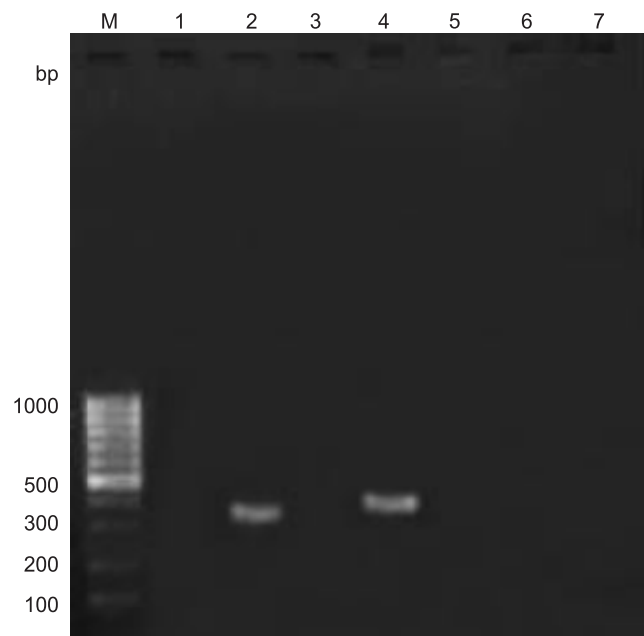


Fig. 1. PCR amplification pattern of mitochondrial 12S rRNA gene with different meat species. Lane M: 100 bp ladder; lane 1: cattle; lane 2: buffalo; lane 3: pig; lane 4: sheep; lane 5: goat; lane 6: chicken; lane 7: negative control.

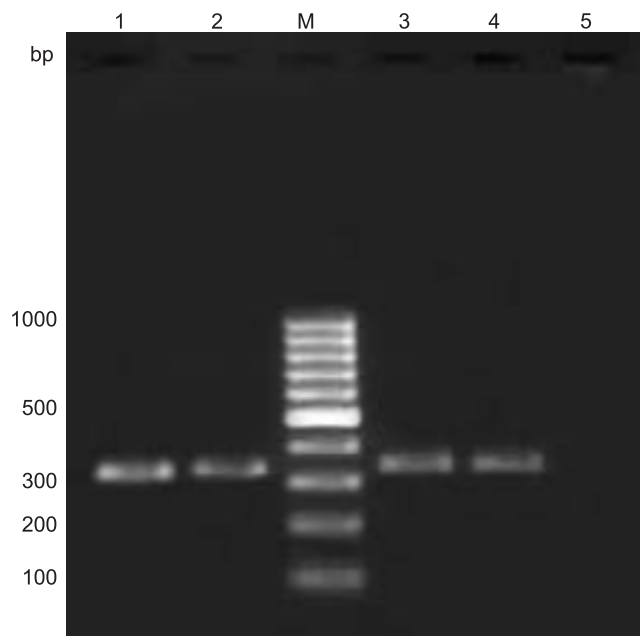


Fig. 2. PCR amplification pattern of mitochondrial 12S rRNA gene in admixed heat treated meat and meat emulsion (at the level 1% adulteration of targeted species meat). Lane M: 100 bp ladder; lane 1: cooked buffalo meat; lane 2: cooked buffalo meat emulsion; lane 3: cooked sheep meat; lane 4: cooked sheep meat emulsion; lane 5: negative control.

The optimized PCR assay using self-designed primer pair was subsequently evaluated successfully for its efficiency to amplify the DNA extracted from the admixed heat treated meat and meat emulsion (Fig. 2). No adverse effect of processing conditions, heat treatment and ingredient used for emulsion preparation was found on PCR amplification. High heat stability and large number of copies of mitochondrial DNA in meat tissue increases the chances of their survival, even in heat processed meat and meat products (Girish *et al.* 2005, Arslan *et al.* 2006, Mane *et al.* 2009). Other workers also reported the effectiveness of PCR assay based on mitochondrial DNA for rapid, robust, sensitive, precise and authentic identification of meat species, even in the heat treated admixed meat and meat products without any adverse effect (Arslan *et al.* 2006, Kesmen *et al.* 2007, Mane *et al.* 2009, Mane *et al.* 2012). It can be concluded from the present study that the developed PCR assay was effective for differentiation of buffalo meat from beef and mutton from chevon in admixed meat and meat products by single PCR reaction using single primer pair. The developed assay can be useful for fast conformational tool in the hands of regulating authority for routine control of authenticity of meat and meat products to protect the consumer from adulteration.

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