Indian Journal of Animal Sciences **91** (1): 15–19, January 2021/Article
https://doi.org/10.56093/ijans.v91i1.113219

PCR based method for authentication of pork in raw and processed products as well as in binary meat mixtures

R THOMAS[1]✉, M SAIKIA[1], S SINGHA[1], Z BARUAH[1], R KALITA[1], N SAHARIA[1] and S RAJKHOWA[1]

Food Quality Control Laboratory, ICAR–National Research Centre on Pig, Rani, Guwahati, Assam 781 131 India

Received: 24 June 2020; Accepted: 30 March 2021

ABSTRACT

In this study, amplification of species-specific marker of mitochondrial DNA origin was carried out to detect pork in raw, processed and meat mixtures containing varying concentrations of pork, viz. 1, 10, 50, 75 and 100%. The species-specific marker for pork was tested in raw pork from different breeds/varieties of pig such as Hampshire, Yorkshire, Ghungroo, Duroc, Rani, and Asha. The size of the amplified product was 290 bp in all the breeds/varieties. The results were consistent in processed pork products, viz. frankfurter sausage, salami, cocktail sausage, pork slice, ham, and pork curry which were subjected to different cooking temperatures ranging from 75 to 121°C. In case of all the mixtures with different concentrations of pork, similar results were observed. Subsequently, this marker was tested for cross-amplification by checking them with beef, carabeef, mutton, chevon, chicken, and duck meat and no amplification was observed. The results suggested that the DNA marker used in this study is highly species-specific and reliable to detect pork adulteration, unambiguously, in raw, processed as well as in meat mixtures containing pork. This technique is rapid, simple and economical as compared to other methods of pork adulteration detection.

Keywords: Binary meat mixture, Meat authentication, Polymerase chain reaction, Processed pork product, Raw pork

Authenticity assessment of meat has become an essential component in food quality control procedures because of the challenging problem of meat adulteration and possible health hazards. The adulterated meat often enters the supply chain and risks the health as well as the sentiments of the people. Such circumstances demand solid detecting techniques to identify the species of meat and it is ideal to develop robust and state of the art techniques to detect such meat adulteration cost-effectively. Several analytical methods have been developed to detect pork adulteration in mixed meat samples and/or identify meat from other animal species as an adulterant. DNA based methods have been widely used by the food technologists and meat scientist in identifying adulteration in meat and meat products. Large numbers of nuclear DNA based procedures are available to identify the origin of meat species such as DNA hybridization (Chikuni *et al.*1990), cloth-based hybridization array system (Murphy *et al.*2007), random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) fingerprints (Calvo *et al.* 2001), species-specific satellite DNA probes (Buntjer *et al.*1998) etc. PCR amplification and sequencing of Cytb (Forrest and Carnegie 1994) and 12S rRNA genes (Girish *et al.* 2004) of mitochondrial origin were also helpful for meat species identification. Girish *et al.* (2005) used restriction fragment length polymorphism (RFLP) of PCR products of Cytb and 12S rRNA genes of mitochondria for meat authentication. Reproducibility of RAPD-PCR is a matter of concern due to the requirement of high stringent conditions (Koh *et al.* 1998, Ilhak and Arslan 2007). However, the mitochondrial DNA markers are proved to be more valuable than nuclear markers in adulteration detection and species identification due to the facts that mitochondrial DNA is maternally inherited, large copy number of DNA having variable regions for PCR amplification is available and possibility to recover DNA even from severely damaged tissues (Rastogi *et al.* 2007).

The present study was carried out to detect pork in raw, processed pork products as well as in binary meat mixtures containing pork as adulterant by using species-specific 12S rRNA and tRNA Val genes of mitochondrial origin.

MATERIALS AND METHODS

Pork samples from different breeds/varieties of pig, viz. Hampshire, Yorkshire, Ghungroo, Duroc, Rani, and Asha were collected from the pork processing plant of ICAR-National Research Centre on Pig, Rani, Guwahati. Also, the processed pork products, viz. frankfurter sausage,

Present address: [1]ICAR-National Research Centre on Pig, Rani, Guwahati 781 131, Assam. ✉Corresponding author e-mail: thomasrlpt@gmail.com

salami, cocktail sausage, pork slice, ham, and pork curry subjected to different cooking temperatures ranging from 75 to 121°C were collected from the institute's pork processing plant. Frankfurter sausages, cocktail sausages and salami were subjected to heat treatment in hot water (80°C) with a holding period of 30 min, so as to have 75°C product core temperature while pork slice and ham were cooked in steam with a holding period of 45 min to have the product core temperature of 75°C. Pork curry was packaged in retort pouches and was cooked at a temperature of 121°C and 15 lb pressure. Samples of beef, carabeef, mutton, chevon, chicken, and duck meat were collected randomly from local retail meat shops. Binary meat mixtures were prepared by combining1%, 10%, 50%, 75% and 100% (w/w) pork with each meat samples, to a final weight of 100 mg (Table 1).

Table 1. Details of combinations used to prepare binary meat mixtures

Binary Meat mixtures	Pork (mg)	Other meat (mg)
Beef +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0
Carabeef +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0
Mutton +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0
Chevon +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0
Chicken +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0
Duck meat +		
1% pork	1	99
10% pork	10	90
50% pork	50	50
75% pork	75	25
100 % pork	100	0

Total DNA was extracted from raw, processed and meat mixtures of pork by phenol–chloroform–isoamyl alcohol method (Sambrook and Russell 2001). Briefly, the meat samples were mixed with lysis buffer containing 10 volume of 10 mM Tris–HCl (*p*H 8.0), 100 mM EDTA (*p*H 8.0), 0.5% SDS and 0.1 mg/mL of Proteinase K and incubated for 3 h at 50°C. The mixture was treated with 50 µg/mL of RNase A for 1 h at 37 °C. It was then extracted with an equal volume of phenol: chloroform: isoamyl alcohol (25 : 24 : 1 vol/vol) and again with an equal volume of chloroform. The DNA was precipitated by ethanol and 1M ammonium acetate solution. Finally, DNA was dissolved in TE buffer. The DNA was also extracted from the samples of beef, carabeef, mutton, chevon, chicken, and duck meat.

The quality of genomic DNA was checked by horizontal submarine agarose gel electrophoresis using 0.8% agarose strength. The quantity of total DNA extracted from various sources was estimated at 260 nm and the purity of DNA was checked by taking ratio of OD readings at 260 nm and 280 nm using UV-Visible spectrophotometer (Cary 60 UV-Vis, Agilent, USA).

Polymerase chain reaction (PCR) was performed in a thermal cycler (ProFlex Base, Applied Biosystems, USA). The species-specific primers used for PCR amplification were designed from different regions of mitochondrial 12S rRNA and tRNA Val genes, as described by Dalmasso *et al.*(2004). The sequences of the primers were PF: 5'-CTACATAAGAATATCCACCACA-3'and PR: 5'-ACATTGTGGGATCTTCTAGGT-3'. The primers were synthesized by Integrated DNA Technologies (IDT). PCR amplification was carried out in 0.2 mL PCR tubes containing 10 µL of PCR master mix, 1 µL (10 pmol) each of forward and reverse primers, 50 ng of DNA. The volume was made up to 25 µL with nuclease-free water. The PCR conditions used were an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 30s, annealing at 50°C for 1 min, elongation at 72°C for 1 min, final elongation at 72°C for 5 min, and hold at 4°C.

Analysis of PCR amplicons was performed using agarose gel electrophoresis. Agarose gel of 1.5% was prepared by dissolving the appropriate quantities of agarose in 1×TAE buffer (*p*H 8.0). Ethidium bromide stock solution was added directly to molten agarose at a concentration of 0.5 µg/ml before pouring the gel. The electrophoretic samples were mixed with 6× loading buffer before loading onto the gel. A 100 bp DNA ladder was run with the samples in agarose gel electrophoresis workstation (BioRad, USA). After electrophoresis, DNA fragments in the agarose gels were visualized and photographed using a Gel Documentation system (Gel Doc EZ, BioRad, USA).

RESULTS AND DISCUSSION

In the present study, the 12S rRNA and tRNA Val genes of mitochondrial genome was utilized for identification of pork which was very specific. Species-specific 12S rRNA-tRNA Val genes of mitochondrial origin were initially tested with DNA extracted from raw pork and it successfully

amplified unique fragments for pork. The size of the amplified product was 290 bp. The mitochondrial 12S rRNA-tRNA Val genes are conserved and hence it is possible to select specific sequences for a particular species (Dalmasso *et al.* 2004). Besides, mitochondrial DNA is maternally inherited because of which only one allele exists in an individual and thus sequence ambiguities are not expected from the presence of more than one allele (Unseld *et al.* 1995). This marker was subsequently tested in different breeds/varieties of pig such as Hampshire, Yorkshire, Ghungroo, Duroc, Rani, and Asha. The results were consistent across different varieties and breeds (Fig. 1). The pork specific primers from 12S rRNA and tRNA Val genes of the mitochondrial genome used in this study were conserved in all the breeds/varieties of pigs such as Hampshire, Yorkshire, Ghungroo, Duroc, Rani, and Asha.

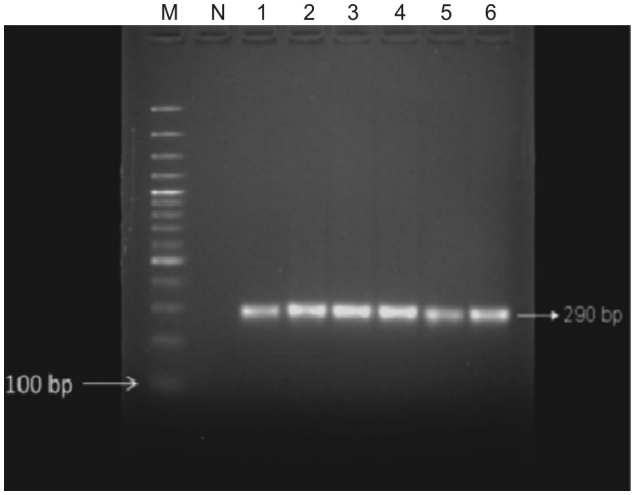

Fig. 1. PCR amplification of DNA from different breeds/ varieties of pig with the species-specific marker. The lanes are M, 100 bp DNA ladder; N, Non-template control; 1, Hampshire; 2, Yorkshire; 3, Ghungroo; 4, Duroc; 5, Rani; and 6, Asha.

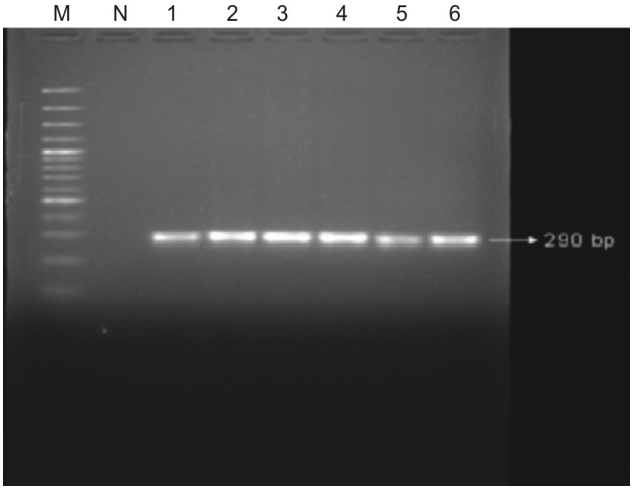

Fig. 2. PCR amplification with the species-specific marker for pork in processed pork products, which were subjected to heat treatments from 75 to 121ºC. The lanes are M, 100 bp DNA ladder; N, Non-template control; 1, Frankfurter sausage; 2, Salami; 3, Cocktail sausage; 4, Pork slice; 5, Ham; and 6, Pork curry.

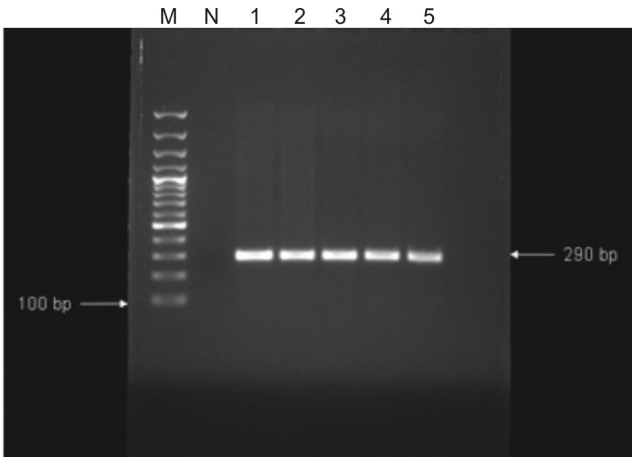

Fig. 3. PCR amplification with species-specific marker for pork in binary mixture containing different concentration of pork. The lanes are M, 100 bp DNA ladder; N, Non template control; 1, 100% pork; 2, 75% pork; 3, 50% pork; 4, 10% pork; 5, 1% pork.

Therefore, it was possible to identify pork irrespective of breeds/varieties.

PCR amplification was done with the DNA extracted from different processed pork products, viz. frankfurter sausage, salami, cocktail sausage, pork slice, ham, and pork curry which were subjected to different cooking temperatures ranging from 75 to 121°C. This marker could successfully amplified from all the processed samples and the size of the amplified product was the same as in raw pork, i.e. 290 bp (Fig. 2). Further, differences in size were not observed in amplicons in any of the binary meat mixtures with different concentrations of pork, viz. 1%, 10%, 50%, 75% and 100% (Fig. 3). Even though, these products were cooked in different temperatures, ranging from 75°C to 121°C, the marker was stable at all these temperature ranges and did amplify in the analysis. Further, processing of pork curry at 121°C temperature and 15 lb pressure also did not affect the PCR amplification of pork specific marker. Therefore, the results indicated successful amplification of the desired DNA segments using DNA extracted from fresh and processed pork samples, confirming the ability of the PCR protocol to amplify the species-specific marker of mitochondrial origin.

This marker was further validated by checking it for cross-amplification in other meat species such as beef, carabeef, mutton, chevon, chicken, and duck meat. It could be seen that the pork primer set amplified successfully the 290 bp mitochondrial DNA fragment from pork, whereas no amplification products were obtained with DNA from beef, carabeef, mutton, chevon, chicken, and duck meat samples (Fig. 4). It was observed that the marker used in this study was highly unique to pork. Moreover, this marker could also detect pork in binary meat mixtures of beef, carabeef, mutton, chevon, chicken, and duck meat, containing 1–100% pork.

The results established the reliability and sensitivity of the technique for detecting pork at different concentrations.

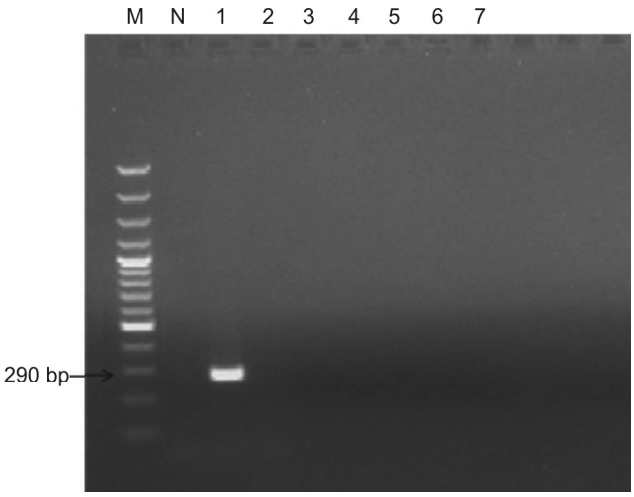

Fig. 4. PCR amplification of species-specific marker from pork and cross-checking with other meat. The lanes are M, 100 bp DNA ladder; N, Non-template control; 1, Pork; 2, Beef; 3, Carabeef; 4, Mutton; 5, Chevon; 6, Chicken; and 7, Duck meat.

Since intentional adulteration for economic profits is generally more than 10%, this technique could be a very useful tool for identification of economically driven meat adulteration. The marker used in this study showed no cross-reactivity with the non-target species and confirmed the absence of any cross-amplification indicating its' specificity. The findings corroborate with the fact that the heat stability and large copy number of mitochondrial DNA in meat tissue contribute to the protection and survivability of the DNA fragments that are sufficient to be amplified by PCR (Girish *et al.* 2004). About 800–1000 of mitochondria are present in an animal cell and each mitochondrion has 6–8 copies of the mitochondrial genome (Gardner and Snustad, 1984). Hence, chances of survival of copies of the mitochondrial genome in a very small amount of sample as well as during extreme conditions of processing and storage are very high (Haunshi *et al.* 2009). Further, it was proved that mitochondrial markers were more efficient than nuclear markers in species identification and authentication purposes (Hopwood *et al.* 1999, Rastogi *et al.* 2007). Therefore, these characteristics make 12S rRNA-tRNA Val genes of mitochondrial origin ideal for identification of species origin of fresh, processed meat and meat products.

Girish *et al.* (2004) developed a method based on forensically important nucleotide sequencing (FINS) which involved PCR amplification of mitochondrial 12S rRNA gene followed by sequencing of amplicon for the identification of pork. The mitochondrial 16S rRNA gene sequence analysis was used for identification of cattle, buffalo, sheep, goat, and pig species in fresh and processed meat (Mane *et al.* 2013). However, these methods involve the sequencing of nucleotides which is time-consuming and require additional cost. PCR-RFLP technique was carried out for identification of different meat species which involved PCR amplification of mitochondrial 12S rRNA gene followed by restriction digestion with restriction enzyme targeting species-specific restriction site (Girish *et al.* 2005, Teixeira *et al.* 2007). Species-specific PCR saves time as it does not involve any post amplification processing like restriction digestion in PCR-RFLP methods. Haunshi *et al.* (2009) used PCR based method for meat species identification by developing species-specific mitochondrial D-loop markers which successfully identified the species of fresh, cooked and autoclaved meat. This indicated the advantages of species-specific PCR over other techniques. Moreover, the result of species-specific PCR was highly reproducible unlike that of Randomly Amplified Polymorphic DNA (RAPD)-PCR (Koh *et al.* 1998).

In conclusion, the PCR amplification of mitochondrial 12S rRNA-tRNA Val genes proved to be a very efficient method for the identification of pork. The marker used in this study could be useful in identifying pork in raw, cooked/processed, as well as in mixed meat samples and the process of detection is simple, economical, and quick as compared to any other method such as RAPD-PCR, hybridization by species-specific probes, sequencing analysis of nuclear or mitochondrial genes, or PCR-RFLP method of species identification. This assay represents a rapid, straightforward, and cost-effective method for the detection of pork adulteration in meat and meat products in a high throughput manner.

ACKNOWLEDGEMENTS

The research work mentioned in this article was carried out under the ICAR-LBS Young Scientist Award project on 'Farm-to-Fork risk profiling of hazards associated with pork supply chain in India' and the World Bank funded APART project. The authors wish to thank Indian Council of Agricultural Research and APART for the financial assistance to undertake this project.

REFERENCES

Arvanitoyannis I S and Van Houwelingen-Koukaliaroglou M. 2003. Implementation of chemometrics for quality control and authentication of meat and meat products. *Critical Reviews in Food Science and Nutrition* **43**: 173–218.

Buntjer J B, Nijman I J, Zijlstra C and Lenstra J A. 1998. A satellite DNA element specific for roe deer. *Chromosoma* **107**: 1–5.

Calvo J H, Zaragoza P and Osta R. 2001. Random amplified polymorphic DNA fingerprints for identification of species in poultry pate. *Poultry Science* **80**: 522–24.

Chikuni K, Ozustume K, Hoishi Kawa T and Kato S. 1990. Species identification of cooked meats by DNA hybridization assay. *Meat Science* **27**: 199–208.

Dalmasso A E, Fontanella P, Piatti T, Civera S, Rosati K and Bottero M T. 2004. A multiplex PCR assay for the identification of animal species in feedstuffs. *Molecular and Cellular Probes* **18**: 81–87.

Forrest A R R and Carnegie P R. 1994. Identification of gourmet using FINS (forensically informative nucleotide sequencing). *Biotechniques* **17**: 24–26.

Gardner E J and Snustad D P. 1984. *Principles of genetics* (*7th edition*). John Wiley and Sons: New York.

Girish P S, Anjaneyulu A S R, Biswas K N, Anand M, Rajkumar

N, Shivakumar B M and Sarma B. 2004. Sequence analysis of mitochondrial 12S rRNA gene can identify meat species. *Meat Science* **66**: 551–56.

Girish P S, Anjaneyulu A S R, Biswas K N, Shivakumar B M, Anand M, Patel M and Sarma B. 2005. Meat species identification by polymerase chain reaction–restriction fragment length polymorphism of mitochondrial 12S rRNA gene. *Meat Science* **70**: 107–12.

Haunshi S, Basumatary R, Girish P S, Doley S, Bardoloi R K and Kumar A. 2009. Identification of chicken, duck, pigeon and pig meat by species-specific markers of mitochondrial origin. *Meat Science* **83**: 454–59.

Hopwood A J, Fairbrother K S, Lockley A K and Bardsley R G. 1999. An actin gene-related polymerase chain reaction test for identification of chicken in meat mixtures. *Meat Science* **53**: 227–31.

Ilhak O I and Arslan A. 2007. Identification of meat species by polymerase chain reaction (PCR) technique. *Turkish Journal of Veterinary and Animal Science* **31**: 159–63.

Koh M C, Lim C H, Chua S B, Chew S T and Phang S T W. 1998. Random amplified polymorphic DNA (RAPD) fingerprints for identification of red meat animal species. *Meat Science* **48**: 275–85.

Mane B G, Mendiratta S K, Tiwari A K and Narayan R. 2013. Sequence analysis of mitochondrial *16S rRNA* gene to identify meat species. *Journal of Applied Animal Research* **41**: 77–81.

Murphy J, Armour J and Blais B W. 2007. Research note: Cloth-based hybridization array system for expanded identification of the animal species origin of derived materials in feeds. *Journal of Food Protection* **70**: 2900–05.

Rastogi G R, Dharne M S, Walujkar S, Kumar A, Patole M S and Shouche Y S. 2007. Species identification and authentication of tissues of animal origin using mitochondrial and nuclear markers. *Meat Science* **76**: 666–74.

Sambrook J and Russell D W. 2001. *Molecular Cloning – A Laboratory Manual* (*3rd edition*), pp. 32–68. New York: Cold Spring Harbour Laboratory Press.

Teixeira L V, Teixeira C S, Bastianetto E and Oliveira D A A. 2007. A buffalo meat products certification by DNA test. *Italian Journal of Animal Science* **6**: 1207–09.

Unseld M, Beyermann B, Brandt P and Hiesel R. 1995. Identification of the species origin of highly processed meat products by mitochondrial DNA sequences. *PCR Methods and Applications* **4**: 241–43.