Research Article

Multiplex PCR-Based Rapid Detection of Six Animal Species in Raw and Processed Meat Using Mitochondrial Genome Sequences

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Abstract | Food adulteration is a global public health risk, and most of developing countries are confronted with this dilemma due to a lack of proper monitoring and policies. The present study aimed to optimize a simple and quick method for detecting adulteration or admixing of different meat species using multiplex-PCR in raw and processed meat targeting a cytochrome b (*cyt b*) gene . A total of 184 samples of raw and processed meat from goat, chicken, cattle, sheep, pig, and donkey were utilized for identification individually and in meat mixture. To simultaneously detect goat (157 bp), chicken (227 bp), cattle (274 bp), sheep (331 bp), pig (398 bp), and donkey (438 bp) meat in a single reaction, a species-specific multiplex PCR has been optimized. The optimized multiplex PCR method was used to identify raw meat and its products containing one or more meat species; the results were consistent with the labeled meat species. The optimized protocol will help to detect and monitor meat species identification and its authentication.

Received | September 20, 2024; **Accepted** | June 05, 2025; **Published** | April 20, 2026
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Citation | Riaz, M.N., S.H. Khattak, S. Mehmood, A. Jamil, K. Jamil, H.M.B. Akhtar, M.F. Rehman and G.M. Ali. 2026. Multiplex PCR-based rapid detection of six animal species in raw and processed meat using mitochondrial genome sequences. *Sarhad Journal of Agriculture,* 42(2): 657-664.
DOI | https://dx.doi.org/10.17582/journal.sja/2026/42.2.657.664
Keywords | Public health, Cytochrome b gene, Multiplex PCR, Meat Adulterations

Introduction

Animal meat is considered to be one of the prime source of protein (20-30%), amino acids mainly lysine, methionine, phenylalanine, leucine, isoleucine along with fat (especially omega-3 polyunsaturated fatty acids), micro vitamins (B6, B12 and vitamin D) and other nutrients including micronutrients (Arscari, 2017; Henchion *et al*., 2017). Recently, meat adulteration is a potential public health concern especially in less developed countries like Pakistan (Li *et al*., 2020). Considering the importance of meat as a prime protein source, now a day's meat adulteration is quite a burning issue worldwide, but in Pakistan adulteration of wholesome with unwholesome meat, poor quality is growing vastly including some ethical and religious issues (Fuseini *et al*., 2017). One of the serious public health concern for food-related items is identifying the adulteration of processed meat with unwanted food ingredients (Haji *et al*., 2023).

Food ingredient authentication is important as it is of human health concern as the ingredients may include the substances that are allergic or toxic (Hoffman *et al*., 2024). Moreover, products labeled with specific meat are often intentionally mixed with other species of low value, owing to the economic advantages (DiPinto *et al*., 2015). This often occurs in countries where beef, mutton and chicken meat are expensive (Cawthorn *et al*., 2013). Furthermore, in countries like Pakistan, where there is less knowledge and high poverty, adulteration of meat with dead, low-quality, or other species' meat, which is prohibited in Pakistan (pig and donkey meat), is normal (Doosti *et al*., 2014). Nevertheless, whether on purpose or by accident, processed meat products are still mislabeled for meat species, particularly pork (Tanabe *et al*., 2007). Adulterations with mono and multispecies have been reported in marketable meat products (Murugaiah *et al*., 2009). It is evident that the rate of adulteration is more in cooked and processed meat rather than raw meat, so it is important to identify fraud in processed meat. Over the past years, a number of techniques for identifying adulterated meat products have been developed. Conventional techniques to identify the species from raw meat include chromatography and DNA hybridization, immune sera diffusion, morphological and histological differences and sensory analysis (Montowska and Pospiech, 2010). However, given that certain circumstances require a lot of time and that proteins might get denatured by heat stress, high pressure, and other processing techniques, these methods could not be sufficient to distinguish between closely related species or be unsuitable for routine application in processed meats (Spanier *et al*., 2004). Conversely, more often than not, DNA-based methods like species-specific PCR, real-time PCR, PCR-restriction fragment length polymorphism (PCR-RFLP), and polymerase chain reaction (PCR) are employed to identify fraudulent meat products (Kumar and Karne, 2017) because of the fact that these methods are simple, rapid, more sensitive and can detect small amounts of DNA (Saez *et al*., 2004). To avoid consumer from any type of possible deception and to ensure food safety, meat legitimacy is essential in food regulatory control provided that proper and accurate detection techniques should be available. In order to address the challenges associated with meat adulteration, the goal of the current study was to standardize a multiplex PCR for identifying meat species, including goat, chicken, cattle, sheep, pig, and donkey in Pakistan.

Materials and Methods

Sample collection

A total of 104 raw meat samples of goat, chicken, cattle, sheep, pig and donkey were collected from Khanewal, Peshawar, Islamabad, Rawalpindi and Mansehra regions. A total of 80 processed meat samples including kababs, nuggets and kofta of different companies were collected from same region. All products were stored at -20 °C before physically analyzed (detection of appearance, odor, and texture and pH determination in meat).

Primer design

The primers used for multiplex PCR and single PCR amplification are listed in (Table 1). All primers were obtained from (Genelink, USA). The cytochrome b gene is widely used for meat adulteration detection because it is part of mitochondrial DNA (mtDNA), which offers several advantages for species identification. Firstly, mtDNA exists in high copy numbers within cells hundreds to thousands per cell compared to nuclear DNA, making it easier to extract and detect, even in processed or degraded samples like cooked or minced meat. This abundance increases the sensitivity of detection methods, allowing identification of trace amounts of adulterated meat. Secondly, the cytochrome b gene contains conserved regions across species, enabling the design of universal primers for amplification, as well as variable regions that differ between species, allowing for species-specific identification. This balance of conservation and variation makes it ideal for distinguishing closely related animals, such as cattle, buffalo, or pork, in a meat sample.

DNA extraction and quantification

Genomic DNA was extracted from 200 mg of each of samples by using the commercially available kit (GeneJet Genomic DNA Purification kit, Thermoscientific, (K 0722) for good quality and non-degraded DNA. Each set of samples was subjected separately for DNA extraction to minimize contamination as multiplex is sensitive and can detect contamination and ultimately gives biased results. Quantification of DNA was done by nano-drop (Thermo Scientific, USA). Moreover, samples were electrophoresed on 1% agarose gel to examine the quality of the extracted DNA.

Table 1: *Primers sequence used in this study.*

S.	Specie	Primer (Cyt)	Sequences (5'→-3')	PCR product (bp)	References
1	Pig	Reverse-5	GCTGATAGTAGATTTGTGATGACCGTA	398	Matsunaga *et al.*, 1999
2	Sheep	Reverse-4	CTATGAATGCTGTGGCTATTGTCGCA	331	Matsunaga *et al.*, 1999
3	Goat	Reverse-4	CTCGACAAATGTGAGTTACAGAGGGA	152	Matsunaga *et al.*, 1999
4	Cattle	Reverse-3	CTAGAAAAGTGTAAGACCCGTAATATAG	274	Matsunaga *et al.*, 1999
5	Chicken	Reverse-2	AAGATACGAATGAAGAAGAATGAGGCG	227	Matsunaga *et al.*, 1999
6	Donkey/Horse	Reverse-2	TCAGATTCACTCGACGAGGGTAGTA	438	Matsunaga *et al.*, 1999
7	Common primer	Forward	AGCTCAATCTGCCTCCGCCAAACAGACCTAAAATC		
8	Pig	D-loop Specific	F- GGT TCT TAC TTC AGG ACC ATC R- GTG TAC GCA CGT GTA TGT AC	294	Jimyeong *et al.*, 2017
9	Donkey	D-loop Specific	F-CATCCTACTAACTATAGCCGTG R- GAATCCTGATAGTGGAGGGA	325	Tahereh *et al.*, 2014

Single PCR assay

The PCR reaction was performed with the volume of 25µL containing 200 µM of each dNTP (Thermo Fisher Scientific, USA), 20 pmol of each primer, 1 U of Taq DNA polymerase (Thermo Fisher Scientific, USA), 2.5 µl of 10× assay buffer 160 mM, 670 mM Tris- HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl2 (Thermo Fisher Scientific, USA) and 5uL of template DNA. The PCR amplification conditions used in reaction were: an initial denaturation at 95 ˚C for 5 min, 40 cycles at 95 ˚C for 20s, 55 ˚C for 20 s, and 72˚C for 30s and final extension 72 ºC for 10 min. To analyze the resultant amplicons, 1.5 % ultrapure agarose gel was used to run the PCR product. The size marker used in the current study was a 100-bp DNA ladder (Thermo Fisher Scientific, USA) and the images were taken using a Gel Doc apparatus.

Multiplex PCR

For multiplex PCR amplification, six primer pairs (goat, chicken, cattle, sheep, pig and donkey) were mixed and multiplex PCR (mPCR) was performed with the same as a single PCR in a thermal cycler (Applied Biosystems, USA). After quantification by nano-drop, MmPCR was standardized by using different PCR profiles. In a set of 8 profiles and 10 different primer concentration, the best profiles along with primer concentration was selected and reconfirmed multiples times (3-5 repeats). To analyze the resultant amplicons, 4% agarose gel was used to run the PCR product.

Detection of pork/donkey in processed meat products

Beef and chicken meat products of different companies were purchased from market. These meat products were kept at -20 ˚C after being separately minced and homogenized. Following the extraction of DNA, species-specific multiplex PCR amplification was performed to determine if contaminated donkey or pig meat was present or absent.

Results

Physical and pH analysis of the different meat species

The physical attributes (color, texture, pH, and smell) of different animal species were examined; meat's typical color ranges from dark red to reddish crimson, depending on the species and age (Table 2).

Table 2: *Physical and pH examination of meat from different species.*

Species	pH	Color	Texture	Odor
Chicken	6.8	Reddish white	Soft	normal
Cattle	6.3	Rosy red	Soft	normal
Sheep	6.9	Reddish	Soft	Sheep odor
Goat	7.0	Dark red	Soft	normal
Pig	6.6	Reddish grey	Soft	normal
Donkey	7.1	Dark Red	hard	Stable odor urine

Specificity of the species-specific primers

The selected species-specific primers were first assessed against six animal species in order to determine the specificity of the primers for species identification. 50 ng of genomic DNA collected from various domestic animal species were used to perform a PCR (Table 3). Goat (157 bp), chicken (237 bp), cattle (277 bp), sheep (331 bp), pig (398 bp) and donkey (438 bp) were detected in a reaction. It was clear from the data that our species-specific primers had a high specificity

and could be used for species identification (Figure 1).

Table 3: *Identification of meat species from raw meat samples using single and multiplex PCR.*

S. No	Sample type	No. of samples	Area/ company	Result
1	Chicken	20	ICT	Chicken
2	Cattle	20	ICT	Cattle
3	Sheep	20	ICT	Sheep
4	Goat	20	ICT	Goat
5	Pig	10	ICT	Pig
6	Donkey	10	ICT	Donkey
7	Mince meat	2		Goat, Cattle, Pig
8	Homemade meat ball	2		Chicken, sheep, donkey

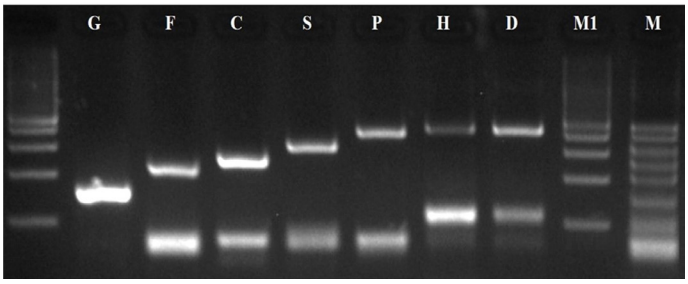

Figure 1: *Agarose gel electrophoresis (4%) of PCR product amplified from the six species. PCR products from G: goat, F: chicken; C: cattle, S: sheep; P: pig; H: horse; D: donkey DNAs were single DNA fragments of 157 bp, 227 bp, 274 bp, 331 bp, 398 bp, 438 bp and 438 bp respectively. M 1 is a Multiplex PCR having all specie's DNA fragments. M: 100 bp Ladder.*

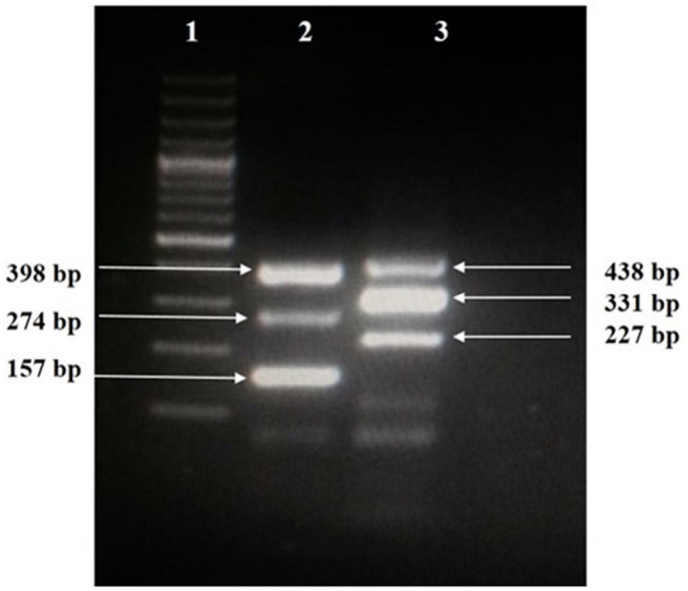

Figure 2: *Agarose gel electrophoresis (2%) of PCR products amplified from DNA of the different mixtures of six meat species. Lane 1 (100bp ladder); Lane 2 (Goat, Cattle, Pig); Lane 3 (Chicken, Sheep, Donkey).*

Multiplex PCR assay was standardized for sheep, goats, cattle, chickens, donkeys, and pig meat identification. As non-halal meats like pork and donkey meats are frequently mixed with chicken, mutton, and beef. The multiplex PCR was successfully optimized for the simultaneous detection of goat, chicken, cattle, sheep, pig, and donkey by optimizing the ratio of primers in PCRas shown in (Figures 1, 2), clear and sharp bands of goat (157 bp), chicken (237 bp), cattle (277 bp), sheep (331 bp), pig (398 bp) and donkey (438 bp) were visualized on gel electrophoresis. The lack of observable cross-reaction with species-specific primers in the DNA samples confirmed the primer's specificity for each species.

Sensitivity of the species-specific PCR

DNA extracted from cattle and pig meats were mixed for use as templates in the ratios of 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9. Figure 3 shows the bands of cattle and pig-specific fragments of 274 and 398 bp, along with the relationships between template DNA amounts and band intensity. In lane 6 (50:50), two bands from cattle and pig DNA indicated similar amounts of intensity of PCR products. When pig DNA increased, from lanes 2-10, the band of 398 bp fragment became intense and that of 274 bp fragment faint.

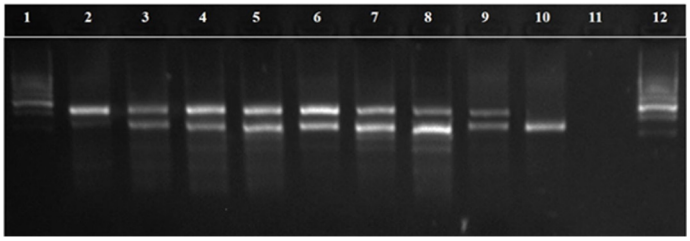

Figure 3: *Agarose gel electrophoresis of PCR products amplified from cattle and pig DNA mixture. Lanes 1, (100bp Ladder); 2, (9:1); 3, (8:2); 4, (7:3); 5, (6:4); 6, (5:5); 7, (4:6); 8, (3:7); 9, (2:8); 10, (9:1); 11, (Blank); 12, (100bp Ladder).*

Detection of pork and donkey species in processed meat products by multiplex PCR

Five commercial meats were subjected to multiplex PCR species identification assays in order to confirm the detection capability of our approach for processed meat products.

The current protocol successfully identifies the species from meat samples even from the mixed meat products (Table 4). The result provided the potential application prospects for detection and monitoring of adulteration of donkey and pork in commercial meat products.

Table 4: *Identification of pork and donkey meat adulteration from processed meat samples.*

S. No.	Sample type processed meat	Species	No. of samples	Company name	Presence of donkey	Presence of pork
1	Chapli Kabab	Chicken	10	A	-	-
2	Seekh Kabab	Chicken	10	A	-	-
3	Nuggets	Chicken	10	B	-	-
4	Kofta	Chicken	10	C	-	-
5	Chapli Kabab	Beef	10	A	-	-
6	Seekh Kabab	Beef	20	B	-	-
13	Shami Kabab	Beef	10	C	-	-

Discussion

The identification of meat species and their origins, as well as the detection of adulteration, holds significant importance from social, economic, ethical, and religious perspectives (Haji *et al.*, 2023; Kesmen *et al.*, 2009). Meat adulteration is a widespread problem in retail markets of Pakistan (Aziz and Khan, 2014). To avoid consumer from any type of possible deception, special methods and techniques are being used to determine and authenticate meat products (Di Pinto *et al.*, 2015). Therefore, there is dire need to develop quick and reliable method for identification of different types of meat species in raw and processed meat. Previous studies demonstrated that DNA-based methods are the favored methods for rapid species identification.

Conventional and real-time PCR based on mitochondrial or nuclear genome sequence have been previously evaluated for qualitative or quantitative identification of raw and processed meat, mostly on an individual basis (Kumar *et al.*, 2015; Kumar and Karne, 2017). This study aimed to establish the rapid protocol for amplifying target gene in meat of selected species using single and multiplex PCR simultaneously.

In this study, two authenticated techniques were evaluated using mt DNA: cyt b gene. First: the conserved region of cyt-b gene was amplified using species specific primers. Second: Multiplex PCR was used to identify meat species from raw and processed meat.

Maintaining sequence conservation within the target species is essential for accurate species identification and preventing false-negative findings (Wang *et al.*, 2021). In this study specie specific primers were evaluated using single PCR and detected specie specific bands corresponding to goat (157 bp), chicken (237 bp), cattle (277 bp), sheep (331 bp), pig (398 bp) and donkey (438 bp) in a reaction. This suggests that all target meat species identified accurately using the species-specific primers that have been selected, with no amplification from non-target species occurring. These results are in accordance with the previous findings (Cheng *et al.*, 2014; Karabasanavar *et al.*, 2013).

Additionally, sensitivity is crucial for identifying meat species (Wang *et al.*, 2020). In the present work, nine distinct ratios of the DNA of each species were amplified under optimal conditions using two species-specific primers (Figure 3). By using a single PCR, each pair of primers specific to a species could be useful in amplifying the target band with 1:9 of the expected size DNA of the relevant species. Similar findings were observed in a previous study (Matsunaga *et al.*, 1999).

Generally, quick and easy detection processes are needed for precise analytical approaches for identifying animal species in meat products (Kumar and Karne, 2017). Six species have been successfully identified at the same time from raw and processed samples using the multiplex PCR technique. This technique can increase efficiency while saving time and money when compared with the conventional PCR method (Rahmati *et al.*, 2016).

Similarly, we standardized a multiplex PCR to quickly and easily detect meat because cheaper meats like pig and donkey meat are frequently mixed in with mutton and beef. The multiplex PCR for the simultaneous identification of goats, chicken, cattle, sheep, pigs, and donkeys was successfully standardized by varying the primer ratios in the PCR. On an agarose gel, distinct and clear bands were observed, as depicted in Figure 1, with expected sizes of 157 bp, 227 bp, 274 bp, 331

bp, 398 bp, and 438 bp, respectively, for goat, chicken, cattle, sheep, pig, and donkey.

In this study amplification with multiplex PCR, oligonucleotide primers clearly revealed band sizes which were specific to corresponding species, similar results were reported previously (Soares *et al*., 2010). The current method has parallel sensitivity with that of earlier approaches (Bai *et al*., 2009) in which no cross reactivity was observed. To avoid unfair competition among food producers, food composition and genuineness is becoming a vital issue.

Quality evaluation in the meat products comprised of many issues, such as fraudulent adulteration of high-quality meat with that of low-quality meat and the presence of unclear meat (Yin *et al*., 2009), usage of vegetable proteins as they are of lower price than animal protein (Zhuang *et al*., 2016). Additionally, the unclear constituents can be health hazardous i.e., bovine spongiform encephalopathy due to mixing of diseased meat tissue and also because some percentage of individuals are sensitive to allergic reactions with specific meat type (Ijaz *et al*., 2020). The most imperative problem of substitution of meat is related to religious concerns and practices because of the reason pork (pig) meat is forbidden in Islam, Judaism and others (Jenkins *et al*., 2010).

Meat adulteration is burning issue of public health as mentioned above, so it is of prime importance to develop or standardized the PCR related technique to quickly distinguish between undesired meats. Present study proved successful in development of the MPCR for goat, chicken, cattle, sheep, pig and donkey. It is also highly recommended to use of this standardized protocol for quick identification of the meat specie than wasting precious time on optimizing in Pakistan. Moreover, dealing bulk of samples, this protocol is highly suitable and accurate in detecting adulterated meat. The established protocol could also opted by the food laboratories, quality control laboratories and researcher for validation and certification of Halal meat production for public consumption in commercial meat markets and industries. However, the study mostly relies on the quality and purity of DNA extracted, which may be compromised in highly processed meats.

Acknowledgements

We are highly obliged "Pakistan Science Foundation" for funding under the grant no. PSF/NSLP/C-NARC (595). This will also help in resolving meat species authentication/ public health issues in Pakistan. This research work could not be completed without generous support of Chairman, PARC for providing institutional facilities in execution and establishment of this facility.

Novelty Statement

This article focuses on the issue of fraudulent sales of Halal meat in Pakistan. To the best of our knowledge, this is the first time a fast detection approach has been developed to resolve the meat adulteration issue in Pakistan.

Author's Contribution

Muhammad Naeem Riaz: Conceptualization and supervision.
Sahir Hameed Khattak: Data analysis and write-up.
Sarfraz Mehmood: Methodology and data analysis.
Aatka Jamil: Methodology.
Khansa Jamil: Paper write-up and paper review.
Hafiz Muhammad Bilal Akhtar: Methodology and formatting.
Muhammad Fazl-ur-Rehman: Methodology.
Ghulam Muhammad Ali: Overall supervision.

Generative AI and AI-assisted technology statement
The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest
The authors have declared no conflict of interest.

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