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Detection of Meat Adulteration by PCR-RFLP: An Update

Mahesh Shanmugasundaram[1*], Rajendran Chellaiah[2], V. A. Sajeev Kumar[3]

Freeze Drying and Animal Products Technology Division
Defence Food Research Laboratory, Siddarthanagar, Mysore, Karnataka, India
*Email: *maheshshanmugasundaram[at]gmail.com*

Abstract: ***Detection of meat species is important for religious, economic, health and traceability issues, which may lead to vetero-legal complications. There are many techniques to address the issue but DNA based methods are gaining popularity due to their specificity and sensitivity. PCR followed by RFLP analysis relies on specific restriction sites recognized by the restriction enzymes, allowing species identification based on the different patterns obtained. This will prevent adulteration of inferior quality meat into superior quality. The advantages of PCR-RFLP are related to its simplicity, relatively low cost compared with other molecular techniques, and suitability for routine analysis. In this review, application of PCR-RFLP technique for the detection of adulteration in meat and meat products including domestic, wild and fish species are discussed.***

Keywords: Meat Adulteration, Identification, PCR-RFLP, Ruminants, Poultry, Game, Pork, Sea food

1. Introduction

Identification of meat species is an important task for its religious, health, quality, economic, traceability and law issues. The substitution with cheaper species is difficult to detect visually after mincing or heat processing and cross contamination due to improper handling and using shared equipments (Zahran, *et al.*, 2015).

In business, certain meats are commonly being adulterated viz., horse meat for beef (UK, Ireland), beef for kangaroo meat (Australia), cat for chicken or rabbit meat, goat for mutton, mutton for venison, and dog & cat meat for chevon etc. Commonly occurring techniques employed for meat species differentiation are physical techniques (change in colour etc), anatomical techniques (dental arrangement etc), histological techniques (diameter of the muscle etc.), chemical techniques (carotene estimation etc.), serological or immunological (ELISA, Electrophoresis, Immunosensor etc.). Immunological techniques are more flourishing for meat detection before heat treatments only (krishikosh. egranth. ac. in). ELISA has high specificity and sensitivity, is rapid, field based, low cost and simple to use but multispecies detection is not available (Jozef *et al*., 2020).

DNA based detection is more reliable and thermally stable than proteins in processed meat. Firstly, DNA hybridization technique was used for meat speciation although laborious and time consuming. These are replaced by more convenient PCR assays which are precise and simple applies to both genomic and mitochondrial DNA. PCR variants, real-time PCR, differential display PCR, LAMP are also used for meat identification.

Real time PCR is more sensitive, specific than PCR and takes less time for obtaining results but expensive and a lab expertise is required. Commercial RT-PCR kits are available. Differential display PCR is a new variant of PCR and has high specificity, sensitivity and is fast. LAMP assays are highly specific, requires short time for detection, require lab expertise and is expensive but the same time can be used both onsite as well in the lab. Restriction fragment length polymorphism (PCR-RFLP) assay is a double step process to identify closely associated species after restriction enzyme digestion of PCR end product. It produces a characteristic band pattern. Which can be used for detection of variation and the use of a reference sample during the assay can be avoided. It is relatively less expensive, and can detect multispecies but with some limitations. This technique requires a lab, specific enzymes, and is time consuming (Jozef *et al*., 2020*).*

The cytochrome b gene can be considered as the universal DNA barcode region of individual species using the 400bp short fragment or 900bp long fragment by PCR-RFLP or sequencing. Species identification can be performed efficiently by using short fragments of *cyt* b, especially in degraded samples or are low in DNA quantity (Andrejevic *et al*., 2019). The interspecies genetic diversity of the same fragments was very high (8.36% to 42.52%), indicating great potential for species discrimination and having wide forensic and judicial applications (Parson *et al*., 2000)

PCR-RFLP analysis for detection of ruminant species

Earlier, mutton and chevon were differentiated by the analysis of satellite I DNA by PCR-RFLP with restriction enzyme (RE) *Apa* I, which has cut site in sheep but not in goat (Chikuni *et al*., 1994). Amplified C*yt b* gene product (359-bp fragment) digested with *Rsa* I, *Taq* I, *Alu* I and *Hinf* I to identify cattle, swine, buffalo, wild boar, goat, sheep, horse, turkey and chicken meat (Meyer *et al*., 1995). Similarly PCR product of *ATPase* subunit 8 and the amino terminal of the *ATPase* subunit 6 proteins digested with *Dnp II* and *SspI*, confirmed the bovine origin of amplified sequence (Tartaglia *et al*., 1998). Further it was used to identify 25 animal species in frozen meat or freeze-dried protein samples using tRNAGlu or *cyt* b and 11 various RE (Wolf *et al*., 1999). PCR-RFLP pattern of *cyt b* genes were used to identify 8 different species of mammals (baboon, cow, pig, dog, cat, bear, deer, raccoon, chicken and wild duck (Nakaki *et al*., 1999). This assay was used to

discriminate Hanwoo meat from meats of Angus and Holstein based on melanocortin gene (Chung *et al.*, 2000).

Cyt b gene with universal primers were used for identification in heat-treated meat products by PCR-RFLP using AM and *Hinf I (*Branciari, R *et al*., 2000) and this protocol was further used to identify species in meat meal and animal feed stuffs (Bellagamba, F *et al*., 2001). Cattle, buffalo, bison and banteng were differentiated using RFLP based on the primer pair specific for mitochondrial and centromeric satellite DNA (Veerkaar *et al*., 2002). Primers were used to amplify pig and horse DNA which could be differentiated by specific REs in ruminant feed. The ruminant feed amplified products obtained from pig DNA contained a restriction site for *Hinf I* whereas the horse DNA-obtained amplicon had a specific RE site recognized by *HypCH4* (Myers *et al*., 2003). Interspecies-specific DNA polymorphisms of the *cyt* b gene were used by PCR-RFLP technology for the discrimination of cattle, sheep, goat, roe buck and red deer (Pfeiffer *et al*., 2004). Some authors amplified the variable region of the *cyt* b gene followed by RE digestion with *Pal* I, *Mbo* I, *Hinf* I and *Alu* I, for species detection in 50 raw or processed food products (Pascoal *et al*., 2004).

Buffalo meat could be detected on *cyt* b gene PCR-RFLP by *Taq* I to obtain products of 108bp and 163bp (Teixeira *et al*., 2007). By using universal primer for *cyt* b amplification and RE digestion with *Alu*I, *Hae*III, and *Hin*fI, meat of cattle, horse, donkey, pig, sheep, dog, cat, rabbit, chicken, and human could be differentiated (Bravi *et al*., 2004). Species identification of beef, buffalo meat, mutton and chevon were based on a 456-bp fragment of the 12S rRNA gene followed by RE with *AzuI, Hha I, Apo I* and Bsp *II* resulting a characteristic band for each species (Girish *et al*., 2005). *Taq I* was used to digest the amplified cyt *b* gene (359 bp) for discrimination between buffalo and cattle meat. Sizes of 191 and 168 bp were generated in buffalo, but none with cattle of 359 bp (Ahmed *et al*., 2007). Similar technique could differentiate between horse and donkey (*Equus asinus*) species using *cyt b* gene (Moustafa *et al*., 2017).

The cytb gene was amplified that yielded products of 359 bp and 464 bp after digestion with *Hae*III and *HinfI* for differentiation of wild and domestic species, even in cooked meat (Partis *et al*., 2000). For specific identification of milk of buffalo and cattle species-specific PCR–RFLP was utilized to amplify a 359 bp amplicon of mitochondrial cytb segment and digested with *Taq*I to generate 191 and 168 bp fragments in buffalo, whereas no fragments were obtained from cattle (Abdel-Rahman*et al*., 2007). For species and halal authentication, PCR-RFLP was applied aiming at *cyt* b genes from beef, pork, buffalo, quail, chicken, goat, and rabbit. PCR products of 359 bp were digested with *Alu* I, *Bsa*J I, *Rsa* I, *Mse* I, and *Bst*U I to differentiate the meats (Murugaiah *et al*., 2009). A 440 bp of the mitochondrial 12S rRNA was PCR amplified after obtaining the reference gene variation in cattle, yak, buffalo, goat, and pig sequences. Two enzymes *Alu* I and *Bfa* I were chosen for species authentication. Goat and pig were differentiated using the *Alu* I enzyme, while cattle, yak, and buffalo were identified by digestion with *Bfa* I giving a high detection sensitivity of cattle DNA in mixed products (Chen *et al*., 2010).

A 360 bp of cytb gene was amplified and digested with *Rsa*I, *Bsa*JI, *BstN*I, *Alu*I, *Taq*I, *Nsi*I and *BstU*I and the RFLP pattern of both raw and processed were compared and this analysis proved that the chicken was present in beef products (Wong *et al*., 2010). A PCR-RFLP using 7 different RE (*Hind* II, *Ava* II, *Rsa* I, *Taq* I, *Hpa* II, *Tru* 1 and *Xba* I) on 710 bp *CO*I gene were able to differentiate cow, chicken, turkey, sheep, pig, buffalo, camel and donkey (Nadia *et al*., 2012).

Adulteration of buffalo meat and meat products were shown by 537 band region of the D-loop with *Bam*H I RE. There was no cross-reaction with cattle, sheep, goat, pig, and chicken in meat and meat products (Mane *et al*., 2012). The contamination in the canned stew samples could be differentiated by digesting the amplified ~195 bp fragments of the variable region in the *cyt* B gene with *Sse9*I restriction enzyme.7 of 7 of Kebab loghmeh, 9 of 10 minced meat, 4 of 8 beef burger and 2 of 5 samples were of other ruminant origin (Amjadi *et al*., 2012). Species differentiation was performed by digestion of PCR products of *cyt* b with *Tsp*509I and *Alu*I. Around 4 (4%), 3 (3%) and 5 (5%) of examined samples (100 in numbers) were contaminated with sheep, goat and donkey meat, respectively (Zahran *et al.*, 2015).

Species identification from raw meat of cow, chicken, turkey, sheep, pig, buffalo, camel and donkey was undertaken by PCR-RFLP using the *CO*I and further digestion with RE *Hind*II, *Ava*II, *Rsa*I, *Taq*I, *Hpa*II, *Tru1*I and *Xba*I (Haider *et al*., 2012). A 497 bp DNA fragments of 16S rRNA from beef, buffalo meat, mutton, chevon and pork was amplified even in heat treated meat products followed by restriction digestion with *Bgl*II, *Hinc* II and *Hinf* I resulting in a characteristic banding pattern (Mane *et al*., 2014). PCR-RFLP was applied on *cyt* b gene for the differentiation of beef, carabeef, chevon, mutton and pork with high specificity by employing two RE *Alu*I and *Taq*I (Kumar *et al.*, 2014). Similarly *cyt b* gene was digested by *Alu*I to differentiate between beef, sheep, pork, chicken, donkey, and horse meats in meat products and the results showed 6 of 68 fermented sausages, 4 of 48 frankfurters, 4 of 55 hamburgers, 2 of 33 hams, and 1 of 20 cold cut meat were found to contain prohibited meat (Doosti *et al*., 2014). For the authentication of meat and meat products beef, buffalo meat, mutton, chevon and pork DNA were subjected to PCR-RFLP. A 497 bp DNA was amplified from mitochondrial 16S rRNA and was subjected to RE *Sau*3AI which gave characteristic banding pattern (Mane *et al.*, 2015). Multiplex PCR products of ND5 and *cyt* B were digested by RE *Alu*I, *Eci*I, and *Fat* I enzymes, in order to differentiate cattle, buffalo, and porcine meat by quantitative and qualitative method (Hossain *et al*., 2016). A set of degenerative primers for specific amplification of 400bp of *cyt* b gene in cow, buffalo, goat, donkey and dog and followed by digestion with *Tfi* I enzyme revealed species specific restriction profile (Asghar *et al*., 2022).

For authentication of species-specific meat between buffalo and cattle, the amplified *cyt* b gene (359 bp) was digested by *Taq*I PCR product of *cyt b* in both donkey and horse (359 bp) were digested by *Alu*I Three fragments 189, 96 and 74 bp were generated in horse, whereas no fragments were

obtained in donkey (359 bp) (Salah abdel-rahman, 2017., Kusec, *et al*., 2017). Size of the PCR amplicons were of 760 bp, 737 bp, 537 bp, 486 bp, 481 bp, 464 bp, 429 bp, and 359 bp by universal primers for identification of 8 species which includes goat, sheep, deer, buffalo, cattle, yak, pig, and camel. Each PCR product could be further digested into fragments of variable sizes by *Ssp* I enzyme (Guan*et al*., 2018). Raw meat samples of buffalo, cow, sheep, goat and chicken were subjected to PCR amplification of a 359 bp of the cyt b gene and digested with *Tas*1 and *Hinf*1 enzymes and DNA fragments of different lengths were obtained. PCR fragment obtained for buffalo remained uncut by enzyme *Hinf* I (Khan*et al*., 2018). A ND4 gene of 952 bp was amplified from cattle, water buffalo, horse, and donkey species. Following digestion with*Saq*AI it was found that all the pastirma collected from various sources were made from cattle meat (Al *et al*., 2020). A 359 bp of the *cyt* b gene region followed by *Alu*1 digestion was used to identify cattle and buffalo (Rahat *et al*., 2020). In India and some African countries camel meat contamination is very common. To detect its authenticity, *cyt* b and 12S rRNA amplicons of 435 and 448 bp were generated. The 12S rRNA amplicon was digested with *Alu* I to generate products of 90, 148 and 210 bp size (Vaithiyanathan *et al*., 2020).

PCR-RFLP assay for detection of wild and hunted species

A PCR-RFLP was used to discriminate between red and sika Deer. The red deer *cyt* b gene was digested by *EcoR* I to 67 and 127 bp fragments while the *cyt* b of sika deer was digested with *BamH* I and *Sca*l which resulted in 48, 146 and 49, 145 bp fragments respectively (Matsunaga *et al*., 1998). PCR-RFLP was used to obtain a 981 bp DNA fragment from *cyt* b gene and was digested with *Alu*I and *Nco*I restriction enzymes. This was used in the identification of cattle, pigs, sheep, chickens, turkeys, rabbits, European hares, dogs, cats, fallow deer, red deer, roe deer and bison (Zimmermann *et al*., 1998). In another study the part of the gene encoding *cyt* b was amplified and digested using enzymes *Hae* III, *Hinf* I, *Rsa* I, and *Tru* 91. Specific restriction profiles allowed a direct identification of ostrich meat in raw and heat-treated samples from meat of other animal sources (Abdulmawjood *et al*., 2002).

Also it was used to identify raw and heat-processed meats from game bird species like quail, pheasant, red-legged partridge, chukar partridge, guinea fowl, capercaillie, Eurasian woodcock and woodpigeon. A 310 bp from the mitochondrial D-loop region was amplified and digested using *Hinf*I, *Mbo*II, and *Hpy188*III endonucleases for species identification (Rojas *et al*., 2009a). PCR-RFLP assay was used to identify wild and domestic meat species using primer pairs based on mitochondrial DNA and RE digestion with *Rsa*1 (Malisa *et al*., 2006). . Meat from sheep and goat were contrasted from sambar and chital using PCR-RFLP using primer pairs based on 12S rRNA gene region and RE *Alu*I, *Rsa*I (Rajput *et al*., 2013). Adulteration of burger with that of dog meat could be identified by PCR of 100 bp *cyt* b gene followed by RE *Alu* I with the ability to detect 0.01% of dog meat using lab-on a-chip detection system (Rahman *et al*., 2015). Macaque monkey meat in commercial meatball products was detected using PCR–RFLP amplicon of the D-loop gene of mitochondria and restriction digestion with *Alu* I and *Cvik* I *(*Rashid *et al*., 2015).

RFLP analysis of a PCR amplified 69 bp gene region followed by RE *Alu*I generated 2 fragments of 43 and 26 bp as determined on a lab –on-a-chip for detection for short length feline DNA in food (Ali *et al*., 2015). The Malayan box turtle is a protected species and subject to illegal wildlife trade for food. A PCR-RFLP assay with a very short amplification of length of 120 bp followed by digestion with *Bfa1*was done. A banding pattern of (72, 43 and 5 bp) was found on separation on a chip based electrophoresis system (Asing *et al.*, 2016). In order to detect adulteration of rabbit, rat, squirrel meat in foods, a multiplex PCR based RFLP was generated. PCR bands of 123, 108, 243, and 141 bp were brought about from rabbit, rat, squirrel and all eukaryotes. The bands were further digested with RE *Bts*I, *Mut*I and *Bts*CI and the sequence of the digested products were 115 & 8 bp for rabbit, 64 & 44 bp for rat, and 176 & 67 bp for squirrel (Ali *et al.*, 2018). In order to detect feline specific adulteration in foods, a DNA pattern (43-and 26-bp) was generated after digesting the 69 bp *cyt* b PCR amplicon and separation using lab-on-a chip platform (Amin, *et al*., 2020). A 359 bp fragment of the cyt b gene amplified by PCR using universal primers followed by three enzymatic digestions could distinguish seven animal species including dromedary, rabbit, goat, turkey, rat, donkey and pork, along with triplex PCR for chicken, dog and cat species (*Gargouri et al.*, 2021)

Identification of poultry species by PCR-RFLP

An actin gene locus was amplified and digested with RE for the differentiation of chicken and turkey with detection limit of chicken meat up to 1%. The chicken signal was clearly detectable with DNA from meat mixtures containing 1% chicken/99% lamb and from meat heat-treated at 120°C (Hopwood *et al.*, 1999). The mitochondrial 12S rRNA gene amplicon was restricted by using *Alo*e and *Sau*3AI to differentiate peacock (*Para cryostats)* from other poultry species (Saini *et al.*, 2007). A RFLP assay was developed for halal authentication of sausages and casings, bread, biscuits and meat balls containing pig derivatives (Aida *et al*., 2007, Erwanto *et al.*, 2012). A method was developed for rapid determination of poultry species (chicken, turkey, ducks and geese) based on analysis of the mitochondrial 12S rRNA region using selected RE *Bsl*I, *Tsp*I, *Mnl*I, *Sau*3AI (Natonek-Wisniewska *et al*., 2009).

Mitochondrial D-loop gene (442 bp) based assay to differentiate the chicken from other meat species by digesting with *Hae*III and *Sau*3AI enzymes to clearly identify chicken (Mane *et al*., 2009). A method was able to differentiate raw ostrich's meat mixed with cattle meat using *Alu*I RE or chicken's with *Hind*III, *Taq*I, *Mbo*I, *Hha*I, and *Bsa*I RE targeting the mitochondrial *cyt* b gene (Abu-Zeid *et al*., 2016). Commercial quail and pigeon meat products were found to be adulterated with chicken meat. A ~440 bp obtained from the 12S rRNA gene was digested with *Alu* I enzyme in order to differentiate the contaminants (He *et al*., 2018). A study was conducted to determine potential adulteration of donkey, chicken or even human tissues or cells in different marketed red meat products. The 12S rRNA region after RE digestion could identify the two

suspected animal species (donkey and chicken) as the adulterant (Omran *et al.*, 2019).

PCR-RFLP assay for detection of marine species

Digestion of the 359 bp of *cyt b* PCR products with *Nci* I, *Sau* 3AI and *Hinf* I endonucleases yielded specific profiles for the fish species *Solea solea*, *Pleuronectes platessa*, *Platichthys flesus* enabling identification of the fish species (Cespedes*et al*., 2008). A segment of the *CO*I gene was digested with *Taq*1 and *Hae*III on the four tuna species *Kasuwonus pelamis*, *Thunnus alalunga*, *Thunnus albacores* and *Thunnus obesus.* The semi nested PCR –RFLP detected contamination in canned tuna of other higher-valued species (Wanniwat*et al*., 2019). Lab-on-a-chip (PCR-RFLP) on *cyt b* gene was developed for the identification of seven catfish species using 3 enzymes *Dde*I, *Hae*III, and *Nla*III. The RFLP patterns for *Clarias batrachus* and *Ictalurus punctatus* were similar, but differed in a single band by *Hae*III (Li *et al*., 2014).

Flatfish was identified by PCR on *cyt* b gene yielding a 464 bp amplicon, followed by RE digestion and the differences in the banding pattern was observed (Carmen *et al*., 2001). Similarly marine fish fillets such as seabass, seabream, umbrine, and dentex were identified by targeting the 359 bp of *cyt*b followed by digestion with *Hae*III (Cocolin *et al.*, 2000). A PCR-RFLP protocol was established for differentiating nine different snapper species by targeting the D-loop of 515 bp length followed by RE using *Tsp*509I. Seven species could be clearly differentiated by 3–5 major bands. The protocol was also found successful in distinguishing the species in frozen, cooked and fried snappers (Sivaraman *et al*., 2018). PCR-RFLP for the *CO*I gene followed by RE digestion with *Mbo*I was proposed to reveal commercial fraud in swordfish trade. *Prionace glauca, Mustelus mustelus* and *Oxynotus centrina* was found in slices labeled as *Xiphias gladius* (Ferrito *et al.*2019). Sea snakes in Thai waters were distinguished by targeting *cyt* b, 12S and 16S rRNA and digested with *Alu* I and *Hinf* I enzymes which generated different sized fragments in different meat mixtures (Suntrarachun *et al*., 2018). Jellyfish of the variety *Rhopilema esculentum kishinouye* and *Stomolophus meleagris* were easily distinguished by restriction digestion of 651bp fragment of the 16S rRNA gene by *Hind* III, *Hpa* I, *Xho* I and *Dra* I in pickled varieties (YuJiang *et al.*, 2019).

6 species of processed tuna fish, raw and smoked Atlantic salmon and rainbow trout were identified by 16S *rRNA* or *CO*I gene amplification followed by RE digestion. Sola and Greenland halibut fishes were identified by amplifying the 12S rRNA and RE by *Aci*I and *Mwo*I for authentication of samples (Quinterio*et al.*, 1998, Carrera *et al.*, 1999b, Carrera *et al.*, 1999a, Céspedes *et al.*, 2000). Puffer fish (*Takifugu rubripes*) was identified by amplifying *cyt*b gene region of 376 bp followed by digestion with *Bst*ZI (Cheng *et al*., 2001). Eels (*Anguilla anguilla, A. rostrata, A. japonica, A. australis*) were identified by using the *cyt*b gene amplicon of 464 bp and further digested by RE *Hae*III, *Hin*fI, and *Mbo*II to yield specific banding pattern (Rehbein *et al*., 2002).

PCR-RFLP of 16S rRNA followed by *Asn* 1 digestion can separate the molluscs of the family *Loliginidae* from those of *Ommastrephidae* with a characteristic 200 bp band and 600–700 bp bands, respectively (Colombo *et al*., 2002). Lab –on-Chip was used to distinguish 10 white fish species by PCR amplification of *cyt*b followed by RE *Dde*l, *Nla*III and *Hae*III (Dooley *et al.*, 2005). The nontranscribed spacer (NTS) of the 5S rDNA was amplified from *Scomber japonicus, S. australasicus, and S. scombrus* mackerel followed by RFLP analysis of the PCR products with *Sca*I (Arahishi *et al.*, 2005). A total of 64 fillet samples were amplified by PCR-RFLP encoding the 16S rRNA and digested by *Vspl* RE in Japan. The generated restriction patterns indicated two different species of hairtails in the fillet samples (Chakraborty *et al*., 2007). A PCR–RFLP of five billfish species *Xiphias gladius*, *Makaira nigricans*, *M. indica*, *Istiophorus platypterus* and *Tetrapturus auda* was carried out *using* the *cyt* b gene with an amplification of 348 bp and digested with *Bsa*JI, *Cac*8I and *Hpa*II enzymes. Two commercial samples of billfish products showed adulteration with other cheaper fish (Sheng *et al.*, 2007). PCR-RFLP technique was developed to identify the species of *Thunnus, Euthynnus, Auxis* and *Sarda* in products of canned tuna. Two amplicons of 126bp and 146bp of *cyt*b gene and five RE were used to analyze the short length fragments (Lin *et al.*, 2007).

PCR-RFLP analysis was used to identify fish species in commercial seafood products. A 464-bp long *cyt* b gene product was digested with *Alu*I, *Hinf*I, *Hae*III, *Dde*I, *Nla*III, *Hinc*II, and *Mbo*II. Out of a total of 65 samples obtained, 10 samples (16.7%) contained other fish species (Mojmir *et al*., 2010). Likewise it was used to identify 62 commercial fish species in Taiwan which included groupers, bream, Sciaenidae, puffer. A 464 bp amplicon of *cyt* b gene were digested with *Dde*I, *Hae*III and *Nla*III and was further resolved on the DNA chip (Chen *et al*., 2014). The intronic region of the parvalbumin gene was used for the differentiation of tuna species *Thunnus albacares* from *T. obesus*, as determined by PCR-RFLP (Abdullah *et al*., 2016). A 570 bp region of the 16S rRNA gene in 16 commercial sea cucumbers were digested with *Dde* I, *Hae* III and *Sty* I and 9 out of 19 commercial products were found to be incorrectly labelled (Zeng *et al.*, 2018).

Identification of Pork samples by PCR-RFLP

A fluorescent PCR-RFLP technique on 12S rRNA gene for identification of porcine, caprine, and bovine species in cooked and autoclaved meat was obtained (Sun *et al*., 2003). Pork was mixed with beef sausage and chicken nuggets and was identified by using the *cyt* b gene and digestion with *Bse*DI. The mitochondrial D-loop gene was used to differentiate Sri Lankan wild boar from village pigs as seen in 17 different restriction sites. In other study D-loop containing the 9bp repeat was digested with *Dra* I enzyme to differentiate the wild and village pigs. Wild boar showed 2 bands one at 150 bp and another at 60 bp (Erwanto *et al*., 2011, Samaraweera *et al*., 2011). PCR-RFLP has been utilized to differentiate pig and wild boar meat (*Sus scrofa*) using 12S rRNA and *cyt b* gene of the mitochondria. The amplification of PCR products was 456 bp of 12S rRNA for both the species and 359 bp and 531 bp for *cyt b* gene of these two meats. *Alu*I, *Hind*III *and Bsaj*I were used to digest

the product. *Alu*I and *Bsaj*I were able to differentiate meat based on restriction pattern while *Hind*III enzyme was unable to restrict the PCR product of both meats (Mutalib *et al.*, 2012).

39 DNA samples from different meatball shops from Indonesia were isolated and amplified for *cyt*b gene. It was digested by *BseD*I into two fragments of 131 bp and 228 bp.9 of the 20 shops surveyed had pork contamination (Erwanto *et al*., 2014). Pork, equine and dog meat were found as adulterates in food products such as burger, kofta, luncheon, sausages in Kalubia market place by PCR-RFLP. Up to 33.3% and 66.7 % contamination was detected in loft and sausage with dog meat (Ahlam *et al*., 2020). Capsules contain gelatin which come from sources like porcine, bovine and fish which is a sensitive issue in Halal, Kosher and in Hindu groups. Multiplex-RFLP gave a specific pattern using *Bsa*AI, *Hpy*188I and *Bco*DI in DNA of gelatin-based bovine, fish and porcine in control experiments. Bovine and porcine DNA was found in 27 and 3 of the 30 different capsuled products. The assay was suitable for detecting 0.1 to 0.01 ng total DNA extracted from pure and mixed gelatins (Sharmin *et al.*, 2018).

2. Conclusion

The PCR-RFLP technique is a simple tool that can be used as a routine assay for detection of contamination in both raw and processed meats. Interpretation of the restriction profiles can be performed visually avoiding tedious sequencing analysis methods. However its applicability is doubtful in admixed meat products at low level of adulteration or substitution due to very complex banding pattern.

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Author Profile

Dr. Mahesh Shanmugasundaram received his Ph. D (Microbiology) from Jiwaji University in 2002. He is currently working as Scientist 'E' in Freeze Drying and Animal Products Technology division in Defence Food Research Laboratory, Mysuru. He contributed towards planning and writing of the paper.

Dr. C. Rajendran obtained his MVSc (ANGRAU), PhD from Indian Veterinary Research Institute (IVRI), Bareilly, Uttar Pradesh. Currently working as Scientist E in Freeze Dried and Animal Product Technology Division, Defence Food Research Laboratory (DFRL), Mysuru. He has contributed towards editing the manuscript.

Dr V. A. Sajeevkumar received his PhD in Chemistry and currently working as Scientist 'F' and Head, Freeze Drying and Animal Products Technology Division at Defence Food Research

Laboratory Mysuru. He has initiated the work on development of various test kits for the detection of adulteration in food products which can help in decision making for acceptance or rejection of food products at consumer level.

Summarized Information About Methods of PCR-RFLP Applied to Meat Species Detection (Amaral *et al*; 2016)

Target Species	Application	Target Gene	References
Pork, cattle, wild boar, buffalo, sheep, goat, horse, chicken, turkey, red deer, roe deer, moose, antelope, chamois, mouflon, and kangaroo	Sausages, marinated and heat – treated meats	Cytochrome b	Meyer et al. (1995)
Red deer and sika deer	Raw and heattreated meats	Cytochrome b	Matsunaga et al. (1998)
Buffalo, cattle, sheep, goat, hare, red deer, fallow deer, moose, antelope, gazelle, wildebeest, chamois, Pyrenean ibex, and kangaroo	Frozen meat and lyophilized Protein extracts	Cytochrome b	Wolf et al. (1999)
Pork	Meat, mortadella, pork sausage, and dry – cured ham	D-loop	Montiel – Sosa et al. (2000)
Ostrich	Raw and heat treated meats	Cytochrome b	Abdulmawjood and Buelte (2002)
Cattle, goat, sheep, pork, quail, wild boar, chicken, turkey, red deer, and roe deer	Chicken nuggets, hamburgers, croquettes, sausages, ham, tortellini, moussaka, paté, ravioli, and cannelloni	Cytochrome b	Pascoal et al. (2004)
Cattle, sheep, goat, red deer, and roe deer	Blood and tissue	D-loop	Pfeiffer et al. (2004)
Cattle, buffalo, sheep, and goat	Raw and heat treated meats, and fried meat products	12S rRNA	Girish et al. (2005)
Pork	Raw meats and fats (halal)	Cytochrome b	Aida et al. (2007)
Cattle, sheep, goat, red deer, fallow deer, and roe deer	Raw and heattreated meats	12S rRNA	Fajardo et al. (2006)
Cattle, sheep, goat, pork, horse, poultry, and deer	Raw meats	Cytochrome b	Maede (2006)
Chicken, duck, turkey, guinea fowl, and quail	Raw meats, heat treated meats, and fried croquettes	12S rRNA	Girish et al. (2007)
Chamois, Pyrenean ibex, mouflon, cattle, sheep, and goat	Raw and heattreated meats	12S rRNA, D-loop	Fajardo et al. (2007a)
Pork and wild boar	Raw meats	MC1R	Fajardo et al. (2008)
Quail, pheasant, redlegged partridge, guinea fowl, capercaillie, Eurasian woodcock, woodpigeon, and song thrush	Raw meats	12S rRNA	Rojas et al. (2008)
Red deer, fallow deer, roe deer, chamois, mouflon, Pyrenean ibex, goat, cattle, sheep, and swine	Raw meats	12S rRNA	Fajardo et al. (2009a, b)
Quail, pheasant, redlegged partridge, chukar partridge, guinea fowl, capercaillie, Eurasian woodcock, and woodpigeon	Raw meats	D-loop	Rojas et al. (2009a)
Cattle, pork, buffalo, quail, chicken, goat, and rabbit	Raw meats	Cytochrome b	Murugaiah et al. (2009)
Chicken, turkey, duck, goose, pheasant, partridge, woodcock, ostrich, quail, and song thrush	Raw and heat treated meats	12S rRNA, Cytochrome b	Stamoulis et al. (2010)
Cattle, chicken, turkey, sheep, pork, buffalo, camel, and donkey	Raw meats and blood	Cytochrome c oxidase subunit 1	Haider et al. (2012)
Pork	Meatballs, streaky beacon, frankfurters, and burgers	Cytochrome b	Ali et al. (2012a)
Cattle, buffalo, goat, sheep, and pork	Raw meats	Cytochrome b	Kumar et al. (2014)
Cattle, sheep, pork, chicken, donkey, and horse Cytochrome b	Raw meats, sausages, frankfurters, hamburgers, and hams	Cytochrome b	Doosti et al. (2014)
Cat 0.01% (w/w)	Raw, heat treated meats and meatballs	18S rRNA	Ali et al. (2015a)
Dog	Burger formulations and commercial burgers	Cytochrome b	Rahman et al. 2015)

Summarized Information about Methods of PCR-RFLP Applied to Meat Species Detection (2016-2022)

Target Species	Application	Target Gene	References
Cattle, buffalo, porcine	Raw meat	ND5, Cytochrome b	Hossain et al. (2016)
Malayan Box Turtle	Raw meat	Cytochrome b	Asing et al. (2016)
Ostrich, cattle, chicken	Raw meat	Cytochrome b	Abu-Zeid et al. (2016)
Donkey and horse	Raw meat	Cytochrome b	Kusec et al. (2017)
Horse and donkey	Raw meat	Cytochrome b	Moustafa et al, (2017)
Snapper	Frozen, cooked and fried	D-loop	Sivaraman et al, (2018)

Rabbit, rat, squirrel	Raw meat	ATP6 and cytochrome b	Ali et al. (2018)
Sea cucumbers	Commercial Products	16S rRNA	Zeng etal. (2018)
Sea snakes	Raw meat	Cytochrome b, 12S &16S rRNA	Suntrarachun et al. (2018)
Capsules with gelatine from animal species	Capsules	16S rRNA	Sharmin et al. (2018)
Quail, pigeon, chicken	Raw meat	12s rRNA	He et al. (2018)
Sheep, goat, deer, buffalo, cattle, yak, pig, camel	Raw meat	Cytochrome b & 12S r RNA	Guan et al. (2018)
Buffalo, cow, sheep, goat, chicken	Raw meat	Cytochrome b	Khan et al. (2018)
Donkey, chicken, human tissues or cells	Red meat	12s rRNA	Omran et al. (2019)
Rhopilema esculentum kishinouye and *Stomolophus meleagris* (Jelly fish)	Raw meat	16S rRNA	Yujiang et al. (2019)
Xiphias gladius (Sword fish)	Raw meat	Cytochrome c oxidase subunit 1	Ferrito et al. (2019)
Tuna species	Raw meat	Cytochrome c oxidase subunit 1	Wanniwat et al. (2019)
Cattle, water buffalo, horse, donkey	Pastirma	ND4	Al et al. (2020)
Cattle, buffalo	Raw meat	Cytochrome b	Rahat et al, (2020)
Camel	Raw meat	Cytochrome b, 12S rRNA	Vaithiyanathan et al. (2020)
Feline	Raw meat	Cytochrome b	Amin et al. (2020)
Dumedary, rabbit, goat, turkey, rat, donkey	Raw meat	Cytochrome b	Gargouri et al. (2021)
Cow, buffalo, goat, donkey, dog	Raw meat	Cytochrome b	Asghar et al. (2022)