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Fraudulent Adulteration/Substitution of Meat: A Review

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Abstract

Meat is defined as the dressed flesh, of certain animals, consumed as food. Most often this includes the skeletal muscles and associated fat and other tissues along with edible organs and offal. Meat and meat products have a great significance in human nutrition and thus for maintenance of consumer health. Meat is very rich source of proteins, containing all the essential amino acids and in most cases is a good source of iron, phosphorus, zinc, selenium, riboflavin, niacin, vitamin B_6, vitamin B_{12}, choline etc. Red meat, such as beef, pork, and mutton contains many essential nutrients necessary for healthy growth and development in children. Red meat is one of the best sources of iron and zinc which is well absorbed by the body. Sizable population of India consumes meat in the form of fresh meat and different meat products. Meat is also utilized as a component in many vegetable based delicious dishes.

Keywords: Meat adulteration, Nutritional value.

Introduction

In Jammu and Kashmir the people are predominantly non-vegetarian, more so in Kashmir valley where meat and meat products are consumed since ages as exemplified by the world famous Kashmiri cuisine, *Wazwan*. The prominent *Wazwan* dishes include *Rista, Gushtaba, Kababs* etc. Almost all the dishes under *Wazwan* are meat-based and generally prepared from mutton. Essentially these are flavorful, ready-to-eat meat products which are usually prepared fresh and served hot as part of splendid meals. The demand for ready-to-eat meat products including *Wazwan* products is increasing day by day mainly due to the socio-economic development and changing life style. Besides their immense local popularity and demand, these products also cater to the fast food requirements of a large number of domestic and foreign tourists and are relished by one and all who visit Kashmir. As meat and meat products represent an important and large component of human food, their quality is of concern to the consumers, the regulatory authorities, the processors and the retailers. The higher demand for meat and meat products accompanied by their escalating cost makes them prone to fraudulent adulteration, substitution and mislabeling. Adulteration literally means debasing something or rendering it impure by mixing it with some inferior or harmful substance. The determination of food authenticity and the detection of adulteration are major issues in the meat industry and are attracting increasing amount of attention. Identification of the species of origin in meat samples is relevant to consumers for the possible economic loss from fraudulent adulterations, medical requirements of individuals who might have specific allergies and for religious reasons. Food allergies to meats such as beef, chicken, turkey, mutton, and rabbit are not unusual. Allergies to meats can be exacerbated in the ill, the young, the old or the immuno-compromised patients. Those following religious dietary codes are sensitive towards meat adulteration. Also there is a ban on the slaughter of cows in many states of India. The fraudulent meat adulteration practice in meat industry introduces unfair competition in meat trade. The consumers nowadays, are more quality conscious and prefer high quality meat products and are, therefore, worried about the meat quality and its integrity all through the food chain. Meat speciation is thus a vital measure to ensure food safety to the consumers as it helps in maintaining the standards related to meat and meat products.

The fraudulent adulteration of costly meat with

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cheap meat is a practice that has been observed all over the world. The meat industry in India is largely unorganized; hence adulteration or substitution of meat in meat products is likely to be practiced. It has been estimated that about 25-30% of meat sold in India is adulterated and the J&K state cannot be an exception to it. In Kashmir valley, the chances of meat adulteration become more frequent during closure of National Highway during inclement weather conditions, as valley is largely dependent on other states for supply of meat animals. Mutton is the costliest meat available in the state and may be adulterated with beef, buffalo meat etc. However, compared to fresh meats, the chances of adulteration are more in processed and comminuted meat products like *Wazwan* products because the comminution, processing and using other ingredients can mask the adulteration effects thus putting the consumers to great risk and immense disadvantage.

Accurate species identification of meat products is important to enforce acts related to livestock products, to maintain livestock product standards, to prevent unfair competition in meat industry, to regard religious and socials customs, to control wild animals poaching etc. The detection of meat species in different foods and feedstuffs deserve special attention too due to the emergence of zoonotic diseases through meat products. Thus, there is a great scope and need, not only for hygienic meat production and processing, but also for the identification of meat species, so as to ensure meat product quality and prevent consumer from being victim of fraudulent adulteration. Therefore, reliable techniques or methods to identify the origin of species in a meat product are necessary for meat authentication purposes.

Several methods exist for determination of the origin of animal species in meat products. These methods are broadly based on physical, chemical and biochemical properties of meat but each is beset with its own limitations. The physico-chemical methods are reliable only in unprocessed raw meats and have no use in ground meats. These methods are affected by age, sex, plane of nutrition etc and are difficult to interpret. In electrophoretic techniques, it is presupposed that the protein composition of meat is similar within the species and are not effected by any factor. However, electrophoretic profile gets influenced by several factors and therefore, results are inadequate and ambiguous in processed and adulterated meat samples. The immunological techniques involves cumbersome process for isolating species specific proteins and face difficulty in distinguishing closely related species which hampers their effectiveness. The available antisera show cross-reactions and during cooking the solubility properties and antigenic 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 or sheep and goats. The methods are also time consuming and ineffective at lower level of adulteration and are qualitative only.

In recent past, DNA as a source of information has been used for speciation of meats. The DNA based technology for such purposes has several advantages. The DNA is omnipresent in all cell types of an individual with identical genetic information irrespective of the sample origin. The information content of DNA is more abundant due to degeneracy of the genetic codes i.e. a specific amino acid is coded by more than one codon. The extraction and analysis of DNA from meat tissues is highly feasible. DNA is extremely stable, survives food processing, conserves structure within all tissues of an individual and allows individual, breed or species identification. The DNA based analysis is superior in terms of specificity, accuracy, reliability and legal acceptability. Two major approaches to identify species of meats by DNA techniques are DNA hybridization and DNA amplification. A number of strategies have been employed in amplification based species identification including use of repetitive sequences, multigene family and use of mitochondrial gene. Compared to use of nuclear DNA, the detection method based on mtDNA can improve the sensitivity further because of their high copy number (about 2,500 copies) of mtDNA against just few copies of genomic DNA per cell. Therefore, mtDNA can be more efficiently used to detect species-specific DNA.

Physico-Chemical Techniques

Singh and Sachan (2011) have given a review of various techniques for meat species identification including the physico-chemical methods employed and stated that physical methods gave the primary idea about the meat species on the basis of quality characteristics of meat. The authors further stated that for meat species specifications, the amount of certain chemicals present in meat of different animal species were estimated. However, the physico-chemical methods were applicable if the meat was in carcass form but the reproducibility and quantitative identification was not possible. The problem would be more complex with ground and processed meat and meat products.

Immunological Techniques

Sherikar *et al*. (1988) studied the use of species specific antisera to adrenal heat-stable antigens for identification of raw and cooked meats of domestic animals by agar gel diffusion and counter immune-electrophoretic techniques and found that one part of meat of a species could be detected in 300 parts of meat mixtures and reported it as rapid and sensitive test.

Govindarajulu (1989) described the use of ELISA for meat speciation in domestic animals (cattle, buffalo, sheep, goat) and mentioned ELISA as a rapid and highly sensitive method for speciation purpose as it could detect up to 2% adulteration and was most suitable method for handling numerous samples at a time.

Thumber (2002) studied meat speciation by

serological techniques. Meat samples from cattle, buffalo, sheep, goat, pig and chicken were utilized for serological analysis. An agar gel precipitation technique (AGPT) was successful for identification of meat species, cross reaction study and detection of adulteration level in meat. Hyper-immune sera were raised in rabbit by intramuscular injection of meat extract. These sera were used to detect level of adulteration. By AGPT, up to 10% level of adulteration of buffalo and cattle meat with sheep and goat meat was detected successfully. The counter-immuno-electrophoresis techniques were also successfully used to detect meat species and were found to be rapid as compared to the AGPT.

Molecular Techniques

Polymerase Chain Reaction

Rao *et al.* (1995) conducted a PCR assay for sex- specific identification of raw meat from domestic animals (cattle, buffalo, sheep and goat). The genomic DNA was extracted from raw muscle tissue of the male and female animals and it was found that the method is simple reliable and accurate for the sex-specific identification of raw meats of these species.

Ganai *et al.* (2000) studied DNA amplification fingerprinting of cattle and buffalo genome by polymerase chain reaction-random amplification of polymorphic DNA (PCR-RAPD) method, utilizing arbitrary oligonucleotide primers. For amplification of genomic DNA of cattle (*Bos indicus*) and buffalo (*Bubalus bubalis*) with short arbitrary oligonucleotide primers, four primers with the sequence, 5' GTGACGTAGG-3′ (G_1), 5'-TGCCGAGCTG-3′ (G_2), 5'-GTGGTGGTGG-3 (G_4) and 5'-GCGAGCGTCCC-3′ (G_7), were used. The two primers, G_4 and G_7, did not yield any amplification product in PCR with templates of cattle and buffalo genomic DNA even after changing reaction conditions. But remaining two primers, G_1 and G_2, yielded the amplification product in cattle as well as buffalo genomic DNA and exhibited highly polymorphic patterns.

Thumber (2002) carried out meat speciation by molecular techniques also. Meat samples from cattle, buffalo, sheep, goat, pig and chicken were utilized for molecular analysis. PCR was found to be a potential technique for meat speciation, consistent and effective tool for heat treated and putrefied meats and more informative than other techniques.

Rastogi *et al.* (2004) conducted species identification and authentication of meat samples (beef, mutton, chevon, pork and chicken meat) by mitochondrial *12S rRNA* gene sequence analysis and conformation sensitive gel electrophoresis. By these methods, the authors could detect adulteration of meat at a level of 10% in heat treated products.

Girish *et al.* (2005) studied mitochondrial *12S rRNA* gene by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to identify beef, buffalo meat, mutton and chevon. PCR amplification yielded 456-bp fragments in each of these species. This technique did not yield satisfactory results with meat mixtures/meats. However, consistent results were obtained with both fresh and processed meat samples.

Jain (2004) used *cyt b* gene variability in detecting meat of domestic animals (cattle, buffalo, sheep, goat, poultry, pig and horse) by Multiplex PCR. Meat was cooked in microwave oven at 100° C and 120° C for 30 min. The meat samples were allowed to putrefy in a natural condition at room temperature for 48 hours. PCR profiles of *cyt b* gene from fresh meat and putrefied meat was compared. Multiplex PCR, using mitochondrial *cyt b* gene species specific primers, successfully gave amplification of DNA from putrefied meat, indicating that putrefication did not inhibit efficiency of amplification of *cyt b* gene region of different species in Multiplex PCR. Detection limits of mixed DNA templates were less than 1 ng.

Kumari (2007) carried out a study to develop a Real Time PCR based test for identification and differentiation of meats of domestic animals particularly of cattle and buffalo meat. DNA extractions were taken from meat samples of cattle, buffalo, sheep, goat and chicken. It was possible to detect and differentiate cattle meat mixed in buffalo meat up to 1: 1000 fraction, by running a duplex PCR followed by cattle specific Real Time PCR. Real Time PCR assay developed in the study was found to be very sensitive and specific to detect adulteration of cattle meat in buffalo meat.

Rastogi *et al.* (2007) used mitochondrial *16S rDNA* gene, *NADH dehydrogenase subunit 4* (*ND4*) gene and nuclear markers viz. the *Actin* gene, for identification and authentication of tissues of animal origin like beef, buffalo meat, mutton, chevon etc. The results suggested that mitochondrial markers were more efficient than nuclear markers for the purpose of species identification and authentication.

Singh *et al.* (2007) detected species of meat through PCR technique using *Actin* gene as a marker. The species involved were cattle, buffalo, sheep, goat, pig and poultry. Both raw and cooked meat was tested. The results showed clear cut differentiation of pig and poultry meat from the cattle, buffalo, sheep and goat meat. But the technique failed to differentiate mutton from chevon and that of beef from buffalo meat.

Mane *et al*. (2009) conducted PCR assay for identification of chicken in meat and meat products, 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. No adverse effects of cooking and autoclaving were found on amplification of chicken DNA fragments. The detection limits were even less than 1% in admixed meat and meat products.

Karabasanavar ***et al.*** **(**2010) studied meat speciation of two species of birds, namely black kite (*Milvus migrans*) and parakeet (*Psittacula krameri*) using PCR assay sequence analysis of mitochondrial *12S*

rRNA gene. They found that PCR amplification of the mitochondrial *12S rRNA* gene and sequence analysis was helpful to solve the problem of identification of an avian species unambiguously.

Gupta *et al*. (2011) used single-nucleotide primer extension assay of mtDNA to authenticate cattle and buffalo meat. The method was rapid and reliable to identify and differentiate cattle and water buffalo meats targeting the mitochondrial *cyt b* region using a Snapshot assay. Snapshot assay was found to detect 1% adulteration in cattle-buffalo meat mixture. Detection of adulteration from degraded DNA obtained from cooked and putrefied samples was one of the very important merits of this technique. The Snapshot assay provided a very sensitive and specific assay to identify and differentiate cattle and buffalo meat. This method was also successful with equal efficiency in fresh, cooked, and putrefied meat.

Mahajan *et al*. (2011) studied meat speciation of beef, buffalo meat, chevon and mutton by PCR-RFLP method using mitochondrial *12S rRNA* gene. Application of this technique on adulterated meat samples could detect meats of any two animal species in proportion of 50:50 and 75:25 except in case of chevon and beef mixture. The technique, however, could not detect any of the two species when proportion of mixture was 90:10 except in case of cattle and buffalo.

Forensically Informative Nucleotide Sequencing

Girish and Nagappa (2009) described the use of forensically informative nucleotide sequencing (FINS) for meat speciation in domestic animals and stated that *12S rRNA* gene sequence analysis was the most preferred method for differentiation of beef, buffalo meat, mutton and chevon by FINS.

Work done Abroad

Physico-Chemical Techniques

Gracey *et al.* (1999) have given a comparative account of the differentiation of meats of domestic animals on various physical characteristics like colour, texture and odour of meat and fat. The differentiation of meats, on these characteristics, was workable only in raw and unprocessed meats and it failed when used in processed meats and meat products. Lawrie and Ledward (2006) differentiated beef and buffalo meat on various histological parameters like muscle fiber diameter, number of muscle fibers per cubic mm, muscle fiber length, density and pattern of the muscle fibers in different meats of animal origin. It was also workable for fresh and unprocessed meats.

Electrophoretic Techniques

Kim and Shelef (1986) studied meat speciation and quantification of different meats in meat mixtures of domestic animal by electrophoretic techniques. The method was based on differential migration of sarcoplasmic proteins under the influence of electric field. The electrophoretic patterns of sarcoplasmic proteins like *creatine kinase* isozyme and myoglobin bands from fresh beef, pork, chicken, and turkey were studied using thin layer agarose gel electrophoresis. Binary mixtures (5/95, 25/75, 50/50, 75/25, 95/5, percentage by weight of each species) were examined by their electrophoretic and densitometric patterns. The relative ratios of the bands, characterizing each species, changed in proportion to the species content in each binary mixture. These ratios made it possible to predict the approximate fraction of each species present in such mixtures.

Immunological Techniques

Isoelectric Focusing

King (1984) reported that certain enzyme staining could be used for better visualization of band patterns of IEF gels in case of lower proportion of contaminants The examples were *coomassie blue* for whole muscle samples, *phosphoglucomutase* for low levels of buffalo, pig or horse meat in beef, *adenylate kinase* for low levels of kangaroo or horse meat in beef and *phosphor gluconate dehydrogenase* for differentiation of mutton from chevon. A wide range of animal species could be identified in cooked meat by staining isoelectric-focusing gels for these enzymes.

Skarpeid *et al.* (1998) reported that IEF was a suitable method for identification of animal species even after cooking at 100°C but not suitable for closely related meat species and frozen meat condition because results were difficult to interpret and had poor reproducibility. The gel profiles were analyzed by multivariate regression to allow the determination of sample composition with prediction errors close to 10%.

Enzyme-Linked Immunosorbent Assay Technique

Patterson and Spencer (1985) studied meat speciation in domestic animals, by three ELISA techniques: Indirect ELISA, Competitive ELISA and Sandwich ELISA. These techniques were classified on the basis of compound fixed, solid support used, concentrations of antigen and antibodies. By visual assessment 0·1% donkey in horse, 0·1% goat in sheep and 1% buffalo in beef were detected. The techniques were rapid and simple to perform and could be used in abattoirs and cold stores with results being available within 1 hour.

Chen and Hsieh (2000) studied the detection of pork in heat-processed meat products by monoclonal antibody-based ELISA. The detection limit was low to 0.5% (w/w) pork in heterologous meat mixtures. The intra-assay and inter-assay coefficients of variation were 5.8 and 7.9%, respectively. The accuracy in analyzing market samples was 100%.

Molecular Techniques

DNA Hybridization

Chikuni *et al.* (1990) applied dot-blots hybridization technique for detection of species-specific DNA fragments in the cooked meats of chicken, pig,

goat, sheep, and cattle. The samples were obtained from the meats that were heated for 30 min at 80, 100 or 120°C. The biotin-labeled chromosomal DNA fragments were hybridized to the sample DNA on nylon membranes. Using this method chicken meat, pork and beef were detected from 50 mg of the commercial canned products.

Ebbehoj and Thomsen (1991) studied the species differentiation of heated meat products by DNA hybridization method. The heated meat products were made from pork and beef. Samples of known composition and heat treatment were investigated. The DNA was hybridized with a ^{32}P-labelled probe made from genomic porcine DNA. The signal intensities from filter-bound DNA probe was determined by laser densitometry of the autoradiograph. The detection limit for heat-treated samples were found to be approximately 0·5% pork in beef.

Buntjer *et al*. (1995) developed a rapid method for species identification in heated meat by using satellite DNA probes. DNA was hybridized to a conjugate of a specific oligonucleotide and *alkaline phosphatase*. Probes were developed for the identification of meat from cattle, sheep/goat, horse, deer, pig, chicken, and turkey. Differentiation from closely related species like turkey and chicken was possible. Admixture of 1-5% of meat of one species in another could be detected.

Hunt *et al.* (1997) developed a simple and non-radioactive slot blot hybridization assay using species-specific oligonucleotide probes for the species identification of rabbit, sheep, pork, beef and goat meats. Clear species discrimination was demonstrated even between the closely related ruminants (goat and sheep). The potential for semi quantization of species in admixture was demonstrated to a detection limit of less than 2.5% adulteration.

Buntjer *et al*. (1999) studied the influence of meat processing on species identification test by DNA oligonucleotide hybridization. Freezing and thawing of meat did not cause a substantial reduction in the hybridization signal. Heating of meat at 100°C or 120°C, however, led to signal reduction caused by DNA degradation, but identification was still possible. Further, no hybridization signal was obtained with corned beef.

Polymerase Chain Reaction

Gouli *et al.* (1999) established a PCR based method for the identification of beef by amplification of bovine 1.709 satellite DNA. The sequence selected for amplification consisted of a 218 bp DNA fragment lying in the 1.709 satellite DNA segment. This method was positive for bovine, buffalo and yak meat DNA, but negative for horse, sheep, goat, camel, swine, deer and mouse meat DNA, etc. At least 33.6 fg of DNA from raw beef samples and 0.32 pg of DNA from cooked or autoclaved beef samples were detected, respectively, by PCR.

Hopwood *et al.* (1999) studied *Actin* gene-related PCR test for identification of chicken in meat mixtures. In this method primers were amplified at a single *Actin* gene locus, giving a positive band with DNA extracted from chicken and turkey, but no amplification with duck, pheasant, porcine, bovine, ovine or equine DNA. The chicken meat was detectable in admixtures containing 1% chicken, 99% lamb and from heat-treated meat at 120°C. Further, the chicken PCR product was differentiated from turkey by restriction enzyme digestion.

Matsunaga *et al.* (1999) applied PCR to identify six meats (cattle, pig, chicken, sheep, goat and horse), which were used as raw materials for products. By mixing seven primers (one common forward primer and six respective reverse primers of respective species) in appropriate ratios, species-specific DNA fragments were identified by only one Multiplex PCR. The products showed species-specific DNA fragments of 157, 227, 274, 331, 398 and 439 bp from goat, chicken, cattle, sheep, pig and horse meats, respectively. Cattle, pig, chicken, sheep and goat fragments were amplified from cooked meat heated at 100°C or 120°C for 30 min, but horse DNA fragments could not be detected from the 120°C heated sample. Detection limits of the DNA samples were 0.25 ng for all meats.

Calvo *et al.* (2001) developed and evaluated a PCR procedure to detect pork in heated and unheated meat, sausages, canned food, cured products, and pates using a DNA-specific porcine repetitive element by nonspecific PCR amplification. Degree of contamination could be partially quantified by detecting up to 0.005% pork in beef and 1% pork in duck pate.

Rodriguez *et al.* (2001) reported that PCR amplification of the nuclear 5S rDNA gene could be used for the identification of goose and mule duck by Multiplex PCR using common forward primer and species-specific reverse primers. The different sizes of the species-specific amplicons, separated by agarose gel electrophoresis, allowed clear identification of goose and mule duck samples. This genetic marker was found to be useful for detecting fraudulent substitution of the mule duck liver for the more expensive goose liver.

Calvo *et al.* (2002) developed and evaluated a PCR procedure to detect beef in heated and unheated meat, sausages, and canned food using a specific and sensitive method. The degree of contamination up to 0.01% raw beef in pork was detected. Similarly 1% beef was detected in cooked meat mixture. Specific PCR amplification of a repetitive DNA element seemed to be better for identification of beef in processed and unprocessed meat and meat products.

Lockley and Ronald (2002) described a novel one-step method for the differentiation of chicken and turkey species. The technique used the PCR and primers that exploited intron variability in cardiac *Actin* gene to generate single products of a characteristic size for each species. No cross-reactivity with porcine, ovine or bovine DNA templates was apparent and analysis of chicken and turkey admixtures indicated that it was possible to detect 1% turkey in 99% chicken and vice

versa.

Hird *et al.* (2003) studied rapid detection of chicken and turkey in heated meat products using the PCR followed by amplicon visualization with vista green. DNA amplification in the polymerase chain reaction was done using species specific primers, chicken forward (CF), chicken reverse (CR), turkey forward (TF) and turkey reverse (TR). The production of an amplicon was detected after the end of the PCR in less than 5 min using vista green and a fluorescence plate reader. The presence of fluorescence denoted the presence of the target species in the sample.

Walker *et al.* (2003) designed and evaluated four assays based upon PCR amplification of short interspersed elements (SINEs) for species-specific detection and quantitation of bovine, porcine, chicken, and ruminant DNA. Using SYBR Green (a nucleic acid dye) based detection, the minimum effective quantitation levels were 0.01, 0.1, 1 and 5 pg of starting DNA template using bovine, porcine, chicken, and ruminant species-specific SINE-based PCR assays, respectively. Bovine DNA was detected at 0.005% ; porcine DNA at 0.0005% and chicken DNA at 0.05% in a 10-ng mixture of bovine, porcine, and chicken DNA templates.

Walker *et al.* (2004) designed and evaluated a series of class specific (*Aves*), order-specific (*Rodentia*), and species-specific (*equine, canine, feline, rat, hamster, guinea pig and rabbit*) PCR based assays for the identification and quantification of DNA using amplification of genome specific short and long interspersed elements. Using SYBR Green (a nucleic acid dye) based detection, the minimum effective quantification levels of the assays ranged from 0.1 ng to 0.1 pg of starting DNA templates. The species-specificity of the PCR amplicons was further demonstrated by the ability of the assays to accurately detect known quantities of species-specific DNA from mixed (complex) sources.

Aslaminejad *et al.* (2010) studied the development and use of quantitative competitive PCR assay for detection of poultry DNA in sausages. PCR is well known to be quantitative if internal DNA standards are co-amplified together with the target DNA. A DNA competitor differing by 83 bp in length from the poultry target sequence was constructed and used for PCR together with the target DNA. Specificity of the new primers was evaluated with DNA from cattle and sheep. The results of quantitative competitive PCR assay showed that the percentage of contamination was in the range of 23.87-52.06%.

PCR using Mitochondrial DNA

The use of mitochondrial DNA (mtDNA) for meat speciation offered two main advantages. Firstly, mtDNA is present in thousands of copies per cell (as many as 2,500 copies), especially in the case of post-mitotic tissues such as skeletal muscle (Greenwood and Paboo, 1999). This increased 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). Secondly, the large variability of mtDNA targets as compared with nuclear sequences facilitates the discrimination of closely related animal species even in the case of mixture of species.

Brown *et al.* (1982) reported that there were many mtDNA molecules within each mitochondrion, making mtDNA a naturally amplified source of genetic variation and it evolved faster than nuclear DNA. It was found that mitochondrial gene coding for proteins evolved 5-10 times faster than the nuclear genes. Further the *tRNA* gene was found to evolve 100 times faster in mitochondrial genome than in nuclear genome.

Hayashi *et al.* (1985) studied the recombination of mammalian mtDNA using mouse and rat somatic cell hybrid clones and rat cybrid clones. Genetic and physical analyses showed that the mtDNAs of the hybrids and cybrids were simple mixtures of the two parental mtDNAs. It was found that mtDNA does not recombine. But some evidence of recombination events were also reported (Hagelberg *et al.*, 1999).

Fei *et al.* (1996) designed Multiplex PCR primers based on mitochondrial D-loop DNA sequences and identified cattle, pig, and chicken meats. Using this method, several meat products were analyzed. Mixing of beef, pork or chicken in meat products was revealed by identification of each single specific DNA fragments. When three sets of primers were employed together, beef, pork and/or chicken specific DNA fragment from these mixed meat products could be identified by only one PCR reaction. The detection limits in a mixed DNA sample were approximately 0.1% beef in pork, 0.001% pork in beef and 1% chicken in pork, respectively.

Buntjer and Lenstra (1998) demonstrated that PCR with fluorescently labeled mammalian-wide interspersed repeat primers generated fingerprints that were suitable for rapid identification of known and unknown species on an automatic sequencing apparatus and with computer assisted data processing. The method allowed the analysis of processed meat samples and offered a convenient alternative to sequencing of mtDNA.

Tartaglia *et al.* (1998) developed a PCR based assay for the identification and detection of bovine specific mtDNA sequences from feedstuffs. The amplified product codes for the whole *ATPase* subunit 8 and the amino terminal portion of the *ATPase* subunit 6 proteins. This method could detect mtDNA in feedstuffs containing less than 0.125% of bovine derived meat and bone meals. *Dnp II* and *Ssp I* RE digestions confirmed the bovine origin of amplified sequence.

Nakaki *et al.* (1999) studied PCR-RFLP pattern of *cytochrome b* (*cyt b*) genes and compared it to identify eight species of mammal (baboon, cow, pig, dog, cat, bear, deer and raccoon dog) and two species of bird (chicken and wild duck). The PCR products of 700 bp were digested with two RE *Hae III* or *Hinf I*, and the difference was observed among mammals and birds as polymerase chain reaction-restriction fragment length

polymorphism (PCR-RFLP).

Bellagamba *et al*. (2001) studied restriction site of PCR products of mitochondrial *cyt b* gene to identify species in meat and foodstuffs. PCR was used to amplify a variable region of mitochondrial *cyt b* gene. PCR products of 359 bp amplicon were digested with *restriction endonuclease*, which generated species-specific electrophoresis pattern. The sequencing of PCR products was used as confirming analysis and PCR-RFLP analysis revealed the presence of meat meal in animal feedstuffs and distinguished species of interest.

Cheng *et al.* (2001) determined the amount of genetic variation in a 376 nucleotide region of the mitochondrial *cyt b* gene in fresh, frozen and steam sterilized meats of puffer (*Takifugu rubripes*) and diversity of sequence between fresh, frozen and steam sterilized meats was absent. *Restriction endonucleases BstZ I* cut the amplified region of *cyt b* gene while *Aat II* did not. It was found that the sequence and restriction site analyses could be used to authenticate species of different processed meats of puffer *T. rubripes*.

Verkaar *et al.* (2002) described two complementary methods for detection and differentiation of bovine species. These were based on mutations in mtDNA and centromeric satellite DNA, respectively. The analysis of satellite DNA was especially relevant for the identification of animals that were of hybrid origin.

Bellagamba *et al.* (2003) described a DNA monitoring method to examine fishmeal contamination with mammalian and poultry products. A PCR method based on the nucleotide sequence variation in the *12S ribosomal RNA* gene of mtDNA was developed and evaluated. Three species-specific primer pairs were designed for the identification of cattle, pig, and poultry DNA. The specificity of the primers used in the PCR was tested by comparison with DNA samples for several vertebrate species and confirmed to be species specific. The PCR specifically detected mammalian and poultry adulteration in fishmeal containing 0.125% beef, 0.125% sheep, 0.125% pig, 0.125% chicken and 0.5% goat.

Bottero *et al.* (2003) used vertebrate primers, designed in the *16S rRNA* gene of mtDNA for detecting animal tissues in feedstuff. These primers were able to amplify fragments between 234 and 265 bp. The fragments were specific for bovine, porcine, goat, sheep, horse, rabbit, chicken, trout, and European pilchard. The specificity of amplicons was confirmed by sequence analysis. The assay proved to be rapid and sensitive with detection limit as low as 0.0625%.

Chapman *et al*. (2003) developed a Multiplex PCR assay utilizing both nuclear and mitochondrial *cyt b* gene loci simultaneously for identification of white shark body parts, including dried fins. The method was found to be highly fruitful in diagnosis accuracy and was highly sensitive. It also helped in designing genetic assays for detection of body tissues of threatened species in food products.

Cheng *et al*. (2003) developed a PCR assay to identify beef, pork, mutton and chicken meat and bone meal in animal diets. Four pairs of primers that targeted highly conserved regions of mtDNA were used. These gene fragments at the targeting region for the four species were 271 bps, 225 bps, 212 bps and 266 bps in size, respectively. The method was effective even at 1% level of adulteration and was a quick and sensitive method.

Kremar and Rencova (2003) developed a sensitive method for the identification of bovine, ovine, swine and chicken specific mtDNA sequences based on PCR. The method allowed the detection of the target species in concentrate mixtures even at 0.01% level. The identification of a sample containing 0.1% of bovine, ovine, swine, and chicken meat-and-bone meal was also confirmed by it.

Meyers *et al*. (2003) developed a PCR primer set capable of amplifying a mtDNA segment of multiple species (cattle, sheep, goat, deer, and elk). The primer set also amplified DNA derived from the rendered remains of pigs and horses, which were exempt from the feed ban. In pig DNA, *restriction endonuclease* site was recognized by *HinfI restriction endonuclease* enzyme, while in horse, by *HypCH4III restriction endonuclease* enzyme. The method was helpful in detection of prohibited meat species in food products.

Rajapaksha *et al*. (2003) developed a PCR assay to differentiate buffalo meat from the meat of Ceylon spotted deer *(Axis axisceylonensis),* Ceylon sambhur *(Cervus unicolor),* cattle, goat, pig, and sheep. A set of primers was designed according to the sequence of the mitochondrial *cyt b* gene of *Bubalus bubalis* and, by PCR amplification, a band of 242 bp was obtained with buffalo DNA. A band of 649 bp was observed for all animal species tested. It could identify rotten (10 days post slaughter), dried and cooked (above 100°C).

Kusama *et al*. (2004) designed primers to detect little amounts of meat and bone meal in ruminant feed. Mitochondrial subunit 8 of the *ATP synthase* gene was used as a target sequence. PCR-based assays revealed amplification of DNA from mammals, ruminants, and individual species using these primers. The method allowed detection of the presence of meat and bone meal in ruminant feed from 0.1 to 0.01% level.

Mendoza *et al.* (2004) developed a semi-quantitative method based on real-time PCR for detection of ruminant DNA, targeting an 88-bp segment of the ruminant short interspersed nuclear element Bov-A_2. This method was specific for ruminants and was able to detect as little as 10 fg of bovine DNA.

Chisholm *et al.* (2005) developed Real Time PCR assays specific for Horse and donkey meat, applicable to the detection of low levels of Horse or donkey meat in commercial products. Primers, designed to the *cyt b* gene, were 3' mismatched to closely related and other species. The assays were highly sensitive and detected the presence of 1 pg of donkey template DNA or 25 pg of horse template DNA when assessed using

dilutions of DNA in water.

Ahmed *et al.* (2007) studied the application of species specific PCR using mitochondrial *cyt b* gene species specific repeats in domestic animals. The experimental material for the study was raw meat of the domestic animals. The results from the study showed that the technique was rapid and effective in species identification. Further the method was sensitive in detecting the adulteration in raw meat below 5%.

Teixeira *et al.* (2007) studied buffalo meat products certification by PCR method using mitochondrial *cyt b* gene. Experimental material for the study was bovine meat mixtures. The method was able to detect mixtures at the concentrations of up to 1% of mixture. It was found that the autoclaved meat was good with respect to sensitivity.

Rojas *et al.* (2009) studied identification of raw and heat-processed meats from game bird species by PCR-RFLP analysis of mitochondrial D-loop region. PCR-RFLP analysis was applied to the identification of meats from quail, pheasant, red-legged partridge, Chukar partridge, guinea fowl, capercaillie, Eurasian woodcock and woodpigeon. PCR amplification was carried out using a set of primers flanking a conserved region of approximately 310 bp from the mitochondrial D-loop region. Restriction site analysis based on sequence data from this DNA fragment permitted the selection of *Hinf*I, *Mbo*II, and *Hpy188*III *endonucleases* for species identification. The restriction profiles obtained, allowed the identification of all game bird species meat. Consistent results were obtained with both raw and heat-processed meats.

Erwanto *et al.* (2011) studied pork authentication in beef sausage and chicken nugget by PCR-RFLP of *cyt b* gene using *BseDI* enzyme. Pork sample in various levels (1, 3, 5, 10 and 25%) was prepared in a mixture with beef and chicken meats and processed for sausage and nugget. The primers CYTb1 and CYTb2 were designed for the mitochondrial *cyt b* gene and PCR successfully amplified fragments of 359 bp. To distinguish existence of porcine species, the amplified PCR products of mtDNA were cut by *BseDI* restriction enzyme. The result showed pig mtDNA was cut into 131 and 228 bp fragments. The PCR-RFLP species assay yielded excellent results for identification of porcine species. It was a potentially reliable technique for pork detection in animal food processed products for Halal authentication.

Zarringhabaie *et al.* (2011) studied molecular traceability of the species origin of meats using Multiplex PCR for mitochondrial *cyt b* gene. The experimental material was meats from buffalo, goat, cattle and sheep. After mixing different portions of the mentioned meat sources, this method was able to trace less than 10% of the other species of meat in the mixture. The method was found to be simple, cheap, rapid, and efficient.

Forensically Informative Nucleotide Sequencing (FINS)

Barttlett and Davidson (1992) used forensically informative nucleotide sequencing (FINS) for identification of animal origin of biological specimens (canned, partially cooked, pickled, salted or smoked).The nucleotide sequencing was subjected to a phylogenetic analysis using a database, and the most closely related species was identified. FINS was found to be a rapid, reliable and reproducible procedure. The method filled the need for an accurate method of determining the species identity of a specimen when it was not possible by conventional means.

Murray *et al.* (1995) studied mitochondrial D-loop variation in 15 species of ungulates by PCR followed by RFLP analysis. The study included moose, caribou, mule deer, black-tailed deer, white-tailed deer, wapiti, Pronghorn antelope, Bighorn sheep, Stone's sheep, domestic sheep, Moulflon sheep, Mountain goat, domestic goat, domestic cattle and bison. The results of the study indicated that there may be sufficient species specific variation in the D-loop region of the mitochondrial genome of the ungulate species, with the exception of deer species, to establish the species origin of the mitochondrial haplotypes.

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