

Duplex-PCR for Identification and Differentiation of Cattle and Buffalo Processed Meat

Rohita Gupta*, D.N. Rank, C.G. Joshi

College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand – 388001, India.

(Received 3 May 2011/ Accepted 5 May 2011)

Abstract

A simple and reliable duplex polymerase chain reaction (duplex-PCR) technique is proposed to identify and differentiate cattle and water buffalo DNA using primers that were tested on mitochondrial DNA (mtDNA) extracted from meat muscle samples. Different levels of autolysis were experimentally produced by putrefaction and heating the samples at various temperatures and conditions to simulate the various meat processing technology. The optimized PCR amplified 113 bp and 152 bp fragment of *cyt b* gene from mtDNA. This test was successful in detecting up to 1 pg adulteration in cattle-buffalo meat mixture. The test is a valuable tool for meat authentication and screening of cooked, putrefied and mixed samples of cattle and buffalo.

Keywords: Meat speciation; Polymerase chain reaction; Cattle; Buffalo, *cyt b*

Introduction

The determination of food authenticity and the detection of adulteration are major issues in the food industry, and attract an increasing amount of attention. Therefore, reliable techniques to identify the species of origin of components in a food product derived from animals are necessary for food authentication purpose. Identification of the species of origin in meat samples is relevant to consumers for the possible economic loss from fraudulent adulterations, medical requirements of individuals that might have specific allergies, and religious reasons (Miguel *et al.*, 2004).

The extensive development of nucleic acid based technologies over the past decade reflects their importance in food analysis. Various Polymerase chain reaction (PCR) based approaches were attempted in the past for meat authentication, but only a limited number of studies targeted buffalo as one of the species under study. In recent years, works have been developed that use PCR coupled with techniques such as hybridization, nucleotide-sequencing, single-strand conformation

polymorphism (SSCP), random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), or forensically informative nucleotide sequencing (FINS), for differentiation of water buffalo meat from cattle meat (Rajapaksha *et al.*, 2003, Rastogi *et al.*, 2004, Girish *et al.*, 2005, Chen *et al.*, 2008, Murugaiah *et al.*, 2009, Chen *et al.*, 2010, Wang *et al.*, 2010). Standard PCR is the most widely used molecular biology technique for its simplicity, availability and its cost effectiveness as compared to other advanced techniques.

In the present paper, duplex-PCR is proposed to identify and differentiate cattle and water buffalo meat. A common primer is used along with two specific primers that allow two different DNA fragments to be amplified, one specific to cattle and the other to water buffalo. These are used to identify meat and meat products from the two species. This work presents a specific, sensitive, effective and inexpensive alternative to the existing methods.

Materials and methods

Sample preparation and DNA isolation

Thirty fresh muscle meat samples for each species were collected from a local slaughter house and

*Corresponding author: Rohita Gupta

Address: College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand – 388001, India

E-mail address: rohitagupta2001@gmail.com

stored at -20 °C until use. Meat samples (3g) from each species were minced and mixed at various levels. Further divided into five replicates and subjected to various experimental procedures of cooking and putrefaction. Meat samples were cooked at 100 °C and 120 °C in dry (hot air oven) and moist heat (water bath and autoclave) for 45 min to simulate various methods of cooking. Different levels of autolysis were also produced by allowing the meat samples to putrefy for a variable period (48 hours to 72 hours) of time at room temperature in unpreserved conditions to stimulate the autolysis in meat. Mitochondrial DNA, along with genomic DNA was extracted by using the method described by Ausubel *et al.* (1987). The quantity and quality (A260/A280 ratio, i.e absorbance at wavelengths of 260 and 280) of DNA was assessed by using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA).

Polymerase chain reaction

Small fragments of *cyt b* gene of mitochondrial DNA extracted from fresh, cooked and putrefied samples were amplified. For this purpose species specific primers described by Rea *et al.* (2001) for identification of cattle and buffalo DNA in Italian cheese, were tested on DNA extracted from meat samples. A common forward primer (5'-CTT CTT ATT CGC ATA CGC AAT CTT ACG ATC- 3') and species specific reverse primers, cattle specific (5'-TGC TCT AAT CCC CCTA CTA CAC ACC TCC A- 3') and water buffalo specific (5'-TAT GAT GTT CCG GCC ATT CAG CCA ATG CC- 3') were used, as described by Rea *et al.* (2001).

Various combinations of primers and DNA of cattle and buffalo origin were tested in a final volume of 25 µl containing 1x PCR master mix (MBI Fermentas, Canada) 10 pmole of each primers and 90-100 ng of DNA template (cattle and/or buffalo). Amplification was performed in Master Cycler gradient thermocycler (Eppendorf, Germany) with the following cycling conditions; after an initial heat denaturation at 95 °C for 5 min, 35 cycles were programmed as follows: 95 °C for 30 sec, 65 °C for 30 sec, 72 °C for 30 sec and final extension at 72 °C for 5 min. This optimized PCR amplified 113 bp product for cattle and 152 bp product for buffalo, which was confirmed by using Genesnap and Genetool programmes (Syngene, UK) and running the products parallel to 100 bp MW marker.

To measure the sensitivity of the presented test, 100 ng of DNA sample was 10 fold serially diluted and tested. The sensitivity and discriminating power was also evaluated by serial dilution of mixed meat samples (cattle and buffalo).

Results

Amplification/cross-reaction was not observed when DNA samples of sheep, goat, pig, horse and chicken were used (data not shown). Positive signals up to 1pg were observed when tested on 10 fold serially diluted test samples. The sensitivity and discriminating power was also evaluated by serial dilution of mixed meat samples (cattle and buffalo) and was found to be capable of detecting 1% adulteration in cattle-buffalo meat mixture (Fig. 1), on further dilution the signals ceased abruptly. The assay was not tested for better sensitivity and possibility of the same cannot be ruled out. False positive and false negative results were not encountered, demonstrating the reliability of the procedure and repetitive test proved the reproducibility of the method.

Discussion

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 and 120 °C in dry (hot air oven) and moist heat (water bath and autoclave) for 45 minutes to simulate cooking. Proper cooking was evident from discolored meat. Many times meat samples are brought to the laboratory for speciation after one or two days of slaughter under unpreserved conditions. Looking to the reality of the situation that exists, different levels of autolysis were produced 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. Polymerase chain reaction 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

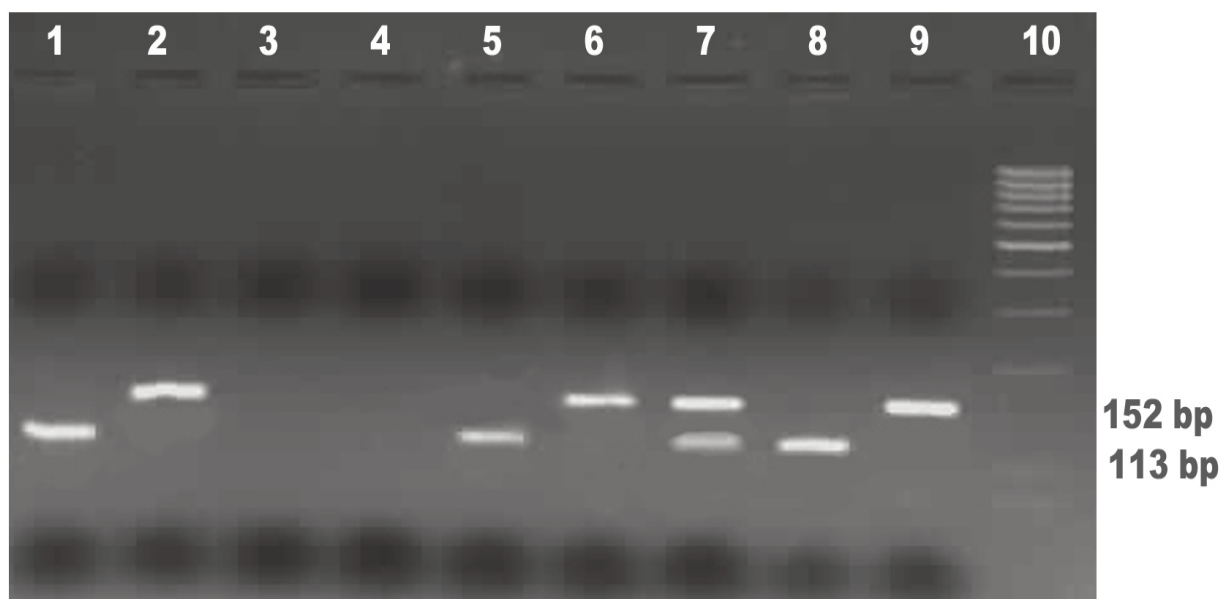


Fig. 1. Lane 1: Cattle DNA with common forward primers and cattle specific reverse primers. Lane 2: Buffalo DNA with common forward primers and buffalo specific reverse primers. Lane 3: Cattle DNA with common forward primers and buffalo specific reverse primers. Lane 4: Buffalo DNA with common forward primers and cattle specific reverse primers. Lane 5: Cattle and Buffalo mixed DNA with common forward primers and cattle specific reverse primers. Lane 6: Cattle and Buffalo mixed DNA with common forward primers and buffalo specific reverse primers. Lane 7: Cattle and Buffalo mixed DNA with common forward primers and cattle specific and buffalo specific reverse primers. Lane 8: Cattle DNA with common forward primers and cattle specific and buffalo specific reverse primers. Lane 9: Buffalo DNA with common forward primers and cattle specific and buffalo specific reverse primers. Lane 10: 100bp DNA molecular weight marker.

does not inhibit amplification of *cyt b* gene region.

Mitochondrial DNA was used 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), especially in the case of post-mitotic tissues such as skeletal muscle (Greenwood and Paboo, 1999). This increases the probability of achieving a positive result even in the case of samples suffering 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).

Conclusion

It can be concluded that cattle and buffalo meat could be reliably identified and differentiated using duplex PCR at optimized conditions and can be applied with equal efficiency to fresh, cooked and putrefied meat.

References

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Sei-

- dman, J.G., Smith J.A., Strehl, K., 1987. Current Protocols in Molecular Biology. Green publishing associates and Wiley- Interscience, New York, pp. 2.2.1.
- Bellagamba, F., Moreti, V.M., Cominicini S., Valfre, F., 2001. Identification of species in animal feedstuffs by polymerase chain reaction–restriction fragment length polymorphism analysis of mitochondrial DNA. Journal of Agricultural and Food Chemistry 49, 3775-3781.
- Chen, D., Bai F., Zhou, M.L., Zhang, X.Y., Wu, D.J., 2008. Differentiation of *Bos grunniens*, *Bos taurus*, and *Bubalus* from meat products mixture based on mitochondrial 12S rRNA gene. Yichuan 30, 1008-1014.
- Chen, S.Y., Liu, Y.P., Yao, Y.G., 2010. Species authentication of commercial beef jerky based on PCR-RFLP analysis of the mitochondrial 12S rRNA gene. Journal of Genetics and Genomics 37, 763-769.
- Dias Neto, E., Caballero, O.L., Vidigal, T., Pena, S., Simpson, A., 1994. Producing randomly amplified polymorphic DNAs from degraded DNA. Workshop of Genomic Fingerprinting. Madrid, Spain 20, 94-95.
- Greenwood, A., Paabo, S., 1999. Nuclear insertion sequences of mitochondrial DNA predominate in hair but not in blood of elephants. Molecular Ecology 8, 133-137.
- Girish, P.S., Anjaneyulu, A.S.R., Viswas, K.N., Rajkumar, N., Anand, M., Shivakumar, B.M., Sharma, B. 2005. Meat species identification by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of mitochondrial 12S rRNA gene. Meat Science 70, 107-112.
- Martinez, I., Man, Y., 1998. Species identification in meat products by RAPD analysis. Food Research Interna-

- tional 31, 459-466.
- Miguel, A.R., Teresa, G., Isabel, G., Luis, A., Pablo, E. H., Rosario, M., 2004. Identification of beef, sheep, goat and pork in raw and heat treated meat mixtures. *Journal of Food Protection* 67, 172-77.
- Murugaiah, C., Noor, Z.M., Mastakim, M., Bilung, L.M., Selamat, J., Radu, S., 2009. Meat species identification and Halal authentication analysis using mitochondrial DNA. *Meat Science* 83, 57-61.
- Prado, M., Franco, C.M., Fente, C.A., Cepeda, A., Vazquez, B.I., Barros-Velazquez, J., 2002. Comparison of extraction methods for the recovery, amplification and species-specific analysis of DNA from bone and bone meals. *Electrophoresis* 23, 1005-1012.
- Rajapaksha, W., Thilakaratne, I., Chandrasiri, A., Niroshan, T., 2003. Development of PCR assay for identification of buffalo meat. *Asian Australian Journal of Animal Science* 16, 1046-1048.
- Rastogi, G., Dharne, M., Bharde, A., Pandav, V.S., Shouche, Y., 2004. Species determination and authentication of meat samples by mitochondrial 12S rRNA gene sequence analysis and conformation sensitive gel electrophoresis. *Current Science* 87, 1278-1281.
- Rea, S., Chikuni, K., Branciari, R., Sangamayya, R.S., Ranucci, D., Avellini, P., 2001. Use of duplex polymerase chain reaction (duplex-PCR) technique to identify cattle and water buffalo milk used in making mozzarella cheese. *Journal of Dairy Research* 68, 689-698.
- Wang, Q., Zhang, X., Zhang, H.Y., Zhang, J., Chen, G.Q., Zhao, D.H., Ma, H.P., Liao, W.J., 2010. Identification of 12 animal species meat by T-RFLP on the 12S rRNA gene. *Meat Science* 85, 265-269.