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ORIGINAL ARTICLE

Pork Specific Polymerase Chain Reaction Assay for Authentication of Meat and Meat Products

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Abstract

Pork specific polymerase chain reaction assay was developed for authentication of meat and meat products. The primer pair was designed based on the D-loop region of mitochondrial gene. The designed pork specific primer pair was able to amplify the desired 276 bp DNA fragments from meat and meat products. The desired DNA fragments amplified with pork specific primer pair was further confirmed by restriction enzyme digestion with *Hha*I and *Sau*3AI. No adverse effect of heat treatments, ingredients used and processing conditions was observed on PCR amplification. The level of detection of pork was found to be less than 1 percent in admixed meat products having five non-targeted meat species viz. beef, buffalo meat, chevon, mutton and chicken. The experiment was repeated several times and results were found to be repeatable.

Keywords: Pork, authentication, mitochondrial gene, PCR assay, admixed meat products.

Introduction

Rapid urbanization and industrialization have led to growth of ready to eat food products including meat and meat products. These changes also increase the chances of adulteration with meat of inferior and *taboo* species in meat and meat products (Mane *et al.*, 2006). The fraudulent adulteration by unscrupulous meat trader or manufacturer will affect the future prospects of meat industry due to related consumers' religious sentiments, economic and health implication (Girish *et al.*, 2005; Mane *et al.*, 2007; Mane *et al.*, 2009).

Nowadays, the PCR assays has been employed for the identification of species origin of meat and meat products targeting genomic and mitochondrial DNA (Girish *et al.*, 2005; Arslan *et al.*, 2006; Mane *et al.*, 2012a; 2012b). The detection of species origin of meat employing species-specific primer pairs was employed for authentication of mammalian and poultry species (Meyer *et al.*, 1994; Arslan *et al.*, 2006; Mane *et al.*, 2009; 2012a; 2012b).

In most of the previous studies, PCR assays specific to meat species were employed targeting genomic DNA admixtures as a model. However, in the present study mitochondrial DNA was targeted for specific identification of pork in directly isolated DNA from heat processed admixed meat and meat products. Species-specific PCR assay was developed for quick and authentic identification of chicken, beef and buffalo meat, even in heat processed admixed meat products containing the non-targeted species earlier (Mane *et al.*, 2009; 2012a; 2012b). The aim of developing pork specific PCR assay in the present study was to create consumers' confidence in ready to eat meat product, which was lacking presently and subsequently avoids the future implications in the meat trade at national and international market. So that keeping the above facts in view, the present study was designed to develop simple, sensitive and cost effective applicable method for authentication of meat and meat products processed under different manufacturing conditions by self designed primer pair based on mitochondrial gene.

Materials and Methods

Samples collection and storage: The fresh meat samples of cattle (ox), buffalo, sheep, goat, pig and chicken were collected from the municipal/local slaughterhouses. After collection, samples were kept at – 20 ^{o}C till further processing. The blood samples were collected from specific breeds of species under investigation from different sources. 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 preserved at -20 ^{o}C till DNA isolations. The authentic DNA samples of certain breeds were also collected from different labs of this institute.

DNA extraction, evaluation of quality and purity: The DNeasy® Blood and Tissue Kit (Qiagen, USA) was used for extraction of genomic DNA from meat and blood as per the instructions given by manufacturer. The same kit was also used for extraction of DNA from processed and cooked meat and meat products. The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis using 0.8 % agarose. The purity of genomic DNA was checked by using spectrophotometer taking Absorbance 260-280.

Design of oligonucleotide primer pairs: In the present study, primer pairs was designed based on D-loop region of mitochondrial gene sequences of pig and their comparison with DNA sequences of the different species retrieved from the National Centre for Biotechnology Information (NCBI) GenBank. Specific primer pairs for pig was designed using primer designing soft-ware (DNA-STAR Inc., USA). The primer pairs designed were synthesized from Metabion International, Germany. The details of the primer pairs employed in the present study were presented below:

Forward Primer: 5' GCA AAC CAA AAC GCC AAG TAC T 3'

Reverse Primer: 5' GGT GGT GAT ATG CAT GTT GAC TG 3'

PCR amplification and electrophoresis of PCR amplified DNA fragments: The reaction mixture was prepared in a 500 µl PCR tube (AXYGEN, USA) in a total volume of 50 µL containing 5 µL of 10 X PCR buffer, 200 µM each of dNTP, 1-2 Units of Taq DNA polymerase (Qiagen, USA), 10-20 pmol each of forward and reverse primer, 1 µL of DNA template (20-30 ng) and remaining nuclease free water (Fermentas, USA). The PCR conditions programmed on master cycler gradient thermocycler (Eppendorf, Germany) were as follows: initial denaturation at 94 ^{o}C for 2 min followed by 35 cycles of denaturation at 94 ^{o}C for 0.5 min, annealing at 60 ^{o}C for 0.5 min and extension at 72 ^{o}C for 1 min. Then final extension was done at 72 ^{o}C for 5 min. The PCR product was kept at -20 ^{o}C for further use.

The submarine horizontal agarose gel electrophoresis was used for analysis of PCR products. Two percent agarose was used for preparation of gel. For that 0.4 g of agarose (Ambion, USA) was put in 20 mL of 1X TBE solution (Fermentas, USA) 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 80 V. The PCR product was finally analyzed using UV transilluminator and documented by gel documentation system (Alpha Imager, USA). The ready to use 100 bp ladders (Fermentas, USA) was used for present study.

Restriction enzyme (RE) digestion of PCR amplified DNA fragments: The DNA fragments amplified by the PCR assay were further confirmed by digestion of PCR products with restriction enzymes. The restriction enzymes were selected for the amplified desired DNA fragments based on available gene sequences of D-loop regions in NCBI database using software (DNA-STAR Inc., USA). The *Hha*I and *Sau*3AI restriction enzyme were used for the confirmation of DNA fragments obtained by PCR assay of pork DNA. For RE digestion first, the PCR products were purified by PCR purification kit (Qiagen, USA) and then restriction digestion reaction was assembled by adding the given reagents in following order: Nuclease free water, PCR product (10-20 µl), 10X enzyme buffer (EB) and restriction enzyme. The reaction mix was incubated overnight at 37 ^{o}C in water bath. The

digestion reaction was stopped by adding 6X loading dye. The products were kept at -20 ^{o}C till electrophoresis. The digested products were subjected to electrophoresis in 2.5 % agarose gel along with 100 bp ladder (Fermentas, USA). Finally, after electrophoresis, the gel was observed for desired band pattern and documented by gel documentation system.

Specificity and sensitivity of PCR assay: The specificity of PCR assay was tested with DNA of other meat species used in the present study, while the sensitivity of the assay was tested in admixed meat and meat products containing less than 1 % pork in meat and meat products under different heat treatments. The non-targeted species in the admixed meat and meat products were buffalo meat, beef, chevon, mutton and chicken.

Effect of processing and cooking methods on PCR amplification: The Meat emulsion was prepared as outlined by (Mane et al., 2009) and three meat products were prepared for the study having different processing or cooking requirements. Different level of admixtures of pork with other species (equal proportion) used in the present study was formed with homogenous blending of meat emulsion carefully. Then the products were heat treated by employing following time-temperature combination i.e. dry heat in oven at 180 ^{o}C for 30 min to prepare the meat kabab and meat patty; steam cooking at 100 ^{o}C for 45 min to prepare the meat block and autoclaving at 121 ^{o}C, 15 psi for 15-20 min. After cooking products were stored at -20 ^{o}C for further use.

Results and Discussion

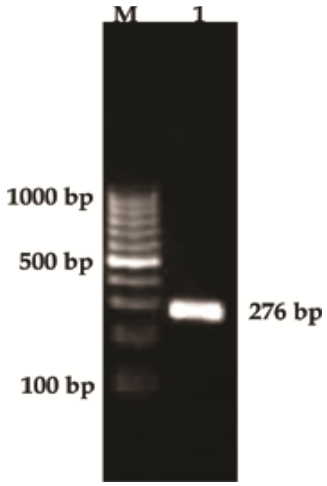
The basic aim of the study was to develop simple, sensitive and cost effective applicable method for detection of pork in meat and meat products processed under different manufacturing conditions by PCR assay using the self designed pork-specific primer pair. The mitochondrial DNA was targeted to design the pork specific primer pair due to their maternal inheritance, thus no sequence ambiguities will to be expected from the presence of more than one allele (Unseld *et al*., 1995). Further the variable regions of the mitochondrial gene have thousands of copies per cell (Greenwood and Paboo, 1999) as against single copy of genomic DNA. The above peculiarities along with very small mound size of mitochondria increases the probability of achieving a positive result even in the severely fragmented DNA due to intense processing conditions (Bellagamba *et al.*, 2001; Girish *et al*., 2005). Thus, making it ideal for identification of species origin of processed meat and meat products (Mane *et al.*, 2009; 2012a; 2012b). In the previous studies (Hopwood *et al.*, 1999; Rastogi *et al.*, 2007) reported that mitochondrial markers are more efficient than nuclear markers for the purpose of identification and authentication meat species.

Optimization of PCR assay for amplification of pork DNA: The PCR assay was successfully optimized for the amplification of 276 bp DNA fragments from D-loop mitochondrial gene. The self-designed primer pair specific for pig was employed for amplification of DNA extracted from pork without any non-specific amplification even after repeated testing (Fig. 1).

The designed pork-specific primer pairs based on D-loop region of mitochondrial gene was found to be highly precise and accurate for the identification of pork DNA. The purpose of employing species-specific primer pairs was to achieve the desired goal of high specificity for authentication of market meat products. In the earlier studies, various research workers reported that species-specific PCR assay authentically identified the species origin of meat and meat products (Meyer *et al.*, 1994; Arslan *et al*., 2006; Mane *et al.*, 2009; 2012a; 2012b).

Characterization of PCR amplified desired DNA fragments: The authenticity of species-specific PCR assay was confirmed by restriction analysis of PCR amplified desired DNA fragments with various restriction enzymes. In the present study, restriction enzymes were selected based on the available gene sequences in the NCBI database by restriction site analysis using MapDraw programme of lasergene software (DNA-STAR Inc, USA). Further amplified desired pork DNA fragments from D-loop region of mitochondrial gene was characterized by digestion with *Hha*I and

Fig. 1: Amplification of optimized pig specific DNA fragments (276 bp) by primer pair based on mitochondrial D-loop gene by PCR assay.



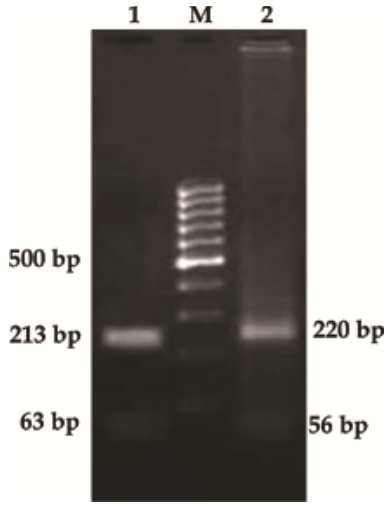
Legends: **Lane M**: 100 bp Ladder; **Lane 1**: Amplified 276 bp DNA fragments of pig Mitochondrial D-loop gene

and *Sau*3AI restriction enzyme (RE) having restriction site at 63 bp and 220 bp, respectively. The digestion of PCR amplified desired DNA fragments with these enzymes resulted in DNA fragment of 213 bp and 63 bp sizes with *Hha*I, and 220 bp and 56 bp sizes with *Sau*3AI (Fig. 2), respectively.

The RE digestion of PCR amplified DNA fragments is generally used for further confirmation of PCR assay. Earlier, various researchers used restriction endonucleases for further confirmation of PCR amplified desired DNA fragments from meat and meat products (Hopwood *et al.*, 1999; Rastogi *et al.*, 2007; Mane *et al.*, 2009; 2012a).

Testing specificity of PCR assay: The optimized pork specific PCR assay was cross checked for their specificity with DNA extracted from cattle, buffalo, sheep, goat, pig and chicken meat. After repeated testing, single DNA fragments of 276 bp was amplified from pork DNA without any cross reaction with other meat species employed in the present study (Fig. 3). The developed PCR assay was found to be very specific for the pork identification. In the cross testing with other meat species, amplification was only observed in the

Fig. 2: Pork DNA fragments amplified by optimized PCR assay digested with restriction enzyme *Hha*I and *Sau*3AI



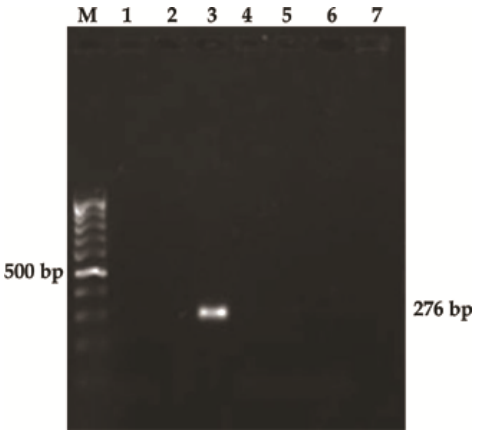
Legends: **Lane M**: 100 bp Ladder; **Lane 1**: DNA fragments of DNA amplified by pork specific primer pair after restrictive enzyme digestion with *Hha*I; **Lane 2**: DNA fragments of DNA amplified by pork specific primer pair after restrictive enzyme digestion with *Sau*3AI

pork. This indicates the importance of species specific PCR assay as a valuable tool for identification of meat species in presence of other meat species DNA (Hopwood *et al.*, 1999).

In the earlier study, the importance of species-specific PCR assay in detection of species origin of meat and meat products was tried and reported for pork (Calvo *et al.*, 2002; Ilhak and Arslan, 2007), buffalo meat (Mane *et al.*, 2012a), beef (Guoli *et al.*, 1999; Mane *et al.*, 2012b), mutton, chevon and chicken (Mane *et al.*, 2007, Ilhak and Arslan, 2007, Mane *et al.*, 2009). They reported that species-specific PCR assay was very sensitive and valuable for authentic identification of species origin of meat and meat products.

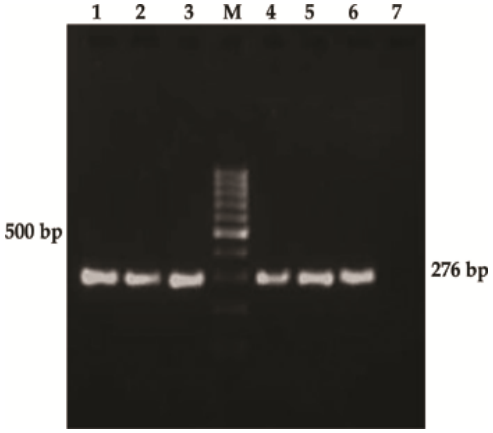
Testing of efficiency of PCR assay under different manufacturing conditions: The optimized PCR assay was further evaluated successfully for its efficiency to amplify the DNA extracted from fresh meat, cooked meat, autoclaved meat, raw meat emulsion, cooked meat emulsion and autoclaved meat emulsion. No adve-

Fig. 3: Specificity of pork specific primer pair with DNA of different meat species.



Legends: **Lane M**: 100 bp Ladder; **Lane 1**: Cattle; **Lane 2**: Buffalo; **Lane 3**: Pig; **Lane 4**: Goat; **Lane 5**: Sheep; **Lane 6**: Chicken; **Lane 7**: Negative Control

Fig. 4: Efficiency of pork specific PCR assay under different processing conditions



Legends: **Lane M**: 100 bp Ladder; **Lane 1**: Fresh meat; **Lane 2**: Cooked meat; **Lane 3**: Autoclaved meat; **Lane 4**: Raw meat emulsion; **Lane 5**: Cooked meat emulsion; **Lane 6**: Autoclaved meat emulsion; **Lane 7**: Negative Control

rse effect of heat treatments, processing conditions and ingredients used for emulsion preparation was observed on PCR amplification. Even in the meat emulsion autoclaved at 121 ^{o}C, 15 psi for 15-20 min after repeated testing has no adverse effects on PCR amplification (Fig. 4). This indicates that the developed pork specific primer pair was found to be very confirmative for the authentication of meat and meat products in all heating/processing conditions.

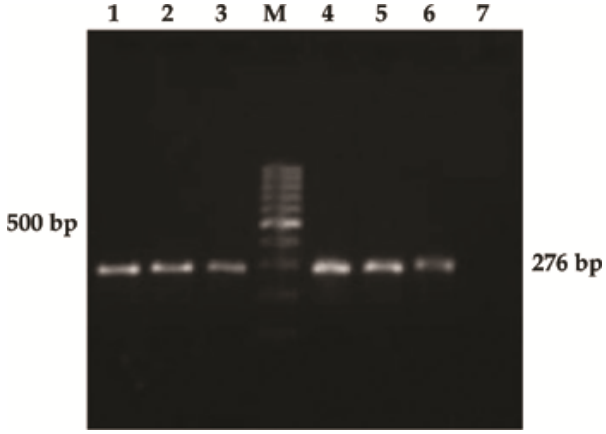
The high heat stability, small structure and large numbers of mitochondrial DNA copies in meat tissues contributing to the survival of sufficient number of DNA copies, even when subjected to extreme processing conditions of autoclaving (Girish *et al.*, 2005; Mane *et al.*, 2009; 2012a). However, a single desired sequence fragment is sufficient for PCR amplification. In the earlier studies, (Hird *et al.*, 2004; Rodriguez *et al.*, 2004; Arslan *et al.*, 2006; Kesmen *et al.*, 2007) successfully amplified desired DNA fragments from heat treated meat and meat products from mitochondrial gene without any adverse remarks, except in pan-fried meat.

Testing of efficiency and sensitivity of PCR assay in admixed minced meat and meat products: The PCR assay was tested for its sensitivity to detect the level of pork in admixed minced meat and meat emulsion under different processing/manufacturing conditions. It was found that the developed PCR assay able to detect less than 1 % of pork adulteration, even in the autoclaved meat emulsion (Fig. 5).

The developed PCR assay was also successfully employed for detection of pork in admixed emulsion based meat products viz patty, kabab and block containing less than 1 % level of pork in presence of meat from non-targeted (buffalo, cattle, sheep, goat and chicken) (Fig. 6). The results of the present study, clearly showed that species-specific PCR assay was highly sensitive and specific for identification of very low percentage of pork adulteration, even in admixed meat products subjected for different processing conditions. Previously in the similar work (Calvo *et al.*, 2002) successfully developed swine-specific primers for detection of pork in wide range of meat and meat products.

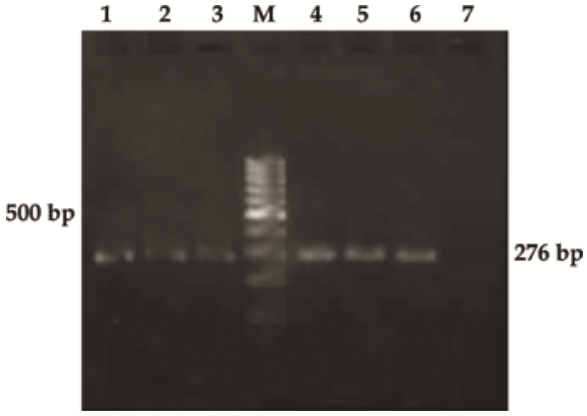
However, pork in admixed meat products was successfully targeted in the present study. In recent study, (Mane *et al.*, 2009; 2012a; 2012b) able to detect the less than 1 % level of chicken, beef and buffalo meat adulteration in admixed meat emulsion and emulsion based heat treated meat products under different manufacturing conditions.

Fig. 5: Sensitivity of pork specific PCR assay under different manufacturing conditions at 1% level of adulteration.



Legends: **Lane M**: 100 bp Ladder; **Lane 1**: Fresh meat; **Lane 2**: Cooked meat; **Lane 3**: Autoclaved meat; **Lane 4**: Raw meat emulsion; **Lane 5**: Cooked meat emulsion; **Lane 6**: Autoclaved meat emulsion; **Lane 7**: Negative Control

Fig. 6: Application of pork specific PCR assay for 5% and 1% level of adulteration in admixed meat products



Legends: **Lane M**: 100 bp Ladder; **Lane 1& 4**: Meat kabab; **Lane 2 & 5**: Meat patty; **Lane 3 & 6**: Meat block; **Lane 7**: Negative Control

(Note: Lane-1, 2 and 3 contains 1% and 4, 5 and 6 contains 5% level of pork in meat products).

Conclusion

The developed pork-specific PCR assay was found to be precise, sensitive and rapid method for routine analysis of admixed meat and meat products under different processing conditions. Thus, it can be concluded that the species-specific PCR assay is very simple and useful tool for routine assessment of authenticity of meat and meat products to protect the consumers from fraudulent practices of meat adulteration/substitution.

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