A rapid method for authentication of Buffalo (*Bubalus bubalis*) meat by Alkaline Lysis method of DNA extraction and species specific polymerase chain reaction

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Abstract Buffalo (*Bubalus bubalis*) meat is a major item of export from India but export of beef i.e. meat from cattle (*Bos indicus*) is prohibited. Also, adulteration of buffalo meat with that of beef (meat from cattle) is a common fraudulent practice because of prohibition on cow slaughter in most states of India. Food analysts require precise identification techniques to implement such regulations. In the present study, a method of DNA extraction by Alkaline lysis from meat samples and speciation of buffalo meat using species specific Polymerase Chain Reaction (PCR) has been reported. Alkaline lysis technique is a rapid method which involves triturating meat with four volumes of 0.2N NaOH, dilution of resultant liquid extract with eight volumes of 0.2N NaOH, heating the mix 75 °C for 20 min followed by neutralization with eight volumes of 0.04N Tris HCl. Entire procedure of DNA extraction takes less than 30 min and it is economical as it involves less expensive chemicals. Method was successfully applied in animal byproducts also viz., liver, heart and kidney. For authentication of buffalo meat, pair of primers was designed based on mitochondrial D loop gene nucleotide sequence. PCR amplification using the designed primers gave amplification of size 482 bp in buffalo and no amplification was detected in closely related species viz., cattle, sheep and goat meat samples. Results of the assay were highly repetitive and

reliable. An export sample referred by export regulation authorities was also analyzed by using the Alkaline lysis method of DNA extraction and species specific PCR which enabled authentication of meat within 5 h.

Keywords *Bubalus bubalis* · PCR · DNA · Alkaline lysis · Mitochondrial D loop gene · Speciation

Introduction

Food authenticity issues in the form of adulteration and improper description have been around for a long time and probably for as long as food has been offered for sale (Ioannis et al. 2005). The adulterated food often enters the supply chain and jeopardizes the sentiments as well as health of the people. The driving force behind any adulteration is the revenue maximization, either by using a low cost ingredient to (partially or totally) substitute a more expensive one, or to (partially) remove the valued component in the hope that the adulterated product neither will be perceived nor detected by the authorities and the consumer (Ioannis and Nikolaos 2005). Substantial proportion of population has religious considerations towards the consumption of meat of a particular animal species. Hence, the meat adulteration has got social, religious, and economic and public health concerns. India exported about 0.48 million tonnes of buffalo meat in 2007–08, worth Indian Rupees 35490.78 Million (1 US \$=47 Indian Rupees). Due to religious taboos slaughter of cows is prohibited in most states of India and export of beef (cattle meat) is totally prohibited. Allegations of exporting beef misrepresenting it as buffalo meat surface frequently. Such circumstances demand robust detecting techniques to detect the species of meat and the tests need to be rapid as

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extended waiting period may lead to huge losses to the exporter since meat is a highly perishable commodity.

Molecular techniques are the most reliable method available for speciation and DNA extraction is the vital step in undertaking molecular techniques. Commonly employed methods, for extraction of DNA from meat at present are by using commercial kits and by Phenol: Chloroform; Isoamyl alcohol (PCI) extraction method. Commercial kit based methods are expensive and require centrifugation facility. PCI based methods are comparatively inexpensive but laborious and time consuming.

In this work, Alkaline lysis: a method of DNA extraction from meat and byproducts has been standardized. Apart from bringing down the cost exponentially this method lessens the extraction time to less than 30 min and it is less laborious involving only single tube with minimal pipetting requirements, which avoids cross contamination problems. Similar method has been used for extraction of DNA from whole blood, buccal epithelial cells, semen and forensic stains (Rudbeck and Dissing 1998). Alkaline lysis method was also used for sexing of chick embryos using whole blood, embryonic tissue and feather bulbs by Haunshi et al. (2008). There are no reports of utilizing the Alkaline lysis method for extraction of DNA from meat.

Meat being a protein rich product an appropriate method was required to be standardized in meat system and the present work focuses on it and results of this evaluation are described herein.

Vast array of techniques have been developed for authentication of meat which includes Electrophoretic methods (Bhilegaonkar et al. 1990), isoelectric focusing (King 1984) and immunological methods viz., counter immunoelectrophoresis (Sherikar et al. 1993) chemometric analysis (Ioannis and van Maria 2003) and ELISA (Martin et al. 1991) Polymerase Chain Reaction (PCR) and its variants are the most preferred techniques for identification of animal species because of their specificity, sensitivity, accuracy and precision. Targeting DNA for the animal speciation offers many advantages over the protein-based techniques. The DNA molecule is more stable and the composition of DNA is same in any cell of the individual. Hence, DNA based techniques namely Polymerase Chain Reaction (PCR) and its variants: Restriction Fragment Length Polymorphism (RFLP) (Girish et al. 2005); Random Amplified Polymorphic DNA (RAPD) finger printing (Saez et al. 2004); DNA Hybridization (Buntjer et al. 1999), PCR Sequencing (Girish et al. 2007); Arbitrarily Primed-PCR (AP-PCR) (Desmarais et al. 1998) and PCR-Single Strand Conformation Polymorphisms (SSCP) (Tejedor et al. 2006) and species specific markers (Haunshi et al. 2009) have been employed for the identification of various animal species in the recent past. The PCR-based techniques provide high level of specificity, sensitivity, accuracy and

precision than the techniques hitherto being used for the purpose of animal species identification. Species specific PCR involves amplification of specific region of the gene by utilizing set of specific primers targeting a species. It takes lesser assay time as it does not involve any post reaction processing except electrophoresis. Primers yield amplicon only in the species for which they have been designed and no amplification is expected in other species.

The present work describes a quicker method for buffalo meat identification by utilizing the DNA extracted by Alkaline Lysis method and species specific PCR by using a set of primers targeting mitochondrial D loop gene specific to buffalo.

Materials and methods

Alkaline lysis (AL) method as reported earlier (Rudbeck and Dissing 1998) was used with modifications for use in the meat system. For standardizing the AL method of DNA extraction, goat (*Capra hircus*) meat was used and extracted DNA was subjected to PCR amplification of mitochondrial 12S rRNA gene. AL method was also compared with the existing methods like Phenol: Chloroform: Isoamyl alcohol (PCI) and commercial kit based DNA extraction method. For standardizing and verifying the cross amplification of buffalo specific primers in other closely related species DNA extracted by PCI method was used.

Meat sample Meat, liver, heart and kidney samples of goat (*Capra hircus*) and meat samples of buffalo (*Bubalus bubalis*) were collected aseptically from the Municipal slaughterhouse, Chengicherla, Hyderabad, India. Samples were kept in refrigeration condition immediately after collection and stored at -20°C till extraction.

Genomic DNA extraction by Phenol: Chloroform: Isoamyl alcohol (PCI) method DNA was extracted from the meat sample by PCI method (Chikuni et al. 1994). About 100 mg crushed/minced fresh meat was mixed with ten volumes of extraction buffer, pH 8.0. Incubated at 37°C for 1 h. Proteinase K (0.1 mg/ml) was added and kept at 50°C in water bath for 3 h. The DNA/RNA solution was mixed with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 3000 rpm for 2–3 min. Aqueous phase was transferred and mixed with an equal volume of chloroform. Centrifuged at 3000 rpm for 2–3 min. Aqueous phase transferred and thoroughly mixed again with an equal volume of chloroform and again centrifuged at 3000 rpm for 2–3 min. Subsequently, aqueous phase was mixed with 0.2 volumes of 1.0 M ammonium acetate solution and two volumes of ethanol and mixed thoroughly.

Spooling DNA transferred to fresh tube using Pasteur pipettes. The extracted genomic DNA was washed with 70% alcohol and centrifuged at 10000 rpm for 5 min. DNA pellets was mixed with IX TE (Tris- EDTA) buffer in micro centrifuge tubes and were kept in water bath at 60 °C for 2 h to inhibit DNase activity and to dissolve pellet properly in the Tris EDTA buffer. After 2 h of incubation, the DNA was cooled and stored at –20 °C for further use.

Genomic DNA extraction using commercial DNA extraction kit DNA was extracted from about 100 mg of meat sample using commercial DNA extraction kit (Bioserve Biotechnologies (India) Pvt Ltd., Hyderabad, India) as per the protocol provided by the manufacturer.

PCR amplification of Mitochondrial 12S rRNA gene Universal primers for mt 12S rRNA gene used for speciation of meat (Girish et al. 2005) was used and sequence of primers are as given below:

Sense - 5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3',
Antisense - 5'-GAG GGT GAC GGG CGG TGT GT-3'

PCR amplification was carried out in 0.2 ml PCR tubes containing 5 µl of 10X PCR buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, 500 mM KCl and 0.1% gelatin), 1 µl of 10 mM dNTP mix, 1 µl (15 pmol) each of forward and reverse primers, 1 U of Taq DNA polymerase, 50 ng of purified DNA and autoclaved Milli- Q water to make a volume up to 25 µl. Conditions on a Master Cycler gradient thermocycler (Eppendorf, Germany) were as follows: 5 min at 94 °C for initial denaturation, followed by 30 cycles of amplification (45 s at 94 °C, 45 s at 60 °C and 1 min at 72 °C) and final extension for 10 min at 72 °C. The PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining.

Designing of primers specific for buffalo (*Bubalis bubalis*) from mitochondrial D loop region The mitochondrial D-loop sequences were downloaded from the GenBank (www.ncbi.nlm.nih.gov) database and aligned using “Megalalign” software (DNASar, Inc., 1996). The primer sites with nucleotide sequence variation between the species and homology within a species (free from SNPs) were located. The oligonucleotide primers were designed against such unique species-specific sites so as to yield appropriate sized PCR products; and the primers were designed using “Primer-Select” software (DNASar Inc., 1996).

Primers designed for PCR amplification of mitochondrial D loop gene was expected to yield amplicon of size 482 bp and the sequence of primers is as follows:

Forward- 5'- TGA CCC TAC TAC TCC GAA TGG -3',
Reverse-5'- GCT GAG TCC AAG CAT CCC -3'

Further, the selected primers were screened for primer specificity (species-specificity) and cross reactivity using basic local alignment search tool “BLAST” (<http://www.ncbi.nlm.nih.gov/blast>). Finally, selected primers were custom synthesized from Bioserve Biotechnologies (India) Pvt Ltd., Hyderabad, India and they were considered for PCR amplification in buffalo DNA sample.

Polymerase Chain Reaction amplification of mitochondrial D loop gene The PCR conditions i.e., primer concentration, dNTPs, MgCl₂ and the cycling program especially the annealing temperature were standardized for each species. Optimized PCR was carried out in reaction mix containing 1X PCR buffer (100 mM Tris-HCl, pH 9.0, 15 mM MgCl₂, 500 mM KCl and 0.1% gelatin), 10 mM dNTP mix, 15 pmol each of forward and reverse buffalo specific primers, 1 U of Taq DNA polymerase, 50 ng of purified DNA and autoclaved Milli- Q water.

Conditions on a Master Cycler gradient Thermocycler (Eppendorf, Germany) were as follows: 5 min at 94 °C for initial denaturation followed by 30 cycles of amplification (45 s at 94 °C, 45 s at 56 °C and 1 min at 72 °C) and final extension for 10 min at 72 °C. The PCR products were analyzed by electrophoresis in 2% agarose gel with ethidium bromide staining. Procedure was repeated in six known samples and three blind samples for confirming the repeatability of the results.

Results and discussion

Extraction of DNA from meat by Alkaline Lysis method Unlike whole blood, buccal epithelial cells, semen and forensic stains which have been used for DNA extraction by Alkaline lysis method in earlier report (Rudbeck and Dissing 1998), meat sample contains high proportion of protein (about 18%). Hence, AL method as per the report did not give optimum results in the trial. Therefore, series of experiments were carried out in which about 500 mg of meat sample was triturated in pestle & mortar with four volumes of 0.2 N NaOH and from this mix 5 µl of extract was mixed with four, six, eight & ten volumes of 0.2N NaOH i.e. in 20, 30, 40 & 50 µl of 0.2N NaOH in a sterile micro centrifuge tubes.

Subsequently, mixed content was heated at 75 °C in water bath for 20 min and then added 180, 270, 360 & 450 µl of 0.04 M Tris HCl (pH 7.75) respectively for neutralization of pH. Second dilution of extract with 0.2N NaOH after initial dilution at eight volumes of 0.2N NaOH reduced the concentration of protein in the solution which helped to yield better PCR results. Dilution of initial extract prepared with the four volumes of 0.2N NaOH followed by

dilution with eight volumes of 0.2N NaOH yielded optimum results for PCR amplification. Concentration of the DNA extract was about 100 ng/ μ l, which is an optimum concentration of DNA required for PCR amplification. The method works on the principle that the alkaline pH and high temperature lyses the protein leaving DNA in the solution.

The procedure of finally optimized method is as follows: About 500 mg of meat was triturated in pestle & mortar with four volumes (4 ml) of 0.2N NaOH. 5 μ l of the extract was again mixed with eight volumes (40 μ l) of 0.2N NaOH in a sterile micro centrifuge tube. Mix was heated at 75 °C in water bath for 20 min and then added eight volumes (360 μ l) of 0.04 M Tris HCl (pH 7.75) for neutralization of pH. 1 μ l of final mix containing about 100 ng DNA was used for PCR. PCI method of DNA extraction took 30 min for completion of DNA extraction compared 4 h by using commercial kit and 8 h by PCI method. Also, AL method does not require any centrifugation unlike that of commercial kit and PCI based methods. Chances of contamination of samples is also less as it is a single tube method which avoids contamination during pipetting and transferring the sample to other tubes

PCR amplification using the DNA extracted by different methods For checking the suitability of DNA extracted from meat, heart, liver & kidney extract by AL method, extract was subjected to PCR amplification using universal primers of mitochondrial 12S rRNA gene (Girish et al. 2005). PCR amplification yielded amplicon of size of about 456 bp and the result of PCR amplification of mitochondrial 12S rRNA gene is given in Fig. 1. The method was equally efficient in meat and all kinds of byproducts also.

DNA extraction from meat by conventional PCI extraction method took about 8 h. Further, it is laborious and involves repeated transfer of solution requiring multiple pipetting steps and multiple tubes to transfer the solution, hence chances of cross contamination between other samples increases. Nonetheless, extracted DNA using PCI method from meat & byproducts gave successful PCR amplification of mt 12S rRNA gene (Fig. 2). Further drawback of PCI method is that it requires the use of corrosive chemicals like Phenol which are injurious to the

Fig. 1 PCR amplification of mitochondrial 12S rRNA gene of DNA extracted from different meat and byproducts by Alkaline Lysis method. Lane 1: Meat; Lane 2: Heart; Lane 3: Liver; Lane 4: kidney; Lane L: 100 bp DNA Ladder

Fig. 2 PCR amplification of mitochondrial 12S rRNA gene of DNA extracted from different meat and byproducts by conventional Phenol Chloroform Isoamyl alcohol method. Lane 1: Meat; Lane 2: Heart; Lane 3: Liver; Lane 4: kidney; Lane L: 100 bp DNA Ladder

user. Additionally this method requires centrifugation at different speeds. However, AL method does not require any centrifugation.

DNA extraction of meat and byproducts was also carried out using the commercial kit of Bioserve Biotechnologies (India) Pvt Ltd., Hyderabad, India. This particular method took about 4 h and DNA was extracted from about 10 mg sample. DNA gave very good amplification of mitochondrial 12S rRNA gene as shown in Fig. 3. Although it is an efficient method but it involves centrifugation and the method is costlier.

Identification of buffalo meat by species specific PCR Initially primers designed for identification of buffalo meat were used for PCR amplification by using DNA extracted by PCI method and it yielded an amplicon of 482 bp as per the primer design. Primers were checked for cross amplification in other closely related species like cattle, sheep and goat and no cross amplification was found in other species except in buffalo (Fig. 4). PCR amplification of mt D-loop for identification of buffalo meat species has been reported (Malisa et al. 2006) but with different primer sequences. DNA extracted from known buffalo meat sample by AL method was also used for PCR amplification of mitochondrial D loop region specific to buffalo for standardization and results were reproducible, reliable and convincing. Test was repeated in ten different buffalo meat samples.

Fig. 3 PCR amplification of mitochondrial 12S rRNA gene using DNA of meat & byproducts extracted by using commercial kit (Bioserve Biotechnologies (India) Pvt Ltd). Lane 1: Meat; Lane 2: Heart; Lane 3: Liver; Lane 4: Kidney; Lane L: 100 bp DNA Ladder

Fig. 4 Species specific PCR amplification in different meat animals using buffalo specific primers. Lane 1: Cattle; Lane 2: Sheep; Lane 3: Goat; Lane 4: Buffalo; Lane L: 100 bp DNA Ladder

Girish et al. (2005) utilized PCR RFLP technique for identification of buffalo meat which involved PCR amplification of mitochondrial 12S rRNA gene (amplicon size 456 bp) followed by restriction digestion with *HhaI* restriction enzyme targeting species specific restriction site, which yielded products of size 247 bp and 209 bp after digestion. A buffalo meat certification method based on PCR amplification of mitochondrial Cytochrome B gene followed by restriction digestion with *TaqI* restriction enzyme yielding digestion products of 108 bp and 163 bp was reported by Teixeira et al. (2007). Mohamed et al. (2007) also reported buffalo meat authentication method based on PCR RFLP of Species Specific Repeat (SSR) sequence. Species specific PCR reported in the present work is advantageous because it saves time as it does not involve any post amplification processing like restriction digestion as in PCR RFLP methods. Another method based on forensically important nucleotide sequencing (FINS) which involved PCR amplification of mitochondrial 12S rRNA gene followed by sequencing of amplicon was reported by Girish et al. (2004) for identification of buffalo meat. But, FINS involve sequencing of nucleotides which is time consuming and involves additional cost. Hence, species specific PCR scores higher over the FINS. Results of species specific PCR was highly reproducible unlike that of Randomly Amplified Polymorphic DNA (RAPD) PCR (Koh et al. 1998). Apart from PCR amplification of mt D loop for buffalo meat species identification, Rajapaksha et al. (2003) exploited mitochondrial Cytochrome b gene for their species identification. The technique could differentiate buffalo from other related animals based on the molecular weight of the PCR product.

Identification of species in export forensic sample A sample of meat was referred to the laboratory of the first author for speciation and authentication. Sample was from consignment of an exporter waiting for clearance from customs officials for export from a port of Andhra Pradesh state, India. Doubts were raised whether meat is of buffalo

or beef; therefore sample was drawn by the customs officials from consignment and was sent under refrigeration temperature to the laboratory. As the consignment was waiting the results were required to be submitted in shortest possible time. The technique standardized in the laboratory i.e. alkaline lysis method for DNA extraction and PCR specific to buffalo meat became handy and hence it was used for confirmation of the sample. DNA was extracted by AL method and upon specific polymerase chain reaction of mitochondrial D loop region and electrophoresis showed amplicon of 482 bp (Fig. 5). The result was achieved in less than 5 h, meat sample was confirmed to be of buffalo, report was submitted to the officials and subsequently consignment was released. Along with species specific PCR of referred sample negative control without DNA sample and positive control with the referred DNA sample with mitochondrial 12S rRNA gene universal primers was also set along as a common marker to ensure that negative results of PCR if any observed were in fact due to lack of template DNA rather than failure of PCR reaction itself. Just to confirm the results of specific PCR, amplicon of mitochondrial 12S rRNA gene was sent for sequencing to commercial DNA sequencing facility of Bioserve Biotechnologies (India) Pvt. Ltd., Hyderabad, Andhra Pradesh, India. Sequencing results obtained were BLAST (Basic Local Search Alignment Tool) aligned in the National Centre for Biotechnology Information data base (www.ncbi.nlm.nih.gov). DNA sequence matched 100% with the accessible buffalo mitochondrial 12S rRNA gene fragment. Hence, result and the reliability of the method was confirmed.

Fig. 5 PCR amplification of forensic export meat sample DNA extracted by Alkaline lysis method with species specific primers of buffalo and mitochondrial 12S rRNA gene. Lane 1 to 2: PCR amplification with buffalo specific primers with 2 µl of AL DNA extract. Lane 3 to 4: PCR amplification with buffalo specific primers with 1 µl of AL DNA extract. Lane 5 to 6: PCR amplification with mt 12S rRNA gene primers (positive control); Lane L: 100 bp DNA Ladder; Lane C: Negative control

Conclusion

In conclusion, the study indicates that the rapid extraction of DNA from meat can be achieved by Alkaline lysis method as it takes 30 min for extraction and method is economical as it involves only simple chemicals like NaOH & Tris HCl. Species specific polymerase chain reaction by using the species specific primers targeting mitochondrial D loop gene, which yield 482 bp amplicon in buffalo meat, enable authentication of buffalo meat. By combining Alkaline lysis method of DNA extraction and species specific PCR developed under the study, buffalo meat could be identified with in 5 h. The techniques were reproducible, reliable, convincing and proved to be robust, which can be handy to the food analysts, especially in cases involving misrepresentation of buffalo meat with beef in both export consignments and in internal trade of buffalo meat.

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