

Identification and differentiation of raw meats of sheep, goat, cattle, pig and poultry by multiplex PCR

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In India, identification of species origin of meat has enormous significance from sociopolitical and economical point of view. DNA based technology has the potential to simplify meat authentication by the use of polymerase chain reaction (PCR) (Saiki *et al.* 1988), which has been used in different forms (Arslan *et al.* 2005, Fajardo *et al.* 2006, Girish *et al.* 2007, Chisholm *et al.* 2008). Due to simpler post PCR analysis and other advantages, cytochrome- b gene based PCR was standardized to identify and differentiate the raw meats of sheep, goat, cattle, pig and chicken.

Meat samples, 6 each, of sheep, goat, cattle, buffalo, pig and chicken were collected locally in sterilized plastic containers and stored at –20°C. Five adulterated samples, viz. 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) and 12 samples without their species identity were included in this study.

DNA from the meat samples was extracted as per Sambrook *et al.* (1989). DNA was washed twice with 70% ethanol, dried for 2–3 h at room temperature,

dissolved in 100–300 µl volume of TE buffer and kept it 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 and DNA was again extracted by using Tris saturated phenol, spinning at 10000 rpm for 15 min, and addition of equal volume of phenol and chloroform: isoamyl alcohol (25: 24: 1), 1/10th volume of 3 M sodium acetate and equal volumes of chilled isopropyl alcohol. The precipitated DNA was washed, air-dried, dissolved and stored. The purity and quantitation of DNA were checked by agarose gel electrophoresis and UV-spectrophotometry respectively.

Polymerase chain reaction (PCR) assay was standardized as per 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 specific to cytochrome- b gene family (Matsunaga *et al.* 1999) was got synthesized commercially (Table 1). The freeze-dried primers were dissolved in nuclease free (NF)

Table 1. 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	CTATGAATGCTGTGGCTATTGTGCGCA	26
Pig	Reverse	GCTGATAGTAGATTTGTGATGACCGTA	27

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water to obtain desirable concentration (1 µg/µl) and were further diluted in autoclaved 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: G: Ch: C: C: S: P respectively, and used together.

PCR reaction was carried out in a final reaction volume of 25 µl. A master mix was prepared and aliquoted 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 10X PCR buffer, 1.5 µl MgCl₂ (25 mM), 0.50 µl dNTPs, 0.50 µl Primer mix (10 pmole/µl), 0.25 ml Taq DNA polymerase (5 U/ml), 0.25 ml DNA template and 19.25 µl Dnase free water. Amplification was carried out at 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec and repeated these steps for 35 cycles. The products of PCR amplification were electrophoresed on 3% w/v agarose gel containing 1% ethidium bromide solution @ 5 µl/100 ml at constant voltage of 80 V for 30 min in 1X TAE. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

PCR, in various forms, was successfully used to identify meats of domesticated animals (Mayers *et al.* 2003, Girish *et al.* 2005, Fajardo *et al.* 2006, Stirtzel *et al.* 2007), meat products (Martinez and Man 1998, Pascoal *et al.* 2004) and fish varieties (Sanjuan and Comesana 2002, Mohindra *et al.* 2007). This study focused on the use of mitochondrial cytochrome-b gene based PCR for the identification and differentiation of meats of sheep, goat, cattle, buffalo, pig and chicken because of the ease of isolating it from the nuclear genome, their high presence inside the cell, the small size, and the simpler post PCR analysis (Wilson *et al.* 1995, Pfeiffer *et al.* 2004).

The yield of DNA extracted from the raw meat ranged from 100 µg to 500 µg per 500 mg of tissue sample. All the DNA samples were found free of proteins and RNA as indicated by the OD ratio (260/280 ~ 1.7 to 1.9) and only such samples were included for this study. By multiplex PCR, cytochrome-b gene fragments were amplified from DNAs of meat samples using species-specific primers. This type of PCR has earlier been used by Fei *et al.* (1996), who designed multiplex PCR primers based on mitochondrial D-loop DNA

sequences and identified meat of cattle, pig and chicken. Similarly, Obrovoska *et al.* (2002) obtained single band for meat species 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.

Multiplex PCR parameters were optimized and best results were obtained in a final volume of 25 µl containing 90–100 ng of DNA at 1.5 mM MgCl₂ concentration, primer mix having concentration 100 pmole/ml and dNTPs (10 mM each) at 60°C as annealing temperature. Initially species specificity of primers was tested by running a conventional PCR using a pair of primers, the forward SIM and species-specific reverse primers. 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 (Nakaki *et al.* 1999, Yan *et al.* 2005, Lanzilao *et al.* 2005, Kitano *et al.* 2007, Haunshi *et al.* 2009). The oligonucleotide common forward SIM and reverse primers designed by Matsunaga *et al.* (1999) were used in this study. All the PCRs amplified the target cytochrome-b gene sequences successfully and produced a single band of size specific to each species. Having confirmed species specificity of each primer independently, a multiplex PCR was designed by mixing all primers in a single reaction but targeting DNA of single species. The PCR reactions were optimized and 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 respectively, and included in the multiplex PCR @ 0.50 µl giving 25 pmoles of reverse primer per 25 µl PCR reaction, species specific amplification was achieved.

Electrophoretic band pattern of PCR products on 3.5–4% agarose gel resulted in clear cut differentiation of species as multiplex PCR amplified fragments produced characteristic band pattern on agarose gel electrophoresis when run on

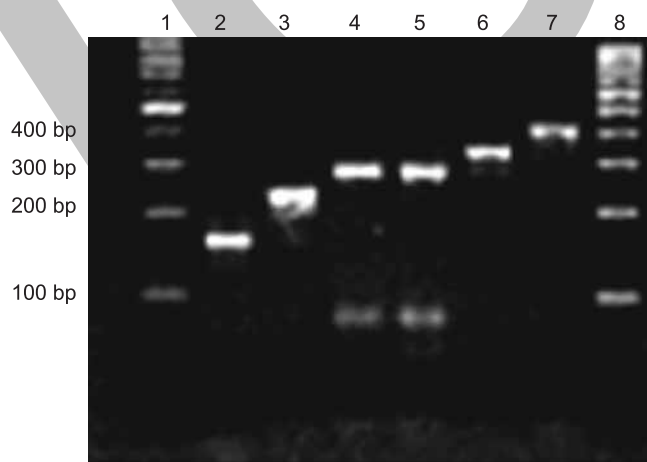


Fig 1. Agarose gel electrophoresis of PCR products amplified from raw meats of goat (2), chicken (3), cattle (4), buffalo (5), sheep (6), pig (7). 100bp ladder (1 and 8).

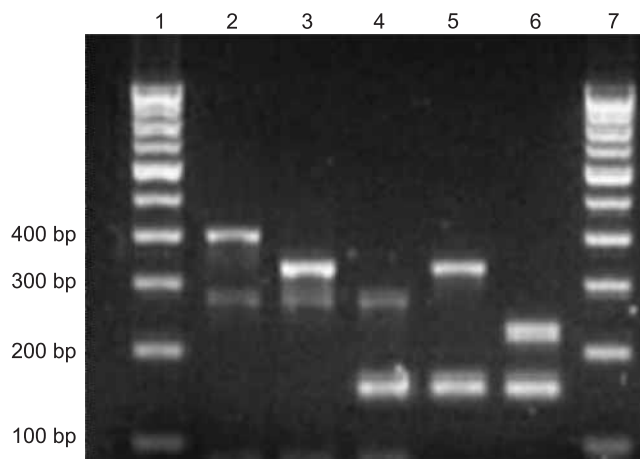


Fig 2. Agarose gel electrophoresis of PCR products amplified from mixed (20: 80) raw meats of cattle and pig (2), cattle and sheep (3), cattle and goat (4), sheep and goat (5), chicken and goat (6). 100bp ladder (1 and 7).

single species and a multiple band pattern when run on DNA mix. PCR products from sheep, goat, cattle, buffalo, pig and chicken produced DNA fragment of 331, 157, 274, 274, 398 and 227 bp respectively. Amplified PCR products from these 6 species thus ranged from 157 to 398 bp respectively (Fig. 1). Fragments of similar sizes have earlier been reported by Matsunaga *et al.* (1999). Meat of two closely related species, sheep and goat, were clearly differentiated, as fragments of these two species were widely different. Similarly, PCR amplicons of pig of 398 bp differentiated this species from cattle because of 274 bp amplicon. The results are of great practical significance to solve the religious disputes in the society. In the present study amplicon of buffalo species PCR was identical to that of cattle, which was obvious, because cattle specific reverse primer was used for buffalo meat.

DNA extracted from 500 mg of adulterated meat samples in the ratios 20: 80 was used as a template for PCR. The amplification resulted in generation of species specific bands of adulterated meat in all the 5 mixed samples examined (Fig. 2). Rodriguez *et al.* (2003) 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%.

DNA extracted from all the unknown meat samples followed by PCR amplification resulted in the identification of species of meat of unknown meat. Out of 12 meat samples, 4 were identified as meat of single animal species (pig/sheep), 5 of mixed meat of two species (4 goat and sheep, 1 goat and cattle) and 3 samples of mixed meat of three species (sheep, goat, cattle).

With the PCR used in the present study, meat of sheep, goat, cattle, pig and chicken were identified and thus demonstrated the suitability of cytochrome b gene to identify and differentiate the raw and adulterated meat. Because, it is a rapid, accurate and easy to perform, the method offers a promising alternative for meat inspection programme intended to assess the species identification of meat and meat products.

SUMMARY

Meat authentication by PCR technique has several advantages. Cytochrome- b gene based multiplex PCR was, therefore, standardized to identify and differentiate the raw meats of sheep, goat, cattle, pig and chicken. Meats of phylogenetically related species like sheep and goat were differentiated as PCR products from meats of these animals produced DNA fragments of 157 and 331 base pairs respectively. Specific differentiation of raw meats of cattle and pig producing DNA fragments of 274 and 398 base pairs respectively has socio-religious significance. The used technique could detect 20% adulteration of meat of one species into the meat of other species and thus fraudulent practice of meat adulteration may be detected by this technique.

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