Haryana Vet. **48** (December, 2009), pp 53-57

Research Article

CYTOCHROME- b GENE BASED PCR FOR IDENTIFICATION AND DIFFERENTIATION OF COOKED MEAT OF SHEEP, GOAT, CATTLE, PIG AND POULTRY

SATISH, ASHWANI KUMAR[1], Y. SINGH, MINAKSHI[2] and G. PRASAD[2]
Department of Veterinary Public Health and Epidemiology
College of Veterinary Sciences, CCS Haryana Agricultural University, Hisar-125 004

ABSTRACT

Species identification of cooked meat or the food products having cooked meat is warranted on several occasions. Cytochrome-b gene based PCR was, therefore, standardized to identify and differentiate the cooked meat of sheep, goat, cattle, pig and chicken. The PCR products from cooked meat samples of these animal species produced DNA fragments of 331, 157, 274, 398 and 227 base pairs respectively and clearly differentiated the species origin of meat. DNA fragments of 331 and 157 base pairs from cooked meat samples of sheep and goats respectively demonstrated clear distinction of phylogenetically closely related species. Specific differentiation of cooked meats of cattle and pig producing DNA fragments of 274 and 398 base pairs, respectively has socio-religious significance. Detection of 20% adulteration of meat in ready to eat meat dish (cooked) may be of use to demonstrate fraudulent practice of meat adulteration.

Key words: Cytochrome-b, PCR, cooked meat, DNA fragments

Identification and differentiation of species origin of meat has enormous significance from economical, sociopolitical and religious points of view. Identification of meat is relatively simple when carcass is intact but becomes difficult if it has been heat processed due to considerable changes in the soluble nature and antigenic determinants of the muscle proteins. Immunological and DNA based methods are suitable approaches employed for identification of such meats. (Chen and Peggy, 2000, Macedo-Silva *et al.*, 2000, Arslan *et al.*, 2006, Kesmen *et al.*, 2009). However, the later approach has advantages because of thermostabilty of DNA and less disruption of nucleic acids during processing. Polymerase chain reaction (PCR) was therefore standardized for identification and differentiation of cooked meat of sheep, goat, cattle, pig and chicken by amplifying conserved fragment of mitochondrial cytochrome-b gene.

MATERIALS AND METHODS

Meat samples: Six raw meat samples each of sheep, goat, pig and chicken from retail meat shops and that of cattle and buffalo from the postmortem cases in the Department of Veterinary Pathology, College of Veterinary Sciences, CCS Haryana Agricultural University, Hisar, were cooked in microwave oven at 120°C for 30 min., wrapped in aluminium foil aseptically and stored at -20°C until further use. Five cooked mixed samples each of cattle and pig (20:80), cattle and sheep (20:80), cattle and goat (20:80), sheep and goat (20:80), chicken and goat (20:80) as adulterated cooked meat samples and 12 unknown samples of cooked meat were also included for identification of species origin of meat.

DNA extraction from meat: DNA from the meat sample was extracted as per the standard protocol described by Sambrook *et al.* (1989). Five hundred mg meat free from adipose tissue was grounded using liquid nitrogen and to this homogenate, 1ml of lysis buffer (mixture of 20 mg/ml Proteinase–K, 300 µl of 10% SDS) was added followed by incubation for 16 h at 55°C. Equal volume of Tris saturated phenol (pH–8.0) was then added and centrifuged at 10,000 rpm at 15°C for 10 min. To the supernatant so collected, half the volume of each Tris-saturated phenol and chloroform: isoamyl alcohol (25:24:1) was mixed and centrifuged and to the supernatant 1/10th volume of 3M-sodium

[1]Corresponding author
[2]Dept. of Animal Biotechnology

acetate (pH–5.5) and equal volume of isopropyl alcohol were added to precipitate DNA which was washed twice with 70% ethanol, dried for 2-3 h at room temperature, dissolved in 100-300 µl volume of TE buffer, kept in water bath at 55-65°C for 45-60 min. and stored at -20°C until used.

RNA contamination in extracted DNA was removed by adding 1 µl of 10 mg/ml RNase-A followed by incubation at 37°C for 1 h. The purity and quantitation of DNA were checked by agarose gel electrophoresis and UV-spectrophotometer respectively.

Multiplex Polymerase Chain Reaction Assay (PCR assay) was performed as per procedure of Matsunaga *et al.* (1999). The reagents used in PCR reaction were Taq DNA polymerase 5 units/µl, 10 X PCR buffer with magnesium chloride, dNTPs 10 mM. A set of primers (Table) specific to cytochrome-b gene family (Matsunaga *et al.*, 1999) was got synthesized by Sigma Aldrich Chemicals Pvt. Ltd, New Delhi, India. The primers supplied in freeze-dried form were dissolved and diluted in autoclaved nuclease free (NF) water to obtain a final concentration of 10 pmole/µl. For the multiplex PCR, primers were mixed in the ratio of 1:0.2:3:0.6:0.6:3:0.6 for SIM: goat: chicken: cattle: cattle: sheep: pig and used together. Cattle primer was used to identify and differentiate meats of both cattle and buffalo.

PCR reaction: PCR was carried out in a final reaction volume of 25 µl. A master mix was prepared and aliquated 24.75 µl in each PCR tube. DNA sample (0.25 µl) was added in each tube to make the final volume. Each PCR reaction mixture contained 2.50 µl of 10 X PCR buffer, 1.5 µl $MgCl_2$ (25 mM), 0.50 µl dNTPs (10Mm each), 0.50 µl primer mix (10 pmole/ µl), 0.25 µl Taq DNA polymerase (5 U/µl), 0.25 µl DNA template and 19.25 µl DNase free water. Amplification was carried out at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds and repeated these steps for 35 cycles.

The products of PCR amplification were put to electrophoresis on 3% w/v agarose gel containing 1% ethidium bromide solution @ 5µl/100 ml at constant voltage of 80 V for 30 min in 10X TAE. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

RESULTS AND DISCUSSION

Species identification of cooked meat or the food products having cooked meat is warranted on several occasions and PCR based methods have successfully been used to identify such meats and meat products (Martinez and Man, 1998, Meyers *et al.*, 2003) and fish varieties (Sanjuan and Comesana, 2002). In this study, mitochondrial cytochrome-b gene based PCR was chosen because the mitochondrial genome is easy to isolate from the nuclear genome, present in high number of copies, smaller size and rapid accumulation of mutations (Moritz *et al.*, 1987, Wilson *et al.*,1995) and the post PCR analysis is much simpler.

DNA yield from the cooked meat samples ranged from 20 µg to 100 µg/500 mg, which was much lower than that of raw meats (100 µg to 500 µg/500 mg) of same species. This may be due to processing technology (cooking, smoking, drying, salting) applied during the manufacture of meat products that may affect to different extents to the integrity of the extractable DNA and results its degradation into small size fragments (Dias Neto *et al.*, 1994, Martinez and Man,

Table

Sequences of SIM and species specific primers

Name	Primer	Sequences (5' – 3')	No. of bases
SIM	Forward	GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA	38
Goat	Reverse	CTCGACAAATGTGAGTTACAGAGGGA	26
Chicken	Reverse	AAGATACAGATGAAGAAGAATGAGGCG	27
Cattle	Reverse	CTAGAAAAGTGTAAGACCCGTAATATAAG	29
Sheep	Reverse	CTATGAATGCTGTGGCTATTGTCGCA	26
Pig	Reverse	GCTGATAGTAGATTTGTGATGACCGTA	27

1998). All the DNA samples were found free of proteins and RNA. The samples having OD ratio at 260/280 between ~ 1.7 to 1.9 and not showing smearing on agarose gel electrophoresis were included for further reactions. DNA from mixed and unknown meat samples were extracted similarly.

Universal primers have been designed to amplify the conserved region of cytochrome-b gene in more than 100 species, including mammals, birds, amphibians, fishes and some invertebrates (Anderson *et al.*, 1982, Irwin *et al.*, 1991, Desjardins and Morais, 1991, Matsunaga *et al.*, 1999). The oligonucleotide primers designed by Matsunaga *et al.* (1999) were got synthesized for this study (Table).

Multiplex PCR was used to identify and differentiate the species of cooked meat samples as employed earlier (Dias Neto *et al.*, 1994, Fei *et al.*, 1996, Koh *et al.*, 1998, Martinez and Man, 1998, Matsunaga *et al.*, 1999, Calvo *et al.*, 2001, Rodriguez *et al.*, 2004). Initially, independent primers were run using a pair of forward primer (common for all species) and species-specific reverse primers. Having confirmed species specificity of each primer independently, a multiplex PCR was standardized by mixing all primers in a single reaction but targeting DNA of single species. The primer concentrations when mixed in 1:0.2:3:0.6:0.6:3:0.6 for SIM: G: Ch: C: C: S: P and included in the multiplex PCR @ 0.50 µl giving 25 pmoles of reverse primer per 25 µl, PCR reaction gave species specific amplification except for cattle and buffalo which produced products of the same size. Obrovska *et al.* (2002) obtained single band of meat of chicken, cattle, pig and horse species by mixing the primers in the ratio of 0.5: 1.5: 0.3: 0.3: 1.0 for SIM: Ch: C: P: H for multiplex PCR while primer concentrations used in this study were the same as reported by Matsunaga *et al.* (1999).

Electrophoretic band pattern of PCR products on 3.5-4% agarose gel resulted in clear cut differentiation of species as multiplex PCR amplified fragments specific to each species producing characteristic band pattern on agarose gel electrophoresis when run on single species and a multiple band pattern when run on DNA mix. The primers in the multiplex PCR amplified target sequences at the efficiency comparable to conventional PCR. PCR products from goat, chicken, cattle, buffalo, sheep and pig produced DNA fragment of 157, 227, 274, 274, 331 and 398 bp respectively (Fig 1). Amplified PCR products from six species thus ranged from 157 to 398 bp respectively. The primer pair specific to meat sample of cattle however, amplified meat sample of buffalo target sequence as well. Therefore, meat samples of cattle and buffalo amplified same size band (274bp). The findings of multiplex PCR on cooked meat samples confirmed the results obtained by Matsunaga *et al.* (1999). Yan *et al.* (2005) also identified 27 meat samples and 4 skin samples by multiplex PCR of the mitochondrial cytochrome-b gene.

DNA extracted from 500 mg of adulterated meat samples in ratio given above was used as a template for PCR. The amplification resulted in generation of species specific bands of adulterated meat in all the five mixed samples examined (Fig 2).

DNA extracted from all the unknown meat samples after PCR amplification resulted in the identification of species origin of meat. Out of 12 meat samples, 4 samples were identified as meat of single animal species (pig/sheep), 5 of two species (4 goat and sheep, 1 goat and cattle) and three samples were identified of mixed meat of three species (sheep, goat, cattle).

Fraudulent practices of adding cheaper meat into meat of higher cost is a common practice. The adulteration of meat of a species of animal into another

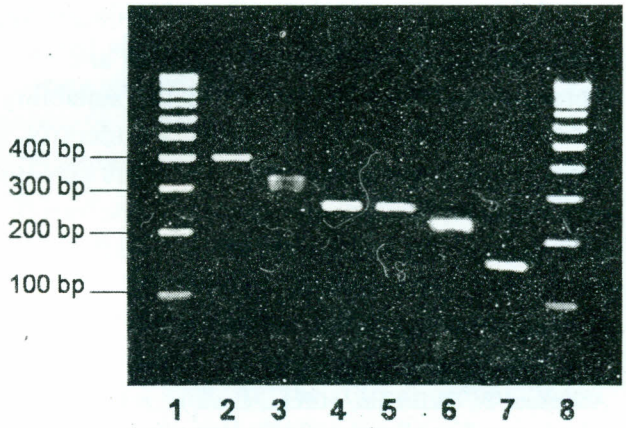

Fig 1. Agarose gel electrophoresis of PCR products amplified from cooked meat samples of pig (2), sheep (3), buffalo (4), cattle (5), chicken (6) and goat (7). 100 bp ladder (1 and 8).

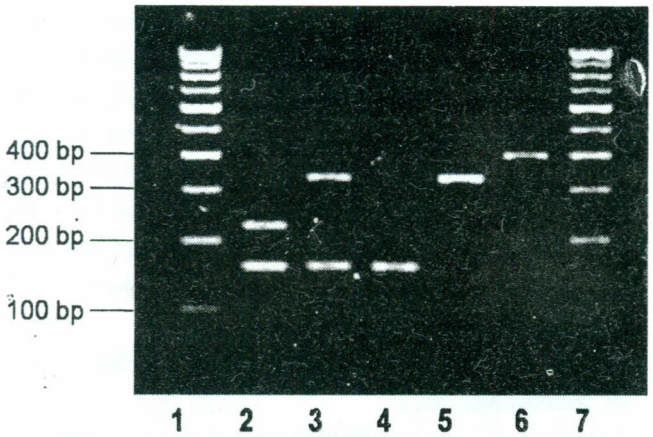

Fig 2. Agarose gel electrophoresis of PCR products amplified from binary mixtures (20:80) of cooked meat samples of chicken and goat (2), sheep and goat (3), cattle and goat (4), cattle and sheep (5), cattle and pig (6). 100 bp ladder (1 and 7)

was evaluated on binary mixtures comprising cattle and pig (20:80), cattle and sheep (20:80), cattle and goat (20:80), sheep and goat (20:80), chicken and goat (20:80). In the present study, species specific bands were obtained from all the five binary mixtures tested. Although it is possible to detect each species when spiked in any other species even at the level of 0.1% (Kesmen *et al.*, 2007), yet this study used 20% adulteration level because the percentage of adulteration is usually near to this level under fraudulent practices to make more profit. Rodriguez *et al.* (2001) demonstrated the clear identification of the presence of chicken in goose and duck at levels of 100, 50, 25, 10, 5, 1 and 0.1%.

With the PCR assay used in the present study, cooked meat of cattle, sheep, goat, pig and chicken were identified and demonstrated the suitability of cytochrome-b gene based PCR to identify and differentiate the meat. This technique offers a promising alternative for meat inspection programme intended to assess the species identification of meat after cooking.

REFERENCES

Anderson, S., de Bruijin, M.H.L., Coulson, A.R., Eperon, I.C., Snager, F. and Young, I. (1982). Complete sequence of cattle mitochondrial DNA. Complete features of the mammalian mitochondrial genome. *J. Mol. Bio.* **156**: 683-717.

Arslan, A., Irfan-Ilhak, O. and Calicioglu, M. (2006). Effect of method of cooking on identification of heat processed beef using polymerase chain reaction (PCR) technique. *Meat Sci.* **72**: 326-330.

Calvo, J.H., Zaragoza, P. and Osta, R. (2001). Technical note: A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. *J. Anim. Sci.* **8**: 2108-2112.

Chen, Fur Chi and Peggy, Hsieh. (2000). Detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. *J. AOAC Int.* **83**: 79-85.

Desjardins, P. and Morais, R. (1991). Sequence and gene organization of chicken mitochondrial genome: A novel gene order in higher vertebrates. *J. Mol. Biol.* **212**: 599-634.

Dias Neto, E., Caballero, O.L., Vidigal, T., Pena, S. and Simpson, A. (1994). Producing randomly amplified polymorphic DNAs from degraded DNA. In: Proceedings of Workshop of Genomic Fingerprinting. Instituto Juan March de Estudios e Investigaciones, Madrid, Spain **20**: 94-95.

Fei, S., Okayama, T., Yamanoue, M., Nishikawa, I., Mnnen, M. and Tsiuji, S. (1996). Species identificication of meat and meat products by PCR. *Anim. Sci. Techno. (Jpn.)* **67**: 900-905.

Irwin, D.M., Kocher, T.D. and Wilson, A.C. (1991). Evolution of the cytochrome-*b* gene of mammals. *J. Mol. Evo.* **32**: 128-144.

Kesmen, Z., Sahin, F. and Yetin, H. (2007). PCR assay for the identification of animal species in cooked sausages. *Meat Sci.* **77**: 649-653.

Kesmen, Z., Gulluce, A., Sahin, F. and Yetin, H. (2009). Identification of meat species by TaqMan-based real-time PCR assay. *Meat Sci.* **82**: 444-449.

Koh, M.C., Lim, C.H., Chua, S.B., Chew, S.T. and Phang, S.T.W. (1998). Random amplified DNA fingerprints for identification of red meat animal species. *Meat Sci.* **48**: 275-285.

Macedo-Silva, A., Barbosa, S.F.C., Alkmin, M.G.A., Vaz, A.J., Shimokomaki, M. and Tenuta-Filho, A. (2000). Hamburger meat identification by dot-ELISA. *Meat Sci.* **56**: 189-192.

Martinez, I. and Man, Y. (1998). Species identification in meat by- products by RAPD analysis. *Food Res. Int.* **31**: 459-466.

Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada J. and Shinmura, Y. (1999). A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Sci.* **51**: 143-148.

Meyers, M.J., Yancy, H.F. and Farrell, D.E. (2003). Characterization of a polymerase chain reaction-based approach for the simultaneous detection of multiple animal-derived materials in animal feed. *J. Food Prot.* **66**: 1085-1089.

Moritz, C., Dowling,T. and Brown, W.M. (1987). Evolution of animal mitochondrial DNA: relevance for population biology and systemics. *Annu. Rev. Ecol. Syst.* **18**: 269-292.

Obrovska, I., Steinhauserova, I. and Nebola, M. (2002). The application of the PCR method to the identification of meat species. *Folia Veterinaria* **46**: 113-118.

Rodriguez, M.A., Garcia, T., Gonzalez, I., Asensio, L., Fernandez,

A., Lobo, E, Hernandez., P.E. and Martin, R. (2001). Identification of goose (*Anser anser*) and mule duck (*Anasplatyrhynchos x Cairina moschata*) foie gras by multiplex polymerase chain reaction amplification of the 5S RDNA gene. *J. Agric. Food Chem.* **49**: 2717-2721.

Rodriguez, M.A., Garcia, T., Gonzalez, I., Asensio, L., Hernandez, P.E. and Martin, R. (2004). PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixtures. *J. Food Prot.* **67**: 172-177.

Sambrook, J., Frisch, E.F. and Maniatis, T. (1989). Molecular Cloning: A Molecular Approach Laboratory Manual. (2nd edn.), Cold Spring Harbour Laboratory Press, New York.

Sanjuan, A. and Comesana, A.S. (2002). Molecular identification of nine commercial flaf fish species by polymerase chain reaction-restriction fragment length polymorphism analysis of a segment of the cytochrome-*b* region. *J. Food. Prot.* **65**: 1016-1023.

Wilson, A.C., Cann, R.L., Carr, S.M., George, M., Gyllensten, J.I., Helm-Bychowski, K., Higuch, R.G., Palumbi, S.R., Prager, E.M., Sage, R.D. and Stoneking, M. (1995). Mitochondrial DNA in relation to evolutionary genetics. *Biol. J. Linn. Soc.* **26**: 375-400.

Yan, Peng, Wu, Xiao-Bing, Shi, Yan, Gu, Chang-Ming, Wang, Ren-Ping and Wang, Chao-Ling (2005). Identification of Chinese alligators (*Alligator sinensis*) meat by diagnostic PCR of the mitochondrial cytochrome-*b* gene. *Biological Conservation* **121**: 45-51.

CONTRIBUTORS MAY NOTE, PLEASE

- **Research/Clinical articles are invited for next issue from the Scientists/Veterinarians engaged in Veterinary profession.**
- **Please follow strictly the format of The Haryana Veterinarian for manuscript writing.**
- **Please send a D.D. of Rs. 100/- in favor of Dean, COVS, as processing fee along with each article.**
- **After revision of article, please return the referee's comments along with hard & soft copies of original & revised manuscripts at the earliest.**
- **Please do not forget to mention your article reference number in all correspondence for a quick response.**
- **We solicit your co-operation.**

Editors