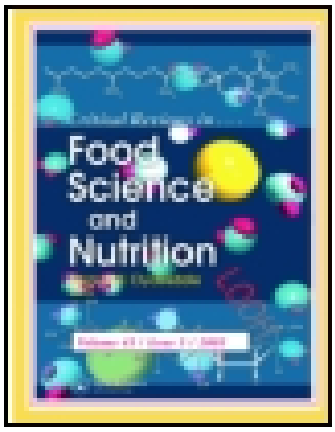

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Identification of Species origin of Meat and Meat Products on the DNA Basis: A Review

Arun Kumar [a] , R. R. Kumar [a] , B. D. Sharma [a] , P. Gokulakrishnan [a] , S. K. Mendiratta [a] & D. Sharma [b]

[a] Division of Livestock Products Technology, Indian Veterinary Research Institute , Izatnagar , Bareilly (U.P.) , 243122 , India

[b] Genome Mapping Laboratory, Central Avian Research Institute , Izatnagar , Bareilly (U.P.) , 243 122 , India

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Identification of Species origin of Meat and Meat Products on the DNA Basis: A Review

Arun Kumar[1], R.R. Kumar[1], B.D. Sharma[1], P. Gokulakrishnan[1]*, S.K. Mendiratta[1] and D. Sharma[2]

[1]Division of Livestock Products Technology, Indian Veterinary Research Institute, Izatnagar, Bareilly (U.P.) 243122, India

[2]Genome Mapping Laboratory, Central Avian Research Institute, Izatnagar, Bareilly (U.P.) 243 122, India

ABSTRACT

The adulteration/substitution of meat has always been a concern for various reasons such as public health, religious factors, wholesomeness and unhealthy competition in meat market. Consumer should be protected from these malicious practices of meat adulterations by quick, precise and specific identification of meat animal species. Several analytical methodologies have been employed for meat speciation based on anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic or immunological principles. However, by virtue of their inherent limitations, most of these techniques have been replaced by the recent DNA-based molecular techniques. In the last decades, several methods based on polymerase chain reaction (PCR) have been proposed as useful means for identifying the species origin in meat and meat products, due to their high specificity and sensitivity, as well as rapid processing time and low cost. This review intends to provide an updated and extensive overview on the DNA-based methods for species identification in meat and meat products.

Keywords meat speciation, adulteration, authentication, meat products, PCR

INTRODUCTION

Meat is one of the most nutritious delicious diets for non-vegetarians. The rising price and decreasing availability of meat steer the tendency of misrepresentation and adulteration of meat and meat products by some meat producers/sellers. One of the most common economic fraudulence widely spread in meat industry is adulteration/substitution of costlier meat with a cheaper or inferior meat. Protection of consumers from these malicious practices of meat adulterations is essential due to various reasons such as economic losses, associated health implications and religious beliefs (Zade, 2002). In order to detect such fraudulent practices of meat adulteration, there is necessity to have a simple, accurate, authentic, precise and sensitive method for meat species identification is of paramount importance.

Various detection methods have been developed till date for identification of meat species. With the advancements in analytical techniques used, authenticity and accuracy of detection methods also increased. New techniques based on molecular approach have raised the hopes of developing better identification methods, which can overcome limitations of existing methods. Earlier numerous methods were used for meat species identification such as anatomical, histological, organoleptic, chemical, biochemical, spectrophotometric, chromatographic, electrophoretic, immunological and immuno-electrophoretic assay etc.

The electrophoretic and immunological techniques are widely used for meat species identification (Hitchcock and Crimes, 1985). The immunological techniques, especially direct and indirect enzyme-linked immunosorbent assay (Andrews *et al.,* 1992; Hoffmann, 1994; Pickering *et al.,* 1995) and monoclonal enzyme-linked immunosorbent assay (Castro *et al.,* 1990;

Gracia *et al.,* 1994; Martin *et al.,* 1991; Chen *et al.,* 2004) are the methods of choice for meat species identification. The electrophoretic techniques widely used for the purpose of meat species identification are SDS-PAGE (Zerifi *et al.,* 1992; Bhilegaonkar *et al.,* 1990), isoelectric focusing (King, 1984; Abraham, 2001), immunoelectrophoresis (Srinivas *et al.,* 1991) and counter immunoelectrophoresis (Reddy and Giridhar, 1995; Necidova *et al.,* 2002).

There are numerous methodologies developed for meat species identification. Chemical methods are time consuming, cumbersome and unable to distinguish between marker and other compounds of similar activity (Hayden, 1979). Identification of meat by electrophoretic techniques such as IEF and SDS-PAGE presume that the protein composition of meat is similar within species. But even electrophoretic pattern of serum and brain protein of same species could be different whereas that of muscle protein of different species (e.g. sheep and goat) could be similar (Koh *et al.,* 1998). Moreover, proteins are denatured during heat and pressure processing, resulting in changed antigenicity and electrophoretic mobility of molecules. Only antibodies raised against heat-stable biomarkers can be used for detection of species in a processed sample (Kim *et al.,* 2004). The limitation of immunological methods for species identification is that the available antisera show cross-reactions. Secondly during cooking the solubility properties and antigens competence of the proteins are altered considerably.

The use of antisera to thermostable antigens has proved to be superior in identification of cooked meat. However, use of such antigens and antisera against them are only partially successful in identification of meats of closely related species of animals like cattle and buffalo from sheep and goats (Bhilegaonkar *et al.,* 1990). Immunoassays do also have limitations, especially in respect to animal feed. Animal feed often contain processed animal proteins

(PAP's) and their detection by immunoassays is mainly of indicative character since most PAP's are insufficiently characterized. These techniques are not full proof in heat treated meat because on heat treatment proteins are denatured altering their three dimensional structures (Mane *et al.,* 2007). For these reasons electrophoretic and immunological analysis have been replaced by DNA-based assay.

DNA BASED METHODS

DNA-based molecular techniques developed over the last two decades have raised the hopes of developing authentic and reliable methods for species identification, due to the stability of DNA at high temperatures, is present in the majority of cells, and the fact that its structure is conserved within all tissues of an individual (Lockley and Bardsley, 2000; Girish *et al.,* 2004; Saini *et al.,* 2007).

DNA HYBRIDIZATION

Initially, DNA hybridization technique was used for meat species identification (Baur *et al.,* 1987; Chikuni *et al.,* 1990; Wintero and Thomsen, 1990; Ebbenhoj *et al.,* 1991; Hunt *et al.,* 1996; Buntjer *et al.,* 1999). Murphy *et al.* (2007) have developed the cloth based hybridization array system for detection of species origin of meat meals in feed grains by amplifying mitochondrial sequences and subsequent probing with species-specific oligonucleotide capture probes embedded on polyester cloth, followed by immunoenzymatic assay of the bound PCR products. This method was sensitive and permitted precise detection of meat meals in animal feeds. However, the process of fixing the probes on polyester cloth, hybridization with amplified probe and subsequent immunoenzymatic assay of the bound PCR products is laborious,

technically demanding and expensive as compared to the species-specific PCR markers. DNA hybridization is less sensitive compared to polymerase chain reaction (PCR) but also less sensitive to sample cross-contamination. DNA hybridization requires relatively inexpensive equipment but it is laborious and time consuming. DNA testing by hybridization is relatively cumbersome and so other DNA based approaches to species identification have been evolved using Polymerase Chain Reaction (PCR).

PCR BASED METHODS

The PCR is an in vitro enzymatic method which allows several million fold amplification of a specific DNA sequence within few hours. This technique was invented by Mullis *et al.* (1986) and is not only useful for molecular biologist and geneticist but also useful for forensic experts and food technologists. Now-a-days PCR assay is gaining immense popularity in identification of species origin of meat and meat products. This is a sensitive, cost effective, precise, authentic and potentially applicable technique for authentication of meat and meat products due to its lesser complexity and fast reliable nature. The PCR assays are also proving useful in solving the issues of traceability of live animals and derived products (Cunningham and Meghen, 2001).

The ingredient required for successful PCR amplification are template DNA, pair of forward and reverse oligonucleotide primers, all four deoxynucleotide triphosphates, a thermostable DNA polymerase enzyme and reaction buffer. The PCR technique involves denaturation, primer annealing and elongation steps for a set number of times depending on the degree of amplification required. This generates billions of copies of desired DNA segment from

picograms quantities of starting DNA in matter of few hours (Chikuni *et al.,* 1994). However, the other parameters are also important for successful amplification of desired PCR products, such as DNA quality, primer concentration, different thermocyclers, brand of DNA polymerase, Mg^{++} concentration, annealing temperature and final extension periods (Meunier and Grimont, 1993; Macpherson *et al.,* 1993).

PCR based method have used the mitochondrial DNA (mt DNA) as well as genomic DNA sequences, however mt DNA sequences have certain unique advantages over the genomic DNA sequences. Animal mt DNA is a small (15-20 kb) circular molecule, composed of about 37 genes coding for 22 tRNAs, 2 rRNAs and 13 mRNAs, the latter coding for proteins mainly involved in the electron transport and oxidative phosphorylation of the mitochondria. The mt genome is arranged very efficiently. It lacks introns, has small intergenic spacers where the reading frames even sometimes overlap. The control region is the primary no coding region, and is responsible for the regulation of heavy (H) and light (L) strand transcription and of H-strand replication. The mt DNA sequences have been widely used in evolutionary genetic studies because they are easily accessible, have a high rate of evolution, and generally follow a clonal pattern of inheritance highly suited to phylogenetic reconstruction.

As a molecular marker, mt DNA possess several advantages over nuclear DNA for studies of speciation in meat products (Lockley and Bardsley, 2000). There are many mt DNA molecules within each mitochondrion, making mt DNA a naturally amplified source of genetic variation. It evolves faster than nuclear DNA (Brown *et al.,* 1982). Different regions of the mitochondrial genome evolve at different rates (Saccone *et al.,* 1991) allowing suitable regions to be chosen for the question under study. The mt DNA tends to be maternally inherited so that

individual normally possess only one allele and thus sequence ambiguities from heterozygous genotypes are generally avoided (Gyllesten *et al.*, 1991); biparental inheritance in marine mussels (Zouros *et al.*, 1992). The mt DNA does not recombine (Hayashi *et al.*, 1985), though some evidence of recombination events has recently been reported (Hagelberg *et al.*, 1999).

The relatively high mutation rate compared to nuclear genes has tended to result in the accumulation of enough point mutations to allow the discrimination of closely related species. It should be noted that mt DNA also exhibits a degree of intra specific variability and so care has to be taken when studying differences between organisms based on single polymorphisms (Chow and Inogue, 1993). The cytochrome b (cyt b) sequences are good tools for studying phylogenetics of closely related species. Within species, control region sequences usually are a better choice, because more relaxed structural and functional constraints lead to a faster average substitution rate. Since D-loop region is a hyper variable region of mitochondrial DNA and hence it is possible to select the sequences, which are specific to particular species. The mitochondrial D-loop region was initially selected to accomplish meat identification because it has the highest substitution rate of all mitochondrial genes, and is the most rapidly evolving region of the mitochondrial genome. The D-loop is included in the control region of the mt DNA and is flanked by the $tRNA^{pro}$ and $tRNA^{phe}$ mt genes (Sbisa *et al.*, 1997). The variable regions of the cyt b gene (Kocher *et al.*, 1989; Matsunaga *et al.*, 1999a and Veerkaar *et al.*, 2002) offer two main advantages:(a) mt DNA 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 increase 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 (b) the large variability of mt DNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species (Hopwood *et al.,* 1999 and Prado *et al.,* 2002).

The heat stability and large copy number of mitochondrial DNA in meat tissue contribute to the protection and survivability of the fragments of DNA that are sufficient enough to be amplified by PCR (Girish *et al.,* 2004). Using an appropriate primer pairs, mitochondrial sequences have been amplified in many species and the resulting differences used for species identification (DiPinto *et al.,* 2005; Herman, 2001). The mitochondrial encoded gene for 12S rRNA was selected in this work for meat species identification because it has an adequate length and grade of mutation, exhibiting a typical mosaic structure of phylogenetically conserved and variable regions (Cronin, 1992). Further, it was proved that mitochondrial markers were more efficient than nuclear markers (RAPD finger printing and Actin gene barcoding) in species identification and authentication purposes (Rastogi *et al.*, 2007). Other workers also suggested that mitochondrial markers are more efficient than nuclear markers for the purpose of identification and authentication meat species (Hopwood *et al.*, 1999).

PCR assays based on its amplification were shown to be more sensitive as compared to single or low copy nuclear DNA targets (Partis *et al.,* 2000). Since, the quantity of PCR products generated corresponds to the copy number of the target DNA sequence (Partis *et al.,* 2000). Girish *et al.* (2005) reported that a higher copy number of mitochondrial DNA ensures a sufficiently high quantity of PCR product, even when small amounts of fresh/or processed meat samples were used.

SPECIES-SPECIFIC PCR

The identification of species origin of meat and meat products by species-specific PCR assay is simple, sensitive and rapid as compared to other PCR assays. However, the number of species to be identified in a single PCR reaction is limited. This PCR assay is employed for the identification of species origin of meat by targeting genomic and mitochondrial DNA. This assay is very powerful for detection of species in mixed meat products. Species-specific PCR assay has been used for species identification in meat and meat products by targeting nuclear DNA by various workers. The pork was identified in fresh and heated beef mixture by amplifying porcine-specific growth hormone gene fragment (Meyer *et al.*, 1994). The mitochondrial DNA which in haploid, lacks recombination and transmitted maternally has been exploited for species identification with the help of species specific primers by various workers. The commonly targeted mitochondrial regions for species identification purpose are 12S rRNA, 16S rRNA, D-loop and Cytochrome b regions.

The mitochondrial DNA assay was applied successfully for the detection and discrimination of chicken, turkey, pig, cow and sheep tissues in animal food ingredients using primers designed on cytochrome b gene by Herman (2001). Beef in meat products was detected using primers designed on mitochondrial cytochrome b gene by Piknova and Kuchta (2002). Raw and heated beef was detected by amplifying 1.709 satellite DNA within 6 hrs (Zhang *et al.*, 1999). Montiel *et al.* (2000) designed highly species-specific primers for D-loop mtDNA for restrictive PCR amplification of 531 bp bands from pork meat and fat in meat mixtures, dry-cured and heated products. They also distinguished wild boar and pork by a simple AvaII

restriction analysis. Bovine, ovine, porcine and poultry species were successfully detected in meat and bone meal using ATPase 6/8 gene by Colgan *et al.* (2001).

Rea *et al.* (2001) developed the duplex-PCR to identify bovine and water buffalo DNA in a single PCR assay in milk and mozzarella cheese (a typical Italian cheese, originally made from pure water buffalo milk). The results of this experiment indicated the applicability of this method, which showed an absolute specificity for the two species and a high sensitivity even down to low DNA concentrations (1 pg). In bovine and water buffalo mixtures of milk and mozzarella cheese, the minimum concentration tested was 1% of bovine in water buffalo milk and water buffalo in bovine milk. Swine-specific DNA primers were successfully designed for detection of pork in wide range of meat and meat products in raw and cooked meats, sausages, cured meat products, hamburgers and patties (Calvo *et al.,* 2001a). This assay was also used for detection of pork in raw and cooked beef and duck pate by Calvo *et al.,* (2002a). It was suggested that specific PCR amplification of a repetitive DNA element is a powerful technique for the identification of beef in processed and unprocessed food, because of its simplicity, specificity and sensitivity (Calvo *et al.,* 2002b). Castello *et al.* (2004) used species-specific PCR assay for detection of bovine and porcine specific mitochondrial DNA fragments of 134 bp cytochrome b and 271 bp ATPase 8-ATPase 6 genes from spray-dried blood products. The ostrich was differentiated from emu by amplifying primers designed on cytochrome b (Colombo *et al.,* 2000).

Walker *et al.* (2004) designed and evaluated species-specific PCR assays for the identification of equine, canine, feline, rat, hamster, guinea pig and rabbit using amplification of genome-specific short and long interspersed elements. Meat of deer species was differentiated

from common domestic animals using primers designed on mitochondrial cytochrome b gene without any cross reaction (Rajapaksha *et al.*, 2002). The effect of different cooking methods was evaluated for PCR amplification of mitochondrial DNA without any adverse remarks except pan frying (Arslan *et al.*, 2006). The detection limit reported by various workers ranged from 0.01 to 5% (Meyer *et al.*, 1994; Buntjer *et al.*, 1995; Calvo *et al.*, 2002a).

Ilhak and Arslan (2007) successfully applied species-specific PCR assay for identification of species origin of meat by amplification of 439, 322, 274, 271, 225, 212 and 157 bp from horse, dog, cat, bovine, sheep, porcine and goat respectively, with detection limit at 0.1% level after 35 PCR cycles in mixed meat. Pork identification in four types of food products, which were sausages and the casings, bread and biscuits, using species-specific polymerase chain reaction (PCR) detection of a conserved region in the mitochondrial (mt) 12S ribosomal RNA (rRNA) gene was developed. Genomic DNA of the food products were successfully extracted except for the casing samples, where no genomic DNA was detected. The extracted genomic DNA was then subjected to PCR amplification targeting the specific regions of the 12S rRNA gene. The genomic DNA from the food products were found to be of good quality and produced clear PCR products on the amplification of 12S rRNA gene of 387 bp from pork species.

The species-specific PCR identification yielded excellent results for identification of pork derivatives in food products and it is a potentially reliable and suitable technique in routine food analysis for halal certification. (Che Man *et al.*, 2007). A species-specific PCR assay was developed for the detection of low levels of pork, horse and donkey meat in cooked sausages. Oligonucleotide primers were designed for amplification of species-specific mitochondrial DNA sequences of each species and detected the presence of 0.01 ng of template DNA in water. When

applying the assay to DNA extracts from sausages samples that were prepared from binary meat mixtures, it was possible to detect each species when spiked in any other species at the 0.1% level. They suggested that this assay can be used to determine mislabeled and/or fraudulent species substitution in comminuted meat products (Kesman *et al.*, 2007). Frezza *et al.* (2008) successfully designed and evaluated species-specific PCR for the detection of bovine, ovine, swine and chicken mitochondrial DNA from heat treated material.

Haunshi *et al.* (2009) reported that species-specific PCR is simple, economical and quick as compared other methods as RAPD, RFLP-PCR and sequencing method of species identification. Mane *et al. (*2009) developed polymerase chain reaction (PCR) assay for specific detection of chicken meat using designed primer pair based on mitochondrial D-loop gene for amplification of 442 bp DNA fragments from fresh, processed and autoclaved meat and meat products. The PCR result was further verified by restriction digestion with HaeIII and Sau3AI enzymes for specific cutting site in amplified DNA fragments. The specificity of assay was cross tested with DNA of cattle, buffalo, sheep, goat, pig, duck, guinea fowl, turkey and quail, where amplification was observed only in chicken without cross reactivity with red meat species. However positive reaction was also observed in quail and turkey. They reported that no adverse effects of cooking and autoclaving were found on amplification of chicken DNA fragments. Thus, the detection limits was found to be less than 1% in admixed meat and meat products. The developed assay was found specific and sensitive for rapid identification of admixed chicken meat and meat products processed under different manufacturing conditions.

MULTIPLEX PCR

The advanced PCR assay was employed by various workers for simultaneous, precise and rapid identification of multiple meat species in single PCR reaction employing multiple primer pairs targeting genomic and mitochondrial DNA. Different primer sets for each species or common forward and species-specific reverse or vice versa have been used for multiple species identification. The nuclear DNA was targeted by various workers for meat species identification by multiplex PCR. Fei *et al.* (1996) designed multiplex PCR primers based on mt D-loop sequences and identified cattle, pig and chicken meats. Behrens *et al.* (1999) used multiplex PCR assay for identification of chicken meat, turkey meat, beef, pork, goat meat, mutton, donkey meat and horse meat. The detection limit was reported to be 1 % in heated and processed meat products. Cantoni *et al.* (2001) amplified the growth hormone gene fragments of 130 bp and 105 bp in cattle and swine respectively. Rodriguez *et al.* (1991) differentiated goose and mule duck in foiegras targeting nuclear 5S rDNA. Matsunaga *et al.* (1998b) differentiated mammalian, poultry and fish species in meat mixture targeting 18S rDNA gene to get specific PCR product of 293, 254 and 267 bp fragments, respectively.

This PCR assay was also employed by Dalmasso *et al.* (2004) for identification of ruminant, poultry, fish and pork materials using 12S rRNA, tRNA Val and 16S rRNA gene to generate species-specific PCR fragments of 104-106, 183, 220-230 and 290 bp respectively, with the detection limit of 0.004% for fish primers and 0.002% for ruminants, poultry and pork primers. Matsunaga *et al.* (1999a; 1999b) targeted mitochondrial cytochrome b gene variability using common forward primer and six reverse primers for identification of goat, chicken, cattle, sheep, pig and horse to get PCR amplified DNA fragments of 157, 227, 274, 331, 398 and 493 bp, respectively, in fresh and cooked meat including autoclaved meat (except horsemeat) heated

at various temperatures. Rodriguez *et al.* (2003) clearly identified goose, mule duck, chicken, turkey and swine in foie gras using common forward primer designed on mitochondrial 12S rRNA gene and species-specific reverse primers to detect approximately 1% of adulteration. Further, Rodriguez *et al.* (2004) developed a PCR assay for the specific and quantitative detection of pork, beef, sheep and goat using common forward primers designed on mitochondrial 12S rRNA and species-specific reverse primers with detection limits of 1% (wt/wt) in raw and heat treated meat mixtures. The detection limits by multiplex assay were reported to be between 0.002% and 3.6% by various workers (Behrens *et al.,* 1999; Matsunaga *et al.,* 1999b; Dalmasso *et al.,* 2004).

Species-specific DNA fragments could be identified in a single PCR reaction by mixing CSP, Chicken-R, Cattle-R, Sheep-R, Pig-R and Horse-R primers in the ratio 1:0.01:0.01:0.01:0.01:0.10 (where '1' represents 0.5 mmol L^{-1}). The specific DNA fragments could be efficiently amplified by CSP-M-PCR with >5 umol L^{-1} species-specific primers, except for 50 umol L^{-1} Horse-R. A detection limit of 0.5 g kg^{-1} was determined for both single species of minced meat and all species of five kinds of minced meat. The CSP-M-PCR method simplified the PCR reaction system and removed the inconsistent amplification efficiency from different primers. This highly sensitive, reproducible and rapid method could potentially be used as a screening or identifying assay to test for the presence of species or ingredients in minced meat and other meat products (Wentao *et al.,* 2008).

Conserved region from mitochondrial 12S rRNA and 16S rRNA genes are very useful region to evaluate the presence of accidently added meat in compound food by multiplex polymerase chain reaction assay for the identification of most species (ruminant, poultry and

porcine). For each food sources (ground meat, sausages and cold cut) 10 samples were collected and DNA extracted successfully. The results demonstrated that none of the samples were contaminated with porcine residuals but 40% of sausages samples and 30% of cold cut samples were contaminated with poultry residuals. Also, the ground meat samples were not contaminated with poultry residuals (Ghovvati *et al.*, 2009).

Yin *et al.* (2009) reported that prevention of possible adulteration of yak meat with cattle meat, based on the sequence of mitochondrial 12S rRNA gene, a multiplex PCR-based approach was proposed for rapid identification of the meat from yak and cattle using three primers designed in this work. Through the combinatorial usage of three primers with a single reaction set, two fragments of 290 and 159 bp were amplified from the cattle meat DNA, whereas only a fragment of 290 bp was obtained from the yak meat DNA. Using the assay described, satisfactory amplification was accomplished in the analysis of raw and heat-treated binary meat mixtures of yak/cattle with a detection limit of 0.1% for cattle meat. The technique was fast and straightforward. It might prove to be a useful tool in the quality control of yak meat and meat products

PCR-RFLP

The Restriction Fragment Length Polymorphism (RFLP) also allows the amplification of conserved region of DNA sequence using PCR and the detection of genetic variation between species by digestion of the amplified fragment with restriction enzymes, which cuts the products sequence at specific site producing different fragments characteristic to the enzymes used and has been used for speciation by exploiting DNA sequence variation within the nuclear and

mitochondrial DNA (Chikuni *et al.,* 1994; Branciari *et al.,* 2000; Girish *et al.,* 2004). But this technique is complex, requires suitably equipped laboratory, strict compliance with analytical techniques and costly enzymes. PCR-RFLP is a convenient, rapid, sensitive and versatile assay for meat species identification (Veerkaar *et al.,* 2002). This assay has greater potential for application in cooked meat.

The nuclear DNA was targeted by various workers for PCR-RFLP identification of meat products. Chung *et al.* (2000) used restriction profile of melanocortnin (MCR) gene as a DNA marker for differentiation of Hanwoo meat from Holstein and Angus meats. Chicken and turkey were differentiated by amplifying actin gene locus followed by restriction enzyme digestion for detection of chicken meat, in cooked meat admixture at less than 1% levels (Hopwood *et al.,* 1999). Mutton and chevon were differentiated by PCR-RFLP analysis of satellite I DNA by *ApaI* restriction enzyme, which has site in sheep but not in goat (Chikuni *et al.,* 1994). Veerkaar *et al.* (2002) applied PCR-RFLP assay for identification of blood and tissue (meat or liver) samples of taurine cattle, zebu, banteng, bison, wisent, water buffalo and African buffalo using primers designed on centrometric satellite DNA.

PCR amplification of the mitochondrial DNA segment followed by restriction enzyme digestion was successfully used to detect multiple meat species viz. cattle, sheep, goats, deer and elk (Myers *et al.,* 2003). This assay was applied for differentiation of closely related species in marinated, heat-treated and fermented meat products using primer pair designed on mitochondrial cytochrome b gene and subsequently digestion with restriction enzymes *(AluI, RsaI, TaqI* and *HinfI)* to detect pig, cattle, wild boar, buffalo, sheep, goat, horse, chicken, turkey and game meats, even in heated admixture of pork with beef at levels below 1% (Meyer *et al.,*

1995). Raw and heat-treated ostrich meat was differentiated from other animal species using primers designed for cytochrome b gene (Abdulmawjood and Buelte, 2002).

Universal primers designed for cytochrome b gene were used to identify species in heat-treated meat products by PCR-RFLP assay using AM and *HinfI* restriction enzymes by Branciari *et al.* (2000). Zimmermann *et al.* (1998) applied PCR-RFLP assay for identification of cattle, pigs, sheep, chickens, turkeys, rabbits, European hares, dogs, cats, fallow deer, red deer, roe deer and bison by amplification of 981 bp DNA fragment from cytochrome b gene (even in roasted meat), followed by digestion with AM and *NcoI* restriction enzymes. Girish *et al.* (2005) successfully applied PCR-RFLP for species identification of beef, buffalo meat, mutton and chevon by amplification of a 456 bp mitochondrial 12S rRNA gene fragment, which were digested with AM, *HhaI*, *Apo* I and *BspTI* restriction enzymes resulting in a pattern that could identify and differentiate each of the above species. In another study, Girish *et al.* (2007) developed PCR-RFLP assay for differentiation of fresh and processed poultry meat products including those cooked at 120^{o}C for 30 min by amplification of 12S rRNA gene followed by restriction digestion with *HinfI, Mph1103I, MvaI* and Eco47I enzymes.

Matsunaga *et al.* (1998a) used primers pairs based on cytochrome b gene for differentiation of deer from domestic animals, while red and sika deer were differentiated by restriction enzyme digestion with EcoRI, *BamHI, ScaI*. Sun and Lin (2003) applied fluorescent PCR-RFLP assay using primer pairs based on mitochondrial 12S rRNA gene as an analytical and quantitative tool for identification of porcine, caprine and bovine species in cooked and autoclaved meat. The detection limit reported by various workers ranged from 1-2% in meat mixtures (Meyer *et al.,* 1995; Hopwood *et al.,* 1999).

Pfeiffer *et al.* (2004) evaluated PCR-RFLP assay to determine the source (i.e., species) of blood traces obtained from a leaf. They reported that PCR-RFLP assay can be useful for the discrimination of cattle, sheep, goat, roe buck and red deer. Malisa *et al.* (2006) developed PCR-RFLP assay for identification of game and domestic meat species by amplifying mitochondrial 246 bp fragment followed by restriction enzyme digestion with Rsa1 and demonstrated the ability of the technique in discriminating between and among wild and domestic species. Saini *et al.* (2007) applied PCR-RFLP assay for differentiation of peacocks *(Pava cristatus)* from other poultry species using universal primers based on mitochondrial 12S rRNA gene of 446 bp fragment, followed by restriction enzyme digestion with *Alu* I and Sau3AI. This assay was recently used for halal authentication of food products such as sausages and casings, bread and biscuits by amplifying 360 bp fragments of mitochondrial cytochrome b gene followed by restriction enzyme digestion with BsaJI (Aida *et al.,* 2007). However, no genomic DNA was detected from the casing, while poor quality of genomic DNA was extracted from bread and biscuits which was not amplified.

Murugaiah *et al.* (2009) developed PCR-restriction fragment length polymorphism (RFLP) in the mitochondrial genes was developed for beef, pork, buffalo, quail, chicken, goat, rabbit species identification and Halal authentication. PCR products of 359-bp were successfully obtained from the cyt b gene of these six meats. AluI, BsaJI, RsaI, MseI and BstUI enzymes were identified as potential restriction endonucleases to differentiate the meats. The genetic differences within the cyt b gene among the meat were successfully confirmed by PCR-RFLP. However, the vast majority of RFLP methods are qualitative and perform best on pure animal tissue since mixtures can produce complicated fingerprints that are not easily interpreted.

REAL-TIME PCR

The real time PCR assay is an advanced technique for quantitative detection of meat species adulteration. This assay is sensitive and has been used for pork identification using primer pair based on growth hormone gene (Wolf and Jurg, 2001). Real Time PCR assay was applied for detection and identification of pork, beef, turkey, chicken and lamb in quantities less than 0.1% even in meat heated at 115°C for 2 h. Mendoza-Romero *et al.* (2004) developed a semi quantitative method based on real-time PCR assay for detection of ruminant DNA, targeting an 88-bp segment of the ruminant short interspersed nuclear element Bov-A2. They reported that this method is specific for ruminants and is able to detect as little as 10 tg of bovine DNA.

Walker *et al.* (2004) designed and evaluated SYBR Green-based PCR assay for quantification of DNA from equine, canine, feline, rat, hamster, guinea pig and rabbit with the minimum effective detection levels ranging from 0.1 ng to 0.1 pg of starting DNA template. SYBR Green real-time PCR assay for detection of bovine meat and bone meal using ruminant-specific Bov-B SINE primers and melting curve analysis to detect 0.1% contamination of bovine and sheep meat and bone meal heated at 133^{o}C for 20 min was reported by Aarts *et al.* (2006). Bellagamba *et al.* (2006) applied a TaqMan real-time PCR assay for quantitative and specific detection of cattle, sheep, goat, swine and chicken in meat and bone meal processed at 130^{o}C, (200 kPa) for 40 min. Real time PCR and melting curve analysis has also been used in species

determination (Lopez-Andreo *et al.*, 2006). Detection by PCR probe technology adds an additional level of specificity, which allows detection in multiplex reactions and avoids detection of non-specific amplification.

Most used are TaqMan® and SYBR® Green technology. TaqMan® technology utilizes in addition to the two primers a TaqMan® hybridization probe. TaqMan® probes are designed to bind between the forward and reverse primer, and contain a fluorophore attached to the 5'-end and a quencher attached to the 3'-end. During PCR, 5'-exonuclease activity of the Taq polymerase degrades the probe and the close proximity between the fluorophore and the quencher no longer exists, which allows fluorescence and subsequently detection. SYBR® Green is a dye that binds to the minor groove of double-stranded DNA. When bound to double-stranded DNA, SYBR® Green is highly fluorescent, but unbound is not. The intensity of fluorescence is proportional to the amount of double-stranded DNA. One advantage of SYBR® Green is that a melting curve can be generated after PCR, and single mutations (Lopez-Andreo *et al.*, 2006) and deletions (Ballin and Madsen, 2007) can be detected.

Laube *et al.* (2007a) developed different TaqManTM®-polymerase chain reaction systems, which allows the detection of even minute amounts of beef, pork, lamb, goat, chicken, turkey and duck in processed foods by amplification of species-specific 108 bp DNA regions (exception: duck, 212 bp) located on the single-copy genes cyclic guanosine monophosphate (cyclic GMP) phosphodiesterase, ryanodine receptor and interleukin-2 precursor. Laube *et al.* (2007b) employed TaqMan™ PCR for quantification to determine the proportion of animal species in relation to the total proportion of meat in the food products down to a concentration of 0.1%.

The real time assay based on mitochondrial cytochrome b gene was developed for detection of unambiguous species in a meat mixture (Hird *et al.*, 2004) and low level of horse or donkey meat in commercial products (Chisholm *et al.*, 2005). Dooley *et al.* (2004) used TaqMan real-time PCR assay for detection of beef, pork, lamb, chicken and turkey with experimentally detection limit below 0.1% in raw meat admixtures. Rapid and highly specific TaqMan real-time quantitative PCR technique based on mitochondrial 12S rRNA to amplify 411bp from pork DNA and 425-428 bp from mammalian species DNA for detection and quantitation of pork in the range 0.5-5% was reported by Rodriguez *et al.* (2005).

Lopez-Andreo *et al.* (2005) reported six TaqMan real-time PCR using minor groove binding (MGB) probes for the detection/quantitation of bovine, porcine, lamb, chicken, turkey and ostrich DNA in complex samples with limit of detection ranged from 0.03 to 0.80 pg of template DNA. They further reported that analysis of experimental mixtures containing two to four different species showed the suitability of the assay for detection of more than 1% of pork, chicken or turkey and of more than 5% of cattle or lamb. Frezza *et al.* (2008) designed and evaluated four species-specific probes for quantification of bovine, ovine, swine and chicken mitochondrial DNA by Real Time PCR to detect short species-specific sequences amplifiable from heat treated material and the detection limit for swine, chicken, ovine and bovine genomic DNA was 0.5 ng, 0.5 ng, 0.01 ng and 0.05 ng, respectively.

RAPD-PCR

The Random Amplified Polymorphic DNA-Polymerase Chain Reaction (RAPD-PCR) also called AP-PCR amplifies the non-targeted DNA using arbitrarily random primers without

regards to the sequences of genome to be fingerprinted (Welsh and McClelland, 1990). The resulting specific and distinct patterns can be used to differentiate meat species (Min *et al.,* 1996). RAPD-PCR is highly discriminatory and provides a simple, fast, reproducible and sensitive fingerprinting method for simultaneous identification of multiple meat species in a single PCR reaction (Saez *et al.,* 2004; Lee and Chang, 1994) under stringent conditions (Koh *et al.,* 1998; Huang *et al.,* 2003). This assay is also suitable for identification of species in meat and meat products under different processing conditions (Calvo *et al.,* 2001b).

The RAPD-PCR assay has been used for identification of species in meat and meat products by various workers. The bovine, caprine, porcine, poultry (chicken and duck), dog, rabbits, rat and human species were differentiated using a single random primer (Lee and Chang, 1994). Korean cattle (beef), deer (venison), sheep (mutton), and goat (chevon) were identified by extracting genomic DNA from muscle tissue (Min *et al.,* 1996). Rao *et al.* (1996) obtained clear and distinct band patterns to differentiate the Indian zebu cattle *(Bos indicus),* buffalo *(Bubalus bubalis),* sheep *(Ovis aries)* and goat *(Capra hircus).* Martinez and Malhedenyman (1998) developed species-specific RAPD fingerprints for wide variety of samples like beef, buffalo meat, pork, lamb, horse, mule, donkey, elk, reindeer, kangaroo, ostrich and lammerull.

Koh *et al.* (1998) differentiated wild boar, pig, horse, beef, venison, dog, cat, rabbit and kangaroo by RAPD-PCR. Martinez and Danielsdottir (2000) used this assay for identification of marine mammalian species. Random amplified polymorphic DNA (RAPD) analysis takes advantage of short arbitrary PCR primers and produces a range of amplified products. The RAPD technique is very powerful where reference material for comparison is available but where little or no information on the DNA sequence is known. RAPD is therefore not only

relevant to domestic animals, but also to rare species, since no prior knowledge of DNA sequences are required. However, RAPD reproducibility has been reported to be poor (Fernandez *et al.,* 2003). RAPD-PCR assay is more accurate and efficient than actin fingerprinting (Rastogi *et al.,* 2007).

Other studies conducted on RAPD-PCR for differentiation include beef of different breeds (Choy *et al.,* 2001); animal species such as bovine, goat, pig, dog, rat, rabbit, chicken (Calvo *et al.,* 2001b); poultry, pig, cattle including mixed meat of ostrich and cattle (Huang *et al.,* 2003); and beef, pork, buffalo meat, mutton and chicken meat (Mane *et al.,* 2006 and 2008). The detection limit was as little as 250 pg of DNA (Calvo et al., 2001b).

SEQUENCE ANALYSIS OF PCR ASSAY

PCR amplification and sequencing of conserved genes and their comparison is one of the precise technique for meat species identification. Characterization of animal species by DNA sequencing depends on availability of known sequences. Much information is present in databases (e.g. Genbank: http://www.ncbi.nlm.nih.gov/) with a large number of sequences from common animal species, breeds, and genetic variations. Sequencing can be used to identify species in unknown samples, even if no reference material is available; it only requires that species have a unique DNA sequence. Sequences obtained from PCR amplicons, and identification through database comparison, has in a number of cases established species identity (Forrest and Carnegie, 1994; Iijima *et al.,* 2006 and Imaizumi *et al.,* 2007).

Identification by sequencing is the most direct and valid way to analyze PCR amplicons and it is often used as a confirmative analysis following results obtained by gel electrophoresis

and real time PCR. Sequencing can easily be applied to individual PCR amplicons; however, if more amplicons are produced in the PCR reaction, a separation of amplicons (e.g. cloning) must be done prior to traditional Sanger sequencing. This process can be laborious and it is therefore advantageous to perform simplex PCR prior to sequencing. However, new sequencing technologies, e.g. pyrosequencing, allows for sequencing of PCR product mixtures, but the large amount of sequences obtained (i.e. >400,000 sequences) and the price per run will limit the usefulness of these technologies at least for a while. We are not aware that these new sequencing technologies have been used so far in meat adulteration analysis as mitochondrial DNA is highly conserved, genes like cytochrome b, 16S rRNA and 12S rRNA have been used by different workers as molecular markers. However, cytochrome b and 12S rRNA are the commonly used molecular markers for identification of meat species. Forrest and Carneige (1994) amplified and sequenced a region of cytochrome b gene to identify buffalo, emu and crocodile meat via a phylogenetic comparison with the help of computer program.

Bradmann *et al.* (2001) reported sequencing of PCR fragment followed by search in an internet accessible database to identify unknown game species. Prakash *et al.* (2000) sequenced and compared 12S rRNA gene to differentiate the tiger skin from bovine skin. Bottero *et al.* (2003) amplified fragments between 234 and 265 bp using primers designed on mitochondrial 12S rRNA gene, even in DNA extracted from severe rendering treatment (at 134.4 to 141.9^{o}C and 3.03 to 4.03 bar for 24 min) for identification of bovine, porcine, goat, sheep, horse, rabbit, chicken, trout and European pilchard. The detection limit of 0.0625% for PCR assay was confirmed by sequence analysis of amplicons. Rastogi *et al.* (2004) used partial sequence analysis of 450 bp of mitochondrial (mt) 12S rRNA gene followed by homology search in

BLAST at NCBI was used for identification of cooked and uncooked meat samples of various known and unknown origin including viscera, and biological fluids like blood and semen using universal primers.

Girish *et al.* (2004) applied the PCR amplification and sequencing to the mt 12S rRNA gene for identification of cattle *(Bos indicus)*, buffalo *(Bubalus bubalis)*, sheep *(Ovis aries)*, goat *(Capra hircus)* and mithun *(Bos frontalis)*. They further reported that there was no effect of routinely used additives or various cooking temperatures (72, 90, 120 and 180°C) on the efficacy of PCR amplification, even closely related species like cattle and buffalo, sheep, and goat could also be differentiated decisively by sequence analysis. Hsieh *et al.* (2005) employed partial sequence analysis of 402 bp PCR amplified fragments of cytochrome b gene for identification of animal species in fresh and processed samples of unknown origin. Recently, Pandey *et al.* (2007) used sequence analysis of 12S rRNA gene sequence for identification of Indian leopard *(Panthera pardus fusca)* by extraction of DNA from scat samples.

OTHER PCR TECHNIQUES

Various other PCR assays were also used for meat species identification. The arbitrarily primed (AP-PCR) assay is similar to RAPD-PCR assay with slight difference in bp size. Ten mer oligos are used in RAPD, while 18 mers oligos or more are used in AP-PCR for PCR amplification, which randomly and/or arbitrarily amplify the DNA in reaction mixtures. Saez *et al.* (2004) reported that AP-PCR allowed identification of five tested species in every sample although more complex patterns including some low intensity bands were generated. The amplified fragment length polymorphism (AFLP-PCR) was applied to discriminate between

Japanese Black and F1 *(Japanese Black x Holstein)* breeds for elimination of falsified breed labeling of meat by Sasazaki *et al.* (2004).

Guha and Kashyap (2005) designed heminested PCR assays by two consecutive amplifications of the mitochondrial 16S rRNA gene for identification of Blackbuck, Goral, Nilgai, Hog deer, Chital, Sambar and Thamin deer. In the first stage, ~550 bp region of the 16S rRNA gene was amplified by PCR from template DNA using universal primers. In the second stage, a species-specific internal region of the 16S rRNA gene was amplified by PCR using the amplicon of the first PCR along with one universal primer and another species-specific primer as the reverse or forward primer.

Fajardo *et al.* (2007) showed adequate specificity and reproducibility against a number of game and domestic meats, even in the DNA extracted from pasteurized (72°C for 30 min) and sterilized (121°C for 20 min) meat. Martin *et al.* (2007) developed PCR assay using primer pair based on targeting mitochondrial 12S rRNA gene for the qualitative detection of four duck species in meat mixtures. They reported that the technique could be applied to raw as well as sterilized muscular binary mixtures, with a detection limit ranging from 0.1% to 1.0% (w/w).

CONCLUSIONS

With increasing demand for animal protein and increasing awareness among consumers regarding composition of food, it becomes essential to correctly authenticate the source of food. In past, researchers have employed several techniques for species identification with every technique having its own advantages and disadvantages. In last decade, DNA based techniques for species identification replaced other conventional techniques and gained popularity due to

conserved DNA molecule in any tissue of an individual and relative stability of DNA at high temperatures. Using DNA based methods have not only increased our detection specificity but also the sensitivity of detection. Advanced DNA based techniques like Real time PCR not only detects the species involved but also quantifies the extent of adulteration. But still, there is huge scope of improvement for the appropriateness of these techniques related to time taken for result forecasting. It usually takes a whole day or two to give results. Therefore, we need to have such rapid techniques that may reduce the result forecasting but at the same time do not compromise the accuracy of results.

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