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Evaluation of multiplex PCR assay for identification and authentication of raw and cooked meats

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Abstract
Proper identification and authentication of food are essential due to economical, public health and religious concerns. Meat speciation has garnered increased prominence in recent years due to surge in deceptive practices involving acts like substitution of premium quality meat with inferior counterparts primarily motivated by economic incentives. This study includes identification and authentication of four commonly consumed meats in India *viz*., Chicken, Mutton, Beef and Pork employing primers which specifically target the mitochondrial genes of these species in the PCR assay obtaining the desired amplicons of 183 bp, 263 bp, 106 bp and 73 bp respectively. Among the studied 260 samples no adulteration was noticed and all the samples were identified correctly employing the multiplex assay. Meat admixtures were prepared to assess the effectiveness of the selected species-specific primers in identification of adulteration. These meat admixtures when subjected to PCR assay revealed these primers could identify adulteration at levels as low as 0.1% for pork and up to 1% for chicken, mutton and beef samples.

Keywords: Identification, meat, adulteration, DNA, multiplex PCR

1. Introduction
The worldwide appetite for top-tier animal protein, including meat and its derived products has been on the rise, owing to the changes in food habits and increased awareness on meat associated health benefits. The challenges related to food authenticity, specifically involving fraudulent adulteration and misrepresentation have been persistent concerns intertwined with the sale of food for as long as it has been a commercial endeavor. With a paramount concern for consumer health, there is a heightened emphasis on rigorously assessing food consumption and ensuring its authenticity to safeguard against illicit and undesirable substitutions, driven by religious, economic and health-related motivations. One such element of immense significance in food quality control procedures is meat speciation. The conventional approaches, which encompass anatomical, histological, organoleptic, electrophoretic, chemical, immunologic and chromatographic methodologies tend to be unwieldy exhibiting limited repeatability and are reputed to have lower sensitivity, making them dependable primarily when dealing with unprocessed meats (Plowman and close, 1988) [1]. A string of protein-based methods were also largely employed for speciation *viz*., electrophoretic methods *viz*., IEF (Isoelectric focusing) (King, 1984) [2], SDS (Sodium Dodecyl Sulphate) – PAGE (Polyacrylamide Gel Electrophoresis (Bhilegaonkar *et al*., 1990) [3] and methodologies related to immunology like PAP (peroxidase antiperoxidase) (Karkare *et al*., 1989) [4], ELISA (Enzyme Linked Immunosorbent Assay) and CIE (Counter immunoelectrophoresis) (Sherikar *et al*., 1993), [2-6]. The effectiveness of methodologies based on lipids and proteins was reportedly low, which has been heavily attributed to the vulnerability of the target biomarkers to modification throughout the processing treatments.
DNA-based methodologies for species identification have garnered broader recognition and trust due to the inherent stability and universal nature of DNA across all cells and tissues. DNA serves as the repository of the complete information within an individual genetically, which remains conserved regardless of the specific organs or tissues (Lockley and Bardsley, 2000) [7]. The incorporation of DNA-based assays stands as a modern intervention in the validation of meat and meat products, laveraging a comprehensive repertoire of available methodologies. To name a few, DNA hybridization and its applications (Chikuni *et al*., 1990 and Ballin *et al*., 2009) [8, 9], FINS (forensically informative nucleotide sequencing) of DNA (Hsieh *et al*., 2005 and Girish *et al*., 2004) [10, 11], PCR (polymerase chain reaction) assay and

its various applications (Matsunga *et al*., 1999) [12], universal primers pairs for PCR-RFLP analysis (Murugaiah *et al*., 2009; Uddin *et al*., 2021 and Gargouri *et al*., 2021) [13-15], PCR-RAPD (Random Amplified Polymorphic DNA finger-printing) (Rastogi *et al*., 2007 and Calvo *et al*., 2001) [16,17], AFLP (Amplified Fragment Length Polymorphism) (Sasazaki *et al*., 2004 and Zhao *et al*., 2018) [18,19], RT- PCR (Real-Time) (Tanabe *et al*., 2007; Liu *et al*., 2021 and Li *et al*., 2021) [20-22] are the most universally accepted and studied techniques, that the researchers had a considerable success in proper speciation of meat with respect to its origin, with minimal effort and toll yet with enhanced sensitivity and specificity. Further, getting into the DNA dimension a layer more, genetic information from nucleus and mitochondria has been successfully utilized in meat speciation studies to discern the origin of meat species in which the DNA sequences from mitochondria were reported to be highly conserved across diverse animal species (Van der Kuyl *et al*., 1995) [23]. The mitochondrial markers have proven to be more efficient than nuclear counterparts in identification of species origin (Rastogi *et al*., 2007) [16], primarily attributed to mitochondrial maternal inheritance, which typically results in the presence of only a single allele in an individual, thereby minimizing the likelihood of sequence ambiguities (Unseld *et al*., 1995) [24]. Mitochondrial-based DNA analysis also facilitates a greater ease to the researchers comparatively with numerous mitochondria present per cell and the multitude molecules of DNA present within each mitochondrion, this intrinsic amplification of genetic material renders mitochondrial DNA a naturally enriched reservoir of genetic variation (Girish *et al*., 2004 and Fajardo *et al*., 2006) [11, 25]. As the mitochondrial gene harbors thousands of copies of variable regions per cell, the likelihood of obtaining a positive result remains significantly higher, even under conditions of severe DNA fragmentation or intense processing (Greenwood and Paboo, 1999 and Bellagamba *et al*., 2001) [26, 27]. Compared to the nuclear DNA, the mitochondrial DNA undergoes a considerably faster rate of evolution, subsequently resulting in a richer array of sequence diversity, which aids in identification of species which are closely related (Brown *et al*., Wolf *et al*., 1999 and Pfeiffer *et al*., 2004) [28-30].

The exponential rise in the restaurant business, coping up with the huge public demands and random, yet global sprouting of ready-to-eat foods, such as hot dogs, burgers, sandwiches, soups, pizzas and many other foodstuffs has been a phenomenal driver to food adulteration. In specific, large parts of the global population resorting to meat associated foods in their daily cuisine although spiking the meat demand, have also ignited the spark of meat adulteration. With meat being an easily accessible commodity for everyone and as many outlets and eateries resort to mixing the meat posing a severe public health threat, there is a dire necessity to have a reliable test for confirmed detection of adulteration. In the current study, simultaneous identification of four commonly consumed meats in India *viz*., Chicken, Mutton, Beef and Pork was carried out using a species-specific Multiplex PCR assay which targeted the mitochondrial genes in identification of both raw and cooked forms of meat.

2. Materials and Methods

2.1 Collection of samples

After standardizing the protocol employed using known samples, meat samples (n=260) including raw, cooked and processed meat samples claimed to be chicken (n=112), mutton (n=74), beef (n=42) and pork (n=32) collected aseptically from various meat retail outlets, restaurants, small scale hotels and roadside vendors in and around Tirupati region. All the collected samples after precise labelling were transported under chilled conditions (4 °C) to the Department of Veterinary Public Health and Epidemiology, C.V.Sc, SVVU, Tirupati and were kept in strict refrigerated condition at -20 °C before subsequent treatment.

2.2 Extraction of DNA

DNA extraction from the gathered samples was carried out employing the PCI (Phenol-Chloroform-Isoamyl alcohol) technique as elucidated in the protocol described by Sambrook and Russel (2001) and Alvardo *et al*. (2017) with slight modifications [31, 32]. About 75 mg of sample was used to extract the DNA initially by digesting it using lysis buffer (EDTA 0.1M; NaCl 1M; Tris Cl 0.1M), 10% SDS and Proteinase K enzyme (10 mg/ml). Further processing by P:C:I (25:24:1) and Isopropanol (for mutton and beef samples) was carried out. Then final washing of the DNA pellets using 70% Ethanol. About 60 µl of nuclease free water was used to dissolve the extracted DNA and was and kept at -20 °C till further processing. To assess the quality of the DNA extracted from the samples, horizontal electrophoresis (Genei, Bengaluru) on a 1% Agarose gel and its visualization through Gel doc system (BIO-RAD, USA) were utilized. The purity and concentration of DNA was checked using 1µl of DNA in Nanodrop (Thermo Scientific, USA) at an absorbance of OD_{260}: OD_{280}.

2.3 Primers for PCR assay

The nucleotide sequences of all four species under study were obtained from various literatures. For chicken samples, the mitochondrial 12SrRNA gene was targeted and mitochondrial ND5 gene was targeted for validating mutton, beef and pork samples. The primer sequences used were given in the Table 1.

Table 1: Species – specific oligonucleotide primers utilized in this investigation.

Species	Mitochondrial gene targeted)	Sequence of Primers 5'-3'		Resultant Amplicon (bp)	Reference
Chicken	12SrRNA	F	TGAGAACTACGAGCACAAAC	183	Dalmasso *et al*. (2004) [33]
		R	GGGCTATTGAGCTCACTGTT		
Mutton	ND5	F	TTCCTCCCTCACACTAGTCACC	263	Uddin *et al*. (2021) [14]
		R	CTGGAACGAATATTATTGAGAAGAAGTC		
Beef	ND5	F	GGTTTCATTTTAGCAATAGCATGG	106	Hossain *et al*. (2017) [34]
		R	GTCCAATCAAGGGTATGTTTGAG		
Pork	ND5	F	GATTCCTAACCCACTCAAACG	73	
		R	GGTATGTTTGGGCATTCATTG		

2.4 Simplex PCR assay
During the preliminary phase of the investigation, the primers were standardized against their particular specific species. The final PCR reaction was carried out in a 25 µl volume containing 12.5 µl of 2 X PCR master mixture, 1µl (10pmol/µl) of each primer of each species, 5.5 µl of template DNA and NFW to makeup the remaining volume. The cycling conditions followed for PCR amplification include an initial Denaturation for 3 minutes at 95 °C followed by 35 cycles of denaturation at 95 °C for 45 seconds, annealing at 60° for 40 seconds, initial extension at 72 °C for 40 seconds and final extension for 5 minutes at 72 °C carried out in a Thermal Cycler (BIO – RAD, USA).

2.5 Multiplex PCR assay: The primers employed in simplex PCR were used for identification of all the species under study simultaneously through a single multiplex PCR assay. This multiplex reaction setup included a 25 µl total reaction consisting of 12.5 µl 2X PCR master mixture, 0.5 µl (20pmol/µl) of each primer (chicken, mutton, beef and pork), 3µl of template DNA and NFW to makeup the remaining volume. PCR amplification was performed following the cycling conditions of that of simplex PCR.

2.6 Specificity and sensitivity estimation of primers
Initially, the specificity of the selected primers and their cross reactivity was checked using NCBI primer BLAST tool. Species specificity and cross reactivity was checked by employing a myriad of primer-DNA combinations. The primers belonging to one species was checked against the DNA extracted from other species in the investigation. In a single reaction, the specificity of a primer belonging to one species was analyzed by adding the template DNA's of all four species. The cross reactivity of the primer belonging to one particular species was also checked by adding template DNAs of other three species individually. The sensitivity of the primers in amplifying the DNA was analyzed by serially diluting the template DNA based on the method used by Guoli *et al*. (1999) [35]. Serial 1:10 dilutions of the DNA template up to 10^{-5} was followed to check the sensitivity. The diluted DNA was then subjected to PCR following the cycling conditions previously mentioned.

2.7 Preparation of meat admixtures
Meat admixtures were prepared using meat samples in various proportions to assess the effectiveness of the primers in discerning meat adulteration. The combinations of various compositions of meat admixtures prepared were given in the Table2. Admixtures of meat were formulated by blending the meat species under study with meat of other species in different proportions (w/w) and made up to 100gm. The meat admixtures were prepared in two combinations. Chicken and pork were admixed together due to physical similarities in terms of colour and presence of subcutaneous fat. Mutton and beef were admixed due to similarities in their colour, consistency and fat type.

Table 2: Compositions of meat admixtures.

Sample (meat admixture)	% of species under study	% of remaining meat admixture
Sample 1	10%	90%
Sample 2	5%	95%
Sample 3	1%	99%
Sample 4	0.1%	99.9%
Sample 5	0.01%	99.99%
Sample 6	0	100%

2.8 Detection of amplified products
The amplified products were subjected to electrophoresis on 1.5% agarose gel run in 1.0 X SBB (Sodium Borate Buffer) along with DNA ladder at 80mA, 5 V/cm for approximately 1 hour. The gel was visualized using Et Br (ethidium bromide) fluorescence at 300 nm wavelength in a gel doc system.

3. Results
3.1 DNA extraction
The DNA extracted using PCI method of all four species under study was suitable in terms of quality and quantity for subsequent PCR assay. The examination of the extracted DNA quality by gel electrophoresis revealed no signs of shearing, confirming its high quality. The purity and concentration of extracted DNA assessed in Nanodrop at an absorbance of OD_{260}: OD_{280} revealed good results with purity ranging from 1.6 to 2.0 and a healthy concentration range of 80 to 120 ng/µl.

3.2 Specificity and Sensitivity estimation of primers
The specificity and possible cross reactivity across the selected four sets of primers was tested by employing a myriad of primer-DNA combinations. The results of the PCR assay revealed that amplification of DNA occurred only when the primers specific to that DNA is present in the reaction mixture. No amplification was noticed when the primers belonging to one species were matched up against the remaining three species under study. This revealed that the primers displayed species-specificity, with no occurrence of cross reactivity with other species. The sensitivity of primers was assessed in terms of least amount of DNA with which the primers can amplify. The four DNA templates belonging to each species under this study were diluted in 1:10 ratio with NFW up to 10-5 dilutions. The PCR amplification was noticed at 10^{-3} dilution for chicken, beef and pork samples with the minimum detected concentration of 0.02 ng/µl, 0.039 ng/µl, and 0.034 ng/µl respectively. In case of mutton, the amplification was noted up to 10^{-4} dilutions with 0.031 ng/µl as the least detected concentration.

3.3 Simplex PCR assay
The simplex PCR assay was executed to standardize the selected primers using DNA extracted from raw meat. The primers generated the desired specific fragment lengths for all four species. The size of the amplicon obtained for chicken samples targeting 12SrRNA was 183bp. The ND5 gene targeted in mutton, beef and pork yielded the amplicon fragments of 263bp, 106bp and 73bp respectively. Fig.1 shows the standardized simplex PCR assay of all four species. After standardization of the primers and the completion of specificity and sensitivity check, identification of the collected samples was conducted using a multiplex PCR assay.

3.4 Multiplex PCR assay: The multiplex PCR assay was standardized in a single reaction containing DNA all four species in this study with amplification conditions similar to that of simplex assay (Fig.1). After standardization of multiplex assay, all the 260 raw, fried and processed meat samples collected in this study were successfully identified. The detailed assessment of samples identified were given in the Table 3. The multiplex PCR assay results demonstrated that none of the meat sample which were sold by its name were adulterated with the other animal species meat.

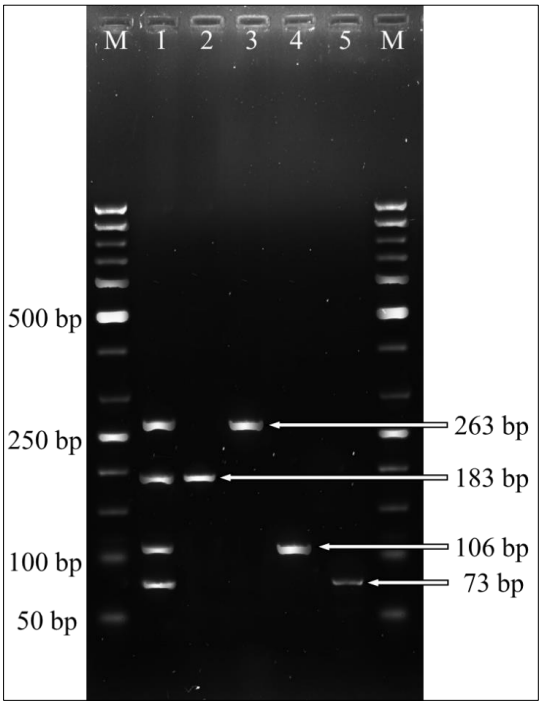

Fig 1: Standardized simplex and multiplex PCR assay of all four species

Lane M: 50bp Molecular Marker; Lane 1: Multiplex reaction of all four species; Lane 2: Chicken-183 bp; Lane 3: Mutton-263 bp; Lane 4: Beef-106 bp; Lane 5: Pork-73 bp.

Table 3: Particulars of number and type of meat samples confirmed by PCR assay

Species under study	Number of samples screened						Total	No. of samples confirmed by PCR assay
	Road side vendors		Small scale hotels		Restaurants			
	Raw	Fried	Steam cooked	Fried	Steam cooked	Fried		
Chicken	5	18	36	15	24	14	112	112
Mutton	7	-	16	16	23	12	74	74
Beef	6	6	26	4	-	-	42	42
Pork	12	-	20	-	-	-	32	32
Total	30	24	98	35	47	26	260	260

3.5 Detection of meat admixtures using PCR assay

Binary meat blends, consisting of chicken-pork and mutton-beef combinations were meticulously crafted in diverse ratios as outlined in Table 2. These mixtures were utilized to assess the detectable extent of adulteration. For determining the level of detection of chicken, admixed meat samples containing various proportions of chicken mixed with pork were subjected to PCR-assay employing species-specific primers which target the 12SrRNA mitochondrial gene of chicken. Amplification of chicken DNA was seen in admixed sample down to 1%. For determining the level of pork detection, admixed meat samples containing varying proportions of pork mixed with chicken underwent a PCR-assay employing species-specific primers targeting the mitochondrial ND5 gene in pig. Amplification of pork DNA was seen in admixed sample down to 0.1%. For determining the level of detection of mutton, admixed meat samples containing various proportions of mutton mixed with beef were subjected to PCR utilizing species-specific primers targeting ND5 gene in sheep. Amplification of mutton DNA was seen in admixed sample down to 1% (Fig. 2). For determining the level of detection of beef, admixed meat samples containing various proportions of beef mixed with mutton were subjected to PCR employing primers which specifically target the ND5 gene in cattle. Amplification of beef DNA was seen in admixed sample down to 1% (Fig. 3).

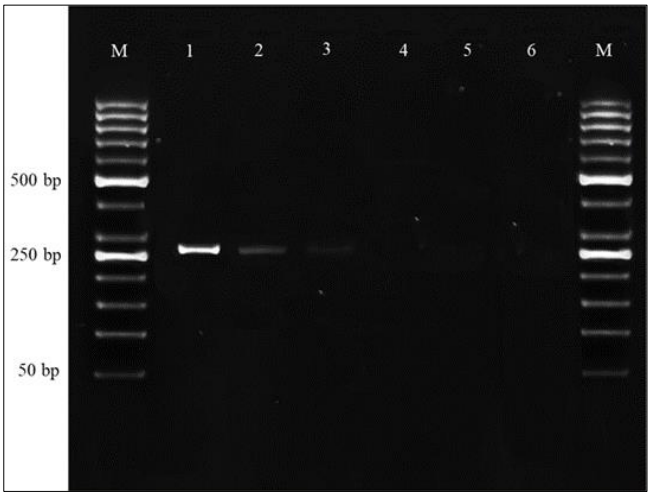

Fig. 2: Mutton admixed with beef in various proportions

Lane M-50 bp Molecular Marker; Lane 1-Mutton 10%, Beef 90%; Lane 2-Mutton 5%, Beef 95%; Lane 3-Mutton 1%, Beef 99%; Lane 4-Mutton 0.1%, Beef 99.9%; Lane 5-Mutton 0.01%, Beef 99.99%; Lane 6-Mutton 0%; Beef 100%.

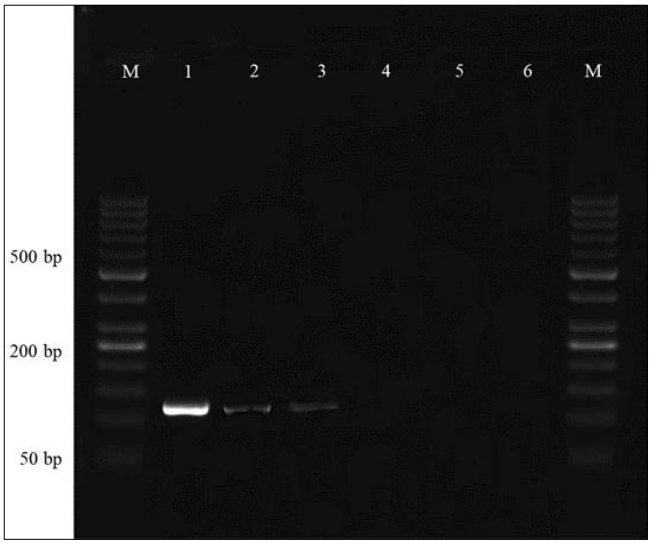

Fig. 3: Beef admixed with mutton in various proportions.

Lane M-50 bp Molecular Marker; Lane 1-Beef 10%, Mutton 90%; Lane 2-Beef 5%, Mutton 95%; Lane 3-Beef 1%, Mutton 99%; Lane 4-Beef 0.1%, Mutton 99.9%; Lane 5-Beef 0.01%, Mutton 99.99%; Lane 6-Beef 0%, Mutton 100%.

4. Discussion

Meat samples belonging to all four species under the study were collected from various fronts in Tirupati. The samples collected were of different types which included both raw and cooked varieties. The extracted DNA was subjected to PCR (Polymerase Chain Reaction) assay using established primers. The sensitivity and specificity of the selected primers was validated.

Initially, simplex PCR was done to standardize the PCR conditions for each species and then a multiplex assay was followed for all four species under study. The DNA extracted from samples underwent a PCR assay utilizing the species-specific primers which target specific sequences unique to that species. In the present study, established primers targeting the mitochondrial 12S rRNA gene have been utilized to identify chicken samples and primers targeting the mitochondrial ND5 gene were employed to distinguish mutton, beef and pork samples.

The primers used in this study for chicken identification were also previously used by Dalmasso *et al*. (2004) [33]; Ghovvati *et al*. (2009) [36]; Parchami Nejad *et al*. (2014) [37]; Mousavi *et al*. (2015) [38] and Galal-Khallaf (2021) [39] yielding an amplicon size of 183bp. The 12S rRNA has also been targeted by using primers which target another specific sequence of the gene for the identification of foods of chicken origin. Koh *et al*. (2011) [40] targeted a sequence of 12S rRNA gene to identify chicken with a 171bp amplicon. Similarly, Abuzinadah *et al*. (2015) [41] and Cahyadi *et al*. (2018) [42] also targeted the mt 12S rRNA gene with another specific sequence of the gene producing amplicon sizes of 95 bp and 611 bp respectively for the identification of chicken meat. Various researchers have also targeted other mitochondrial genes like cytochrome b (Matsunaga *et al*., 1999; Kitpipit *et al*., 2014; Hossain *et al*., 2017; Qin *et al* 2019 and Cai *et al*., 2021) [12, 43, 34, 44, 45]; 16S rRNA gene (Luo *et al*., 2008) [46]; D loop gene (Haunshi *et al*., 2009) for validation of meats of chicken origin [47].

The ND5 gene has been used for recognition of meats of sheep, cattle and porcine origin by Hossain *et al*. (2017) and Uddin *et al*. (2021) targeting species-specific sequences in the gene [34, 15]. Their work has been used a reference for selecting the established primers for detection of mutton, beef and pork samples in thus study. For detection of mutton samples, the established primers were used targeting ND5 gene to yield an amplicon of 263bp which can identify foods of sheep origin. The PCR assay results were in accordance to Uddin *et al*. (2021) [15]. Other mitochondrial genes which have been targeted for detection and authentication of sheep species through PCR assay include D-loop gene (Karabasanavar *et al*., 2011) [48], cytb gene (Matsunga *et al*., 1999; Herman, 2001; Jain *et al*., 2007, Nischala, 2022 and Thomas *et al*., 2021) [12, 49, 50 51, 52], 16S rRNA gene (Ghovvati *et al*., 2009) [36], 12S rRNA gene (Iqbal *et al*., 2020 and Li *et al*., 2021) [53, 22], COX1 gene (Izadpanah *et al*., 2018) [54] and ND2 gene (He *et al*., 2015) [55].

Beef samples were identified by PCR assay employing the primer pair targeting ND5 gene with an amplification of 106 bp. There were also previously used by Hossain *et al*. (2017) and the PCR assay results of this investigation were also in accordance with Hossain *et al*. (2017) [34]. Other mitochondrial genes targeted for identification and authentication of cattle species through PCR include cytb gene (Matsunga *et al*., 1999; Abdul-Hanssan and Tauma, 2014; Foong and Sani, 2013) [12, 56, 57], D-loop (Kotowicz *et al*., 2007; Mane *et al*., 2012; Mousavi *et al*., 2015; Kumar *et al*., 2016 Karabasanavar *et al*., 2017 and Thomas *et al*., 2021) [58, 59, 38, 60, 61, 52], 16S rRNA gene (Ghovvati *et al*., 2009; Cai *et al*., 2021) [36, 45], 12S rRNA (Iqbal *et al*., 2020) [53], COX1 (Spychaj *et al*., 2016, Izadpanah *et al*., 2018) [62, 54] and ND4 gene (Li *et al*., 2021) [22].

Pork samples were identified using established primers pair targeting ND5 gene previously used by Hossain *et al*. (2017) and Uddin *et al*. (2021) [34, 14]. The results of the PCR assay gave an amplicon of 73bp which was parallel to the research work of Hossain *et al*. (2017) and Uddin *et al*. (2021) [34, 14]. A 141bp amplicon was generated by Ali *et al*. (2015) who also targeted a specific region of ND5 mitochondrial gene for the detection of meats of porcine origin. ND5 gene was also targeted by Kesmen *et al*. (2007) in their PCR assay studies for the recognition of porcine species in cooked sausages [63, 64]. Apart from ND5, various works have been done targeting other mitochondrial genes for the identification of pork *viz*., D- loop gene by Haunshi *et al*. (2009), Karabasanavar *et al*. (2014) and Kumar *et al*. (2012) [47, 65, 66], Cytb gene by Matsunaga *et al*. (1999) and Foong and Sani, (2013) [12, 57], 12S rRNA by Ghovvati *et al*. (2009), Sakalar and Abasiyanik (2011), Kumar *et al*. (2012) and Iqbal *et al*. (2020) [36, 67, 53], the COX1 gene by Spychaj *et al*. (2016) [62], Li *et al*. (2021) [22] and the mitochondrial ATPase subunit 6 gene by Lahiff *et al*. (2001) [68] and Safdar *et al*. (2014) [69] respectively.

As no adulteration was noticed in any of the collected samples, meat admixtures were prepared so as to assess the effectiveness of the primers in identification of one species, when present along with other meat species in a certain proportion. These admixtures were prepared by mixing raw meats of two species in various proportions. The DNA from these admixed samples was extracted and subsequently analyzed by PCR assay to know the detection level of that particular species under study. The chicken and pork meats have the physical similarities in terms of colour and presence of subcutaneous fat while the red meats of sheep and cattle are similar in their colour, consistency and fat type. These combinations are commonly practiced adulteration technique

followed by the meat vendors. Hence, these two combinations of meat admixtures were prepared to determine the PCR efficacy using the primers. The samples under this study were prepared in the proportions of 10%, 5%, 1%, 0.1% and 0.01%. following PCR assay, the chicken meat mixed with pork was successfully detected up to 1% level in admixed samples. While pork DNA was amplified successfully even when 0.1% pork was admixed with chicken meat. The level of detection of mutton was 1% in admixtures of mutton and beef. Similarly, for beef samples, the 1% detection limit was noticed in admixed beef and mutton samples. Consistent with the outcomes of the current investigation, Mane *et al*. (2012) also documented a 1% detection limit of buffalo meat admixed with pork, beef, mutton, chevon and chicken using PCR assay [59]. Bhat *et al*. (2016) also used PCR assay to detect the presence of cattle and buffalo meat down to the level of 1% in the mixed meat cooked Rista (Kashmiri mutton product) [70]. Similarly, Partis *et al*. (2000) [71] also found 1% pork in beef meat; Panwar *et al*. (2015) identified 1% LOD for admixed sheep and goat meat samples [72]. In contrast, Meyer *et al*. (1994) [73] identified 0.5% pork in beef using the duplex PCR technique; Ilhak and Arslan (2007) were able to detect 5%, 2.5%, 1%, and 0.5% levels of pork, horse, cat, or dog meat admixed with beef, sheep, and goat meat samples in their PCR assay after 30 cycles and also 0.1% was detected after 35 cycles of amplification [74]. Whereas, Ali *et al*. (2012) documented that PCR assay was sensitive enough to identify as little as 0.01% of contaminated pork in a mixture of beef and chicken in the spiking studies of pork adulteration [75].

5. Conclusion

It can be concluded that the oligonucleotide primers selected were effective enough in simultaneous detection of all the species in this study targeting the specific mitochondrial genes. The identification of meat admixtures using these specific primers helped to the conclusion that these primers were sufficient in detecting the practice of adulteration even in minute quantities. Hence this assay can serve as a standard tool for identification and authentication of meat or its derived products, safeguarding consumers from deceitful and fraudulent practices associated with meat adulteration.

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