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Research article

A multiplex PCR-based approach for reliable identification of different meat species and possible adulteration in meat markets in Chittagong, Bangladesh

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ABSTRACT

Food authenticity issues in the form of adulteration and improper description have been around for a long time in Bangladesh. The adulterated food often enters the supply chain and jeopardizes the credibility of the sellers as well as health of the consumers. Molecular detection has been a reliable tool for animal species identification since many days. Polymerase chain reaction (PCR) is quite useful tool that is highly accurate and relatively faster in differentiating specimens derived from different animal species. The present study was carried out to validate previously described methods to identify different meat species by a multiplex PCR method and a preliminary molecular survey was performed on 60 meat samples for identification of possible adulteration in butcher shop in Chittagong. A multiplex PCR was developed and evaluated on the mitochondrial *cytochrome b* gene to differentiate in six meat species (chicken, duck, sheep, goat, cattle and buffalo). A common forward primer was designed on a conserved DNA sequence of the *cytochrome b* gene and reverse primers on species-specific DNA sequences from each species. By multiplex PCR, DNA bands of 157 bp, 227 bp, 274 bp, 331 bp, 398 bp, 439 bp were visualized that corresponds to meat samples derived from goat, chicken, cattle, sheep, duck and buffalo, respectively. There were no adulterated meat identified among the samples examined during this study, indicating fair economic business in the study area.

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INTRODUCTION

Food adulteration is a common issue in developing countries like Bangladesh. Although there are stringent rules for ensuring food safety and consumer health, these are not enforced properly in this country. Globally, food adulteration has ranged from the simple addition of natural compounds to the much more serious case of contaminant with harmful substances (Defernez *et al.,* 1995). With promotion of global trade, the importance of safe and properly labelled food items is crucial for fair trade and economic progress.

To protect the consumer from frauds, detection of adulteration or fraudulent substitution is essential through the identification of meat species in conventional means (Meyer *et al.,* 1995). DNA and protein based molecular methods are used for species identification (Jones, 1991) and biomolecular techniques such as the polymerase chain reaction (PCR) is an effective technique that is highly accurate, relatively faster and has been reported previously by several authors (Dalmasso *et al.,* 2004; Bottero *et al.,* 2003). Matsunaga *et al.* (1999) have developed a

simple rapid method, called multiplex PCR, for simultaneous detection of six different meat species at a time by using the mitochondrial *cytochrome b (CYTB)* gene. The present study was therefore attempted to develop and validate previously reported methods using modern DNA-based tools to reliably and rapidly identify meats derived from different animal species (chicken, duck, sheep, goat, cattle and buffalo) in local markets of Chittagong, Bangladesh.

MATERIALS AND METHODS

DNA extraction

Fresh raw meat samples from six different livestock animal such as cattle *(Bos taurus),* buffalo *(Bubalus bubalis),* goat *(Capra hircus),* sheep *(Ovis aries),* chicken *(Gallus gallus)* and duck *(Anas platyrhynchos)* were collected. In addition, 60 meat samples from six meat species were collected from 10 local markets of Chittagong metropolitan area. Samples were transported to the laboratory with ice and later stored at -20°C until used. Modified Phenol-chloroform DNA extraction procedure was used to extract DNA from fresh muscle tissue samples (Sambrook *et al.,* 1989). Samples were subjected in 20 vol of 100 mM Tris HCL (pH 9.0) consisting of 100 mM NaCl, 5 mM EDTA and 1% SDS, and grind gently till it got slurry. Then 10 µl proteinase K (Sigma) was added and incubate at 37°C for 2 h. DNA solutions were treated with equal volume of phenol: chloroform: isoamyl-alcohol (25:24:1) and then with an equal volume of chloroform: isoamyl-alcohol (24:1). The solutions were then centrifuged at 12000 rpm for 10 min to separate aqueous layer. The supernatants were then washed with 500 µl of 70% ethanol and centrifuged at 7000 rpm for 10 min. Supernatant were later decanted and DNA pellets were left for air drying. Ultimately, DNA concentrated by ethanol precipitation was dissolved in 20 µl Tris-HCL for use as the PCR template.

Polymerase chain reaction (PCR)

Previously described oligonucleotide primers were used in this study for multiplex PCR amplification (Table 1). A common forward primer all*CYTB*-F and species-specific reverse primers (cattle: ca*CYTB*-R, goat: go*CYTB*-R, sheep: sh*CYTB*-R and chicken: ch*CYTB*-R) were designed from published sequence of cattle, goat, sheep and chicken mitochondrial *(CYTB)* gene (Matsunaga *et al.,* 1999). The reverse primers for other two livestock meats of duck and buffalo (duck: du*CYTB*-R, buffalo: bu*CYTB*-R) were designed from GenBank database of NC_009684 and NC_006295 for duck and buffalo, respectively. These primers were mixed in the ratio of 1:0.2:3:0.6:3:0.6:2 for all*CYTB*:go*CYTB*:ch*CYTB*:ca*CYTB*:sh*CYTB*:du*CYTB*:bu*CYTB*, and used together for the multiplex PCRs of this study (the ratio 1 means 20 pmol primer/50 ml PCR solution).

PCR amplification was performed in 20 µl of a reaction mixture containing 10 µl of 2X PCR master mix (GoTaq Green Master Mix, Promega), 4-60 pmol of primer-mix, and 0.5-1.0 µg of genomic template DNA. Amplification was carried out with an initial denaturation at 94 °C for 5 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, followed by a final elongation at 72 °C for 5 min. The PCR products were subjected to electrophoresis in 2% (wt/vol) agarose gel (Promega) buffered with Tris-EDTA solution (pH 8.0), stained with ethidium bromide, and visualized under a UV trans-illuminator.

Using the assay developed in the current study, an epidemiological survey was preliminarily carried out for the screening of the possible adulteration in meat markets in Chittagong. Total 60 meat samples were chosen randomly from 10 local markets in Chittagong metropolitan area in Bangladesh.

Table 1. Oligonucleotide primers to be prepared from the *cytochrome b* gene

Primers	Species	Sequence (5'-3')
allCYTB-F	-	GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA
caCYTB-R	*Bos taurus*	CTAGAAAAGTGTAAGACCCGTAATATAAG
*buCYTB-R	*Bubalus bubalis*	CTCAAATTCACTCAACCAGACTTGTA
goCYTB-R	*Capra hircus*	CTCGACAAATGTGAGTTACAGAGGGA
shCYTB-R	*Ovis aries*	CTATGAATGCTGTGGCTATTGTCGCA
chCYTB-R	*Gallus gallus*	AAGATACAGATGAAGAAGAATGAGGCG
*duCYTB-R	*Anas platyrhynchos*	GCTGAAAATAAGTTGGTAATTACGGTA

*Prepared for this study.

RESULTS

Meat identification by species-specific primers

The multiplex PCR assay using frozen DNA template revealed characteristic pattern of species-specific bands. Fig. 1 shows 2% agarose gel electrophoresis of PCR products amplified from the six species of meat DNA used in this study. PCR products from goat, chicken, cattle, sheep, duck and buffalo DNAs were visible as characteristic single DNA fragments of 157, 227, 274, 331, 398 and 439 bp, respectively. The six meat species could thus be identified based on the length of PCR products as described by other investigators elsewhere. Notable that all 7 sets of primers (corresponding to six species of animals) were added in each reaction.

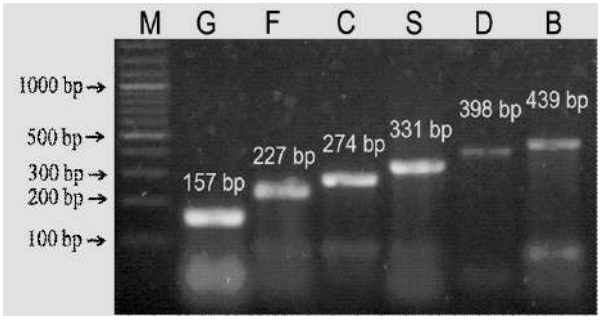

Fig 1: Agarose gel electrophoresis (2%) of PCR product amplified from the six species. PCR products from G: goat, F: chicken; C: cattle, S: sheep; D: duck; B: buffalo DNAs were single DNA fragments of 157 bp, 227 bp, 274 bp, 331 bp, 398 bp, 439 bp, respectively. M is a molecular size marker.

Specificity in mixed meats

The identification of a species from any mixed sample requires multiplex or species-specific PCR assay. To validate the efficiency of the previously described primers and those designed during this study, we have randomly mixed DNA samples from different meat species and examined by multiplex PCR. Fig. 2 shows 2% agarose gel electrophoresis of PCR products of mixed samples with variable number of DNA species in each reaction. The study showed specific characteristic bands of different size (corresponding to different meat species) as revealed in multiplex PCR in Fig. 1.

A total of 60 samples from 6 meat species were analyzed during the study period to assess the possible adulteration or mislabelling. No adulterated meat was identified among 60 samples surveyed. However, one goat sample and one buffalo sample could not be amplified during this study for unidentified reason.

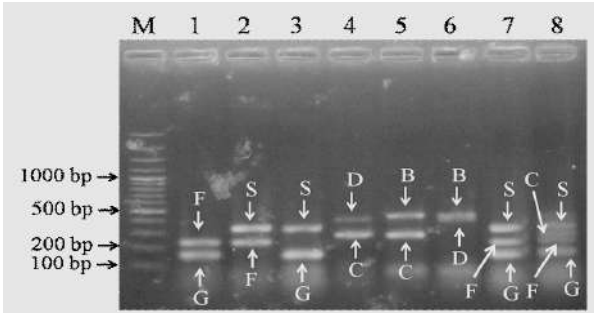

Fig 2: Agarose gel electrophoresis (2%) of PCR products amplified from DNA of the different mixtures of six meat species. Lane 1 (F: Chicken-G: goat), lane 2 (S: sheep-F: chicken), lane 3 (S: sheep-G: goat), lane 4 (D: duck-C: cattle), lane 5 (B: buffalo-C: cattle), lane 6 (B: buffalo-D: duck), lane 7 (G: goat-C: chicken-S: sheep), lane 8 (G: goat-F: chicken-C: cattle-S: sheep). M: molecular size marker.

DISCUSSION

The goal of the study was to validate the previously described multiplex PCR assay for detection of different meat species in a single reaction and to find out the possible adulteration in raw meat in the local meat markets in Chittagong, Bangladesh. While DNA based molecular asaay requires special instruments and setups, it is well recognized that multiplex PCR is a powerful technique for simultaneous identification of various meat species. In this method all primers are amalgamate together for amplification of multiple target regions. Therefore a single run can lead to identifying suspected meat species within a short period of time.

In this study mitochondrial *CYTB* gene were used for species identification by PCR. This is because the *CYTB* gene of mitochondrial DNA (mtDNA) has been found to be a powerful indicator for identifying the species with DNA analysis techniques by several other investigators (Zehner *et al.,* 1998, Parson *et al.,* 2000). This gene is ideal for species identification as it shows limited variability within and much greater variation between species (Caine, 2006). That enables to work out methods allowing differentiation between many species in a single test. Since all mitochondrial genes behave as a haploid locus therefore the problem of heterozygosity can be avoided. Moreover, the mitochondrial genome has a higher rate of mutation compared to the nuclear genome, is maternally inherited, has a less hybridization and has a high copy number, which facilitates PCR amplification and sequence recovery from tissue samples (Saccone *et al.,* 1999). Again, the mitochondrial genome lacks

introns, pseudo genes and repetitive sequences, which facilitate sequence alignments of the amplified genes (Lin *et al.,* 2005). Finally complete mtDNA genome sequences are publicly available; primers can therefore be designed to amplify and sequence any species that has a published mtDNA genome (Folmer *et al.,* 1994). The study was conducted on six domestic animals (cattle, buffalo, goat, sheep, chicken, duck) those are used as common meat species for consumption as the essential protein source throughout the Bangladesh. Therefore they were chosen for the comparative evaluation of adulteration rate in the local markets and slaughter houses.

Primer specificity in the entire DNA of a target species was examined by conventional PCR using a pair of a common forward primer and each species-specific primer. The size of PCR products was as expected with no additional fragment from a target species (data not shown). This result showed that the species-specific primers amplified only one size fragment from a target species. Primer specificity to the other species was examined by multiplex PCR using the same primer mixture in the method. Fig. 1 showed that multiplex PCR resulted in a single band of target size from one meat species and no fragment produced by non-specific amplification.

Fig. 2 showed that specific sizes bands were produced in mixed samples in one reaction. Actually the common forward primer was designed to be shared by the six species; therefore, amplification efficiency of PCR was affected by only species-specific reverse primers. No cross-reaction between the relevant species was observed showing an excellent reliability of the assay to determine adulterated meat species.

The survey in the present study suggested fair economic business in the study area, although sample size is very small. However, PCR amplification of two samples was not possible among 60 samples. This may be due to long time storage of meats with high ambient temperature in market tend to the poor quality of DNA. So, adulteration in these two samples cannot be ruled out. However, further study is needed to clarify the possible adulteration in large scale.

In conclusion, although it requires modern lab equipments, this multiplex PCR based method is quite useful, fast and sensitive for routine analysis of meat species inspection and identification. Using the present method, the six meat species could all be identified at the same time more easily and reliably than conventional methods. The analytical conditions must be improved for quantitative differentiation.

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