P-ISSN: 2304-3075; E-ISSN: 2305-4360
International Journal of Veterinary Science
www.ijvets.com; editor@ijvets.com

Research Article https://doi.org/10.47278/journal.ijvs/2023.012

Multiplex PCR Assay for Simultaneous Detection of Beef Meat Fraud with Different Species

Samar S. Ibrahim[1], Rasha Elsabagh[2], El Sayed A. Abdelrahman[3], Mohamed K. Morsy[4], Essam Ismail El touchy[5] and Ahmed Medhat Hegazy[1*]

[1]Department of Forensic Medicine and Toxicology, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Qalyubia, Egypt
[2]Department of Food Hygiene and Control, Faculty of Veterinary Medicine, Benha University, Moshtohor, Toukh 13736, Qalyubia, Egypt
[3]Al-Basateen Slaughter House, Cairo Veterinary Authority, Cairo, Egypt
[4]Department of Food Technology, Faculty of Agriculture, Benha University, Moshtohor, Toukh 13736, Qalyubia, Egypt
[5]Biotechnology Department, Animal Health Research institute AHRI.ARC, Egypt
***Corresponding author:** ahmed_medhat012@yahoo.com; ahmed.hegazy@fvtm.bu.edu.eg

Article History: 22-764 Received: 12-Dec-22 Revised: 19-Jan-23 Accepted: 25-Jan-23

ABSTRACT

Identification of species in animal-derived foods is a crucial component of its management. Legal, financial, religious, and public health considerations all apply to food adulteration, particularly in the case of beef meat. Food adulteration mostly includes replacement of low price ingredient in meat products to get unlawful higher benefits. To protect consumers from meat adulteration, various methods have been investigated. The DNA-based techniques are rapid, greater resistance to degradation, adequate for the detection of small amounts of DNA in complex handled food varieties. This work was designed to investigate different concentrations of beef meat adulterations with other meat types (equine, sheep, dog, and pork) using multiplex PCR. Fresh meat samples of different animal species cattle, donkey, pork and dog were collected for detection of their adulteration. Meat species samples were minced to make meat mixture for mimicking adulteration. The multiplex PCR assay for five meat species was run effectively, clarifying five unique PCR fragments. These PCR sections compared the particular sizes expected for the five designated species**.** The results showed successful amplification of the target cyt b gene sequences with the expected amplicon sizes (271pb) for cattle, (274pb) for sheep, and (808pb) for dog meat. Amplification of the target mt DNA, and 12S rRNA-tRNA Val gene sequences with the expected amplicon sizes (359pb) for equine, and (290bp) for pork's meat. The developed multiplex PCR assay was sensitive enough to detect 0.5% (w/w) adulterated meat under mixed matrices. It was concluded that the multiplex PCR could greatly minimize the cost for detection of meat adulteration.

Key words: Beef Mt DNA, Cyt-b Gene, Meat Adulteration, Multiplex PCR, 12S rRNA-tRNA Val Gene.

INTRODUCTION

Meat is an important wellspring of different micronutrients, which are fundamental for development. It contains about 20 to 35% protein, all essential amino acids, fatty acids, minerals, and vitamins (Abuelnaga et al. 2020). The highest produced meat categories in the world were bovine, poultry and pig (Fengou et al. 2021). In Egypt, beef meat consumption reached 713,000 million tons in 2019, up 28,000 million tons contrasted with the 2018 (Galal-Khallaf 2021). Accordingly, to the high cost of meats and its heavy processing; the incidence of beef adulteration has become clear.Meat adulteration is a serious problem in many countries. It mostly includes replacement of low price ingredients in meat products to get unlawful higher benefits (Uddin et al. 2021). This may have an adverse consequence on human health because of the spread of diseases such as swine and avian influenza virus, as well as foot and mouth disease (FMD) (Qin et al. 2019). In addition, severe allergic reactions for some people were recorded (Uddin et al. 2021). The illegal activity raises serious concerns about public health, food safety, religious and ethical issues (He et al. 2022).

Cite This Article as: Ibrahim SS, Elsabagh R, Abdelrahman ESA, Morsy MK, Touchy EIE and Hegazy AM, 2023. Multiplex PCR assay for simultaneous detection of beef meat fraud with different species. International Journal of Veterinary Science 12(4): 607-611. https://doi.org/10.47278/journal.ijvs/2023.012

Various methods have been investigated to protect consumers from meat adulteration based on protein and DNA analysis (Fengou et al. 2021). The protein-based analyses have many drawbacks such as instability by heat treatment (Iqbal et al. 2020). The DNA-based techniques are high thermal stability, rapid presence in all cell types, reliable, greater resistance to degradation, adequate for the detection of small amounts of DNA, robust, increased specificity and sensitivity. DNA is an inert molecule; varieties in nucleotide sequences empower them to separate between species (Iqbal et al. 2020). To identify animal species for the purpose of fraud detection; DNA testing is proposed as a standard method. However, conventional PCR is expensive, time-consuming, and invasive (Fengou et al. 2021). Multiplex PCR-a derivative of conventional PCR- has the ability to identify multiple species in one tube by amplifying several DNA sequences in a single PCR reaction (Denyingyhot et al. 2022). It requires PCR primers only without any modification, which reduces the cost of detection and saves time (Liu et al. 2019). It has attracted wide consideration in food safety because of its low cost, high efficiency, sensitivity, and simplicity (Cheng et al. 2022). So that, this work was designed to investigate different concentrations of beef meat adulterations with other meat types (equine, dog, and pork) using multiplex PCR.

MATERIALS AND METHODS

Ethical approval

Ethics approval of this experiment was carried out in accordance with the rules and guidelines of the Institutional Ethics Committee, Benha University, Egypt. Approval Protocol Reference Number: BUFVTM 28-09-22.

Sampling

One hundred and twenty fresh meat samples of different animal species (30 samples for each species) cattle, donkeys, pigs and dogs were collected for detection of their adulteration with illegal substitution with cheaper meats. Meat samples of cattle and pork meats were purchased from Al-Basateen Abattoir, Cairo. Dog meat was purchased from pet's clinic, Cairo. While donkey meat was obtained from Giza Zoo. All samples were collected in a clean disposable polyethylene bag and stored at -20°C for analysis.

Sample preparation

One hundred and twenty unprocessed meat species samples were minced to make meat mixture for mimicking adulteration. Three kind of meat mixtures (cattle – donkey, cattle – dog and cattle – pork). The first mixture containing beef meat adulterated with different concentration (0.5, 5, 10, 30, and 50%) of donkey meat. The second mixture containing beef meat adulterated with different concentration (0.5, 5, 10, 30, and 50%) of dog meat. The third mixture containing beef meat adulterated with different concentration (0.5, 5, 10, 30 and 50%) of pork meat. Each mixture was prepared to final weight of 50g.

DNA Extraction

QIAamp DNA Mini Kit (Catalogue no. 51304) was used for DNA extraction and purification from the samples. It contains silica-membrane-based nucleic acid for purification from various types of samples. The total hands-on preparation time is only 20min as the spin-column procedure does not require mechanical homogenization.

Each sample (25mg) was incubated overnight at 56°C with 180µL of ATL buffer and 20µL QIAGEN protease into the bottom of a 1.5mL microcentrifuge tube till tissue lysis then incubated at 72°C for 10min. The mixture was centrifugated at 4000rpm for 2min to remove drops from the inside of the lid. Transfer the supernatant was diluted with ethanol (96%). The mixture was then put to a QIAamp DNA mini spin column. To enhance the purity of the eluted DNA, the DNA attached to the column was washed twice in two centrifugation stages using two distinct wash buffers. A 50µL buffer AE, pure DNA was eluted from the column. The spectrophotometer was used to determine the content and purity of DNA (Biometra, Germany) at 260 and 280nm, respectively (to obtain the ratio of the absorbance). The DNA fragments partition was done by electrophoretic separation (100V for 60min). To determine the fragment sizes a 100bp plus DNA Ladder (Qiagen, Germany, GmbH) was used.

Primer sequences

Five primer sequences were used for multiplex PCR identification and are listed in Table 1 (Dossti et al. 2014; Tasara et al. 2005; Maede 2006; Abdel-Rahman et al. 2009). Species-specific primers were synthesized by Mid land Certified Reagent Company_ oilgos (USA).

Multiplex PCR

An Emerald Amp GT PCR master mix (Takara) (Code No. RR310) (25µL total sample volume) was used for multiplex PCR amplification. The mixtures involved in PCR reaction contained (12.5µL) PCR master mix (RR310A Kit), 1µL each of forward and reverse primers, 6µL extracted DNA and 4.5µL double distilled water. Species-specific primer sequences consisting of common forward primer for cattle, equine, sheep, dog and pig meats were embraced and used together for the multiplex PCR. The amplification conditions were illustrated in Table 2. Molecular size markers were indicated on each gel. Agarose gel electrophoresis was used to analyze PCR amplification products.

DNA amplification

Finally, the amplified product was separated and electrophorized according to (Sambrook et al. 1989). The electrophoresis grade agarose (1.5g) was prepared in 100mL TBE buffer in a sterile flask. To dissolve all granules; the mixture was heated with agitation in microwave then allowed to cool at 70˚C. Then added 0.5µg/mL ethidium bromide and mixed thoroughly. The warm agarose was poured directly into gel casting apparatus with desired comb in apposition and left at room temperature for polymerization. The electrophoresis tank was filled with TBE buffer. A 20µL each PCR product samples, positive control, and negative control were loaded to the gel. The power supply was 1-5volts/cm of tank length. The run was stopped after about 30min then the gel was transferred to UV cabinet. It was photographed by a gel documentation system and the data was analyzed through computer software. A 100bp DNA ladder was used.

Table 1: Primer sequences of mt DNA of beef, equine, sheep, dog and porcine species-specific genes for PCR identification system

Species	Target gene	Primer Sequence 5'-3'	Amplified Product (bp)	References
Beef	Cyt-b	GCCATATACTCTCCTTGGTGACA GTAGGCTTGGGAATAGTACGA	271	Dossti et al. (2014)
Equine	mt DNA	CCC TCA AAC ATT TCA TCA TGA TGA AA GCT CCT CAA AAG GAT ATT TGG CCT CA	359	Maede (2006)
Sheep	Cyt-b	ATGCTGTGGCTATTGTC CCTAGGCATTTGCTTAATTTTA	274	Dossti et al. (2014)
Dog	Cyt-b	GGA GTA TGC TTG ATT CTA CAG AGA AGT GGA ATG AAT GCC	808	Abdel-Rahman et al. (2009)
Porcine	12S rRNA-tRNA Val	CTA CAT AAG AAT ATC ACC CAC ACA TTG TGG GAT CTT CTA GGT	290	Tasara et al. (2005)

Table 2: Cycling conditions and target genes of the different primers during cPCR

Species	Gene	Primary denaturation	Secondary denaturation	Annealing	Extension	No. of cycles	Final extension
Beef	Cyt-b	94˚C/5min	94˚C/30s	57˚C/30s	72˚C/30s	35	72˚C/10min
Equine	mt DNA			60˚C/40s	72˚C/40s		
Dog	Cyt-b			52˚C/40s	72˚C/50s		
Sheep	Cyt-b			57˚C/30s	72˚C/30s		
Porcine	12S rRNA-tRNA Val			52˚C/30s	72˚C/30s		

RESULTS

A total of 90 beef meat samples were adulterated with different concentrations (0.5, 5, 10, 30, and 50%) of donkey, dog, and pig meat. Each mixture was prepared to final weight of 50g.

DNA extraction

According to the current findings, the isolated DNA was appropriate for PCR amplification. The DNA extraction approach, which relies on DNA binding to a silica matrix in the presence of Chao tropic agents, was deemed successful and capable of removing PCR inhibitors that could otherwise obstruct PCR reactions.

Multiplex PCR specificity

The set of primers maintained the same specificity when multiplex PCR was performed on comparable samples, as demonstrated in Fig. 1. Additionally, the electrophoresis pattern clearly demonstrates the absence of cross-contamination and the species-specific band. Thus, these samples' species were determined using multiplex PCR with species-specific primers.

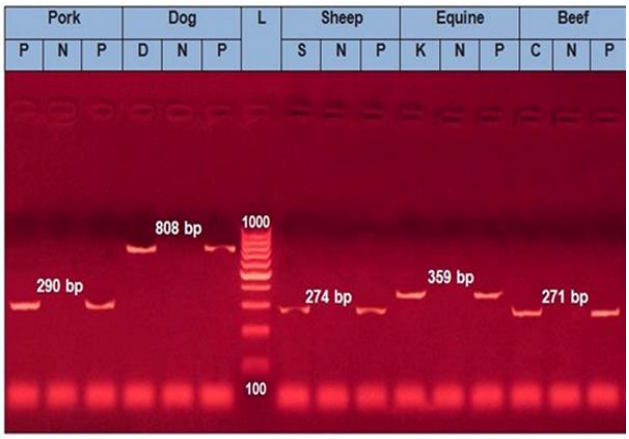

Fig. 1: Multiplex PCR on meat species beef (C), equine (K), sheep (S), dog (D), pork (P), (N) negative control, and (P) positive control.

Testing samples by multiplex PCR

Five distinct PCR fragments were identified by the multiplex PCR for five meat species. These PCR fragments fit the five targeted species' predicted sizes (Fig. 1). For identifying various meat species, PCR was designed to amplify partial length copies of the cyt b, mt DNA, and 12S rRNA-tRNA Val genes. The obtained results showed that the target cyt b gene sequences were successfully amplified with the predicted amplicon sizes (271, 274, and 808) for cattle, sheep, and dog meat, respectively. The target mt DNA and 12S rRNA-tRNA Val gene sequences were successfully amplified with the predicted amplicon sizes (359 and 290bp), respectively, for equine and pork meat.

DISCUSSION

Meat adulteration is a global issue that negatively affects trade on a national and international scale. The authentication of meat has attracted a lot of interest recently due to concerns about health, legal, religion, and economics (Chugunova 2020; El-Jalil et al. 2020; Temisak et al. 2021). For meat products to be of high food quality and safety, species discrimination procedures are essential. Even in highly processed meat products, the molecular authentication of meat species, which rely on the PCR amplification of DNA fragments, has proven to be a viable and extremely sensitive instrument for exact distinction of meat species (Huang and Gu 2022). The protein-based techniques have some limitations due to its high cost and lack of specificity (Zhao et al. 2020).

The present study was designed to investigate different concentrations of beef meat adulterations with other meat types (equine, sheep, dog, and pork) using multiplex PCR. Successful development of the multiplex PCR for the simultaneous identification of five meat species were recorded (Fig. 1). Five distinct PCR fragments that matched the target meat species' anticipated sizes were detected. The sizes of the specific PCR products of beef, equine, sheep, dog, and pork DNA were in accordance with the expected sizes 271, 359, 274, 808, and 290bp with no PCR products from any other

species. The negative control showed no PCR fragments (N). The five sets of primers tested by multiplex PCR produced no unexpected products or cross-reactions, indicating that the genes were sufficiently conserved to allow for the identification of animal meat species. Target species' predicted product size was amplified, indicating the excellent specificity of each primer (Galal-Khallaf 2021). These results agree with (Liu et al. 2019) who detect meat species of cattle, dog, pig, donkey, and rabbit as low as 0.05% (W/W). Our findings also agree with (Wang et al. 2018) who created a multiplex PCR technique and discovered that it was sensitive enough to spot 0.1% (w/w) of contaminated meat and 1pg of pure DNA in mixed matrices. (Galal-Khallaf 2021) mentioned that the multiplex PCR detection limit varied from 0.001 to 0.1ng. Therefore, the procedure that was used in the present study produced satisfactory results. For both qualitative and quantitative detection of beef, equine, sheep, dog, and porcine ingredients; the multiplex test proved a very reliable and accurate approach for meat adulteration (Wang et al. 2019).

The findings showed that the band brightness stayed at a saturated intensity while primer pair concentration was decreased since 0.5, 5, 10, 30, and 50% of beef meat adulteration with other species (Fig. 2, 3 and 4). So that all of the target genes for the various components, can be simultaneously amplified. Different target genes can be amplified well without any cross-reactivity using multiplex detection. At 0.5%, the clean bands are distinguishable. Particularly, the acquired detection limit is strong enough to meet public demand and restrain commercial wrongdoings because the adulteration percentage of 0.5% can only produce insignificant profits (Iqbal et al. 2020).

The simultaneous detection of various species was made possible by the introduction of multiplex PCR, a method for identifying numerous species by mixing multiple primer sets into a single amplification process (Zhao et al. 2021). The multiplex PCR assay is one of the quick, efficient, sensitive, and affordable PCR-based techniques that has been used in a number of meat authenticity investigations (Li and Guan 2019). These techniques can be applied to evaluate the adulteration in commercially accessible meat products, conduct safety checks, and control the quality of food (Wang et al. 2020). The assay that has been created can be useful for a proper investigation of meat products in the food chain. The assay is able to distinguish between cattle/buffalo and sheep/goat species but not between the goat and sheep species or between the cattle and buffalo species (Iqbal et al. 2020).

Conclusion

The current study notes that the multiplex PCR procedure is used to simultaneously detect adulterated components in beef samples, including those from sheep, dogs, donkeys, and porcine. Our strategy shows a number of noteworthy advantages over previously described approaches. Under mixed matrices, the devised multiplex PCR assay was sensitive enough to pick up 0.5% (w/w) adulterated beef. It doesn't require any additional processing aids, such as specialized knowledge, specialized equipment, or reagents, and can be used to concurrently distinguish beef from equine, sheep, canine, and porcine in a single reaction. The cost of analysis can be significantly reduced by multiplex PCR.

Limitation of the study

Further research is required into processed meat products, which could make PCR amplifications more challenging.

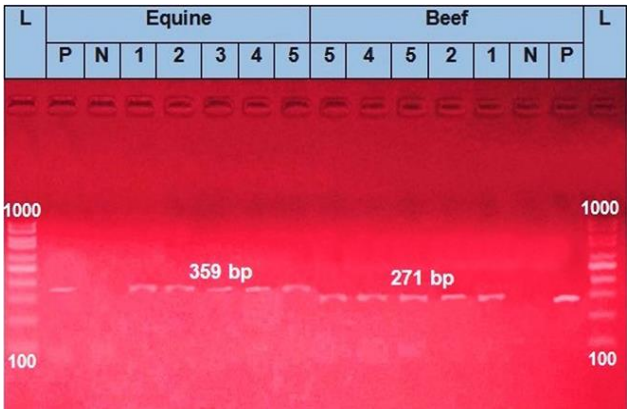

Fig. 2: Multiplex PCR on beef meat adulterated with equine meat with different concentration (1) 0.5%, (2) 5%, (3) 10%, (4) 30%, (5) 50%, (N) negative control, and (P) positive control.

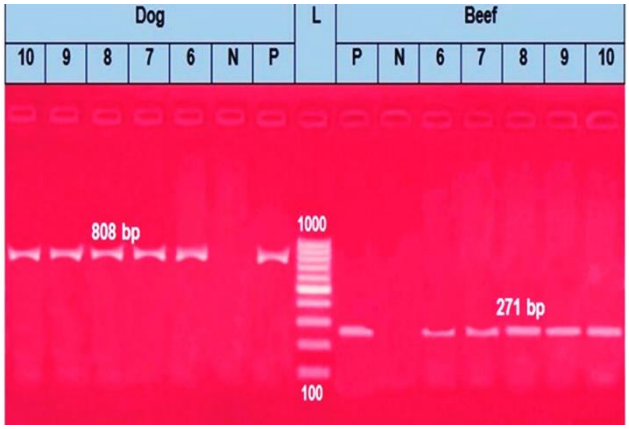

Fig. 3: Multiplex PCR on beef meat adulterated with dog meat with different concentration (6) 0.5%, (7) 5%, (8) 10%, (9) 30%, (10) 50%, (N) negative control, and (P) positive control.

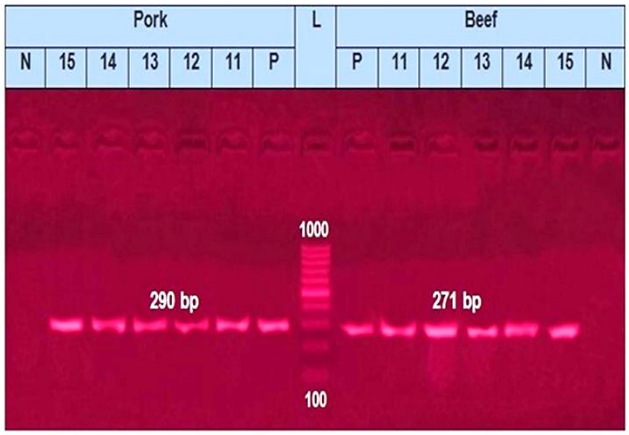

Fig. 4: Multiplex PCR on beef meat adulterated with pork meat with different concentration (11) 0.5%, (12) 5%, (13) 10%, (14) 30%, (15) 50%, (N) negative control, and (P) positive control.

Author contribution

S.S.I., R.E.S., E.S.A., M.K.M. and E.I.E. designed the study, collected the samples and analyzed the data. A.M.H. wrote and revised the manuscript. All authors read and approved the final manuscript.

REFERENCES

Abdel-Rahman S, El-Saadani M, Ashry K and Haggag A, 2009. Detection of adulteration and identification of cat's, dog's, donkey's and horse's meat using species-specific PCR and PCR-RFLP techniques. Australian Journal of Basic and Applied Sciences 3(3): 1716-1719.

Abuelnaga ASM, ABD El-Razik KAE-H, Ata NS, Hedia RH, Elgabry EA-E, Soliman MMH and Marie HSAW, 2020. Bacteriological assessment and multiplex-PCR test for the detection of meat adulteration of different animal species. Food Science and Technology 41: 98-104. https://doi.org/10.1590/fst.11520

Cheng Y, Wang S, Ju S, Zhou S, Zeng X, Wu Z, Pan D, Zhong G and Cai Z, 2022. Heat-treated meat origin tracing and authenticity through a practical multiplex polymerase chain reaction approach. Nutrients 14(22): 4727. https://doi.org/10.3390/nu14224727

Chugunova E, 2020. Using histological analysis to detect mincemeat falsification. International Journal of Veterinary Science 9: 540-545. https://doi.org/10.37422/IJVS/20.071

Denyingyhot A, Srinulgray T, Mahamad P, Ruangprach A, Sa S, Saerae T, Vesaratchavest M, Dahlan W and Keeratipibul S, 2022. Modern on-site tool for monitoring contamination of halal meat with products from five non-halal animals using multiplex polymerase chain reaction coupled with DNA strip. Food Control 132: 108540. https://doi.org/10.1016/j.foodcont.2021. 108540

Dossti A, Ghasemi Dehkordi P and Rahimi E, 2014. Molecular assay to fraud identification of meat products. Journal of food science and technology 51(1): 148-152. https://doi.org/10.1007/s13197-011-0456-3

El Jalil MH, Khamar M, Maaninou S**,** Dahha M, Zinedine A and Ameur N**,** 2020. Antibiotic resistance of *Escherichia coli* strains isolated from broiler meat in Morocco. International Journal of Veterinary Science 9: 305-308.

Fengou L-C, Lianou A, Tsakanikas P, Mohareb F and Nychas G-JE, 2021. Detection of meat adulteration using spectroscopy-based sensors. Foods 10(4): 861. https://doi.org/10.3390/foods10040861

Galal-Khallaf A, 2021. Multiplex PCR and 12S rRNA gene sequencing for detection of meat adulteration: A case study in the Egyptian markets. Gene 764: 145062. https://doi.org/10.1016/j.gene.2020.145062

He C, Bai L, Chen Y, Jiang W, Jia J, Pan A, Lv B, and Wu X, 2022. detection and quantification of adulterated beef and mutton products by multiplex droplet digital PCR. Foods 11(19): 3034. https://doi.org/10.3390/foods11193034

Huang C, and Gu Y, 2022. A Machine learning method for the quantitative detection of adulterated meat using a MOS-based E-nose. Foods 11(4): 602. https://doi.org/10.3390/foods11040602

Iqbal M, Saleem MS, Imran M, Khan WA, Ashraf K, Yasir Zahoor M, Rashid I, Rehman H-U, Nadeem A and Ali S, 2020. Single tube multiplex PCR assay for the identification of banned meat species. Food Additives & Contaminants: Part B 13(4): 284-291. https://doi.org/10.1080/19393210.2020.1778098

Li X and Guan Y, 2019. Specific identification of the adulterated components in beef or mutton meats using multiplex PCR. Journal of AOAC International 102(4): 1181-1185. https://doi.org/10.5740/jaoacint.18-0338

Liu W, Tao J, Xue M, Ji J, Zhang Y, Zhang L and Sun W, 2019. A multiplex PCR method mediated by universal primers for the identification of eight meat ingredients in food products. European Food Research and Technology 245(11): 2385-2392. https://doi.org/10.1007/s00217-019-03350-9

Maede D, 2006. A strategy for molecular species detection in meat and meat products by PCR-RFLP and DNA sequencing using mitochondrial and chromosomal genetic sequences. European Food Research and Technology 224: 209-217. https://doi.org/10.1007/s00217-006-0320-2

Qin P, Qu W, Xu J, Qiao D, Yao L, Xue F and Chen W, 2019. A sensitive multiplex PCR protocol for simultaneous detection of chicken, duck, and pork in beef samples. Journal of food science and technology 56(3): 1266-1274. https://doi.org/10.1007/s13197-019-03591-2

Sambrook J, Fritsch EF and Maniatis T, 1989. Molecular cloning: A laboratory manual. 2nd Ed, Cold Spring Harbor Laboratory Press.

Tasara T, Schumacher S and Stephan R, 2005. Conventional and real-time PCR–based approaches for molecular detection and quantitation of bovine species material in edible gelatin. Journal of Food Protection 68(11): 2420-2426. https://doi.org/10.4315/0362-028X-68.11.2420

Temisak S, Thangsunan P, Boonnil J, Yenchum W, Hongthong K, Oss Boll H, Yata T, Rios-Solis L and Morris P, 2021. Accurate determination of meat mass fractions using DNA measurements for quantifying meat adulteration by digital PCR. International Journal of Food Science & Technology 56(12): 6345-6358. https://doi.org/10.1111/ijfs.15375

Uddin SMK, Hossain MM, Chowdhury ZZ and Johan MRB, 2021. Short targeting multiplex PCR assay to detect and discriminate beef, buffalo, chicken, duck, goat, sheep and pork DNA in food products. Food Additives & Contaminants: Part A 38(8): 1273-1288. https://doi.org/10.1080/19440049.2021.1925748

Wang L, Hang X and Geng R, 2018. Molecular detection of adulteration in commercial buffalo meat products by multiplex PCR assay. Food Science and Technology 39: 344-348. https://doi.org/10.1590/fst.28717

Wang W, Liu J, Zhang Q, Zhou X and Liu B, 2019. Multiplex PCR assay for identification and quantification of bovine and equine in minced meats using novel specific nuclear DNA sequences. Food Control 105: 29-37. https://doi.org/10.1016/j.foodcont.2019.05.016

Wang W, Wang X, Zhang Q, Liu Z, Zhou X and Liu B, 2020. A multiplex PCR method for detection of five animal species in processed meat products using novel species-specific nuclear DNA sequences. European Food Research and Technology 246: 1351-1360. https://doi.org/10.1007/s00217-020-03494-z

Zhao G, Shen X, Liu Y, Xie P, Yao C, Li X, Sun Y, Lei Y and Lei H, 2021. Direct lysis-multiplex polymerase chain reaction assay for beef fraud substitution with chicken, pork and duck. Food Control 129: 108252. https://doi.org/10.1016/j.foodcont.2021. 108252

Zhao J, Li A, Jin X and Pan L, 2020. Technologies in individual animal identification and meat products traceability. Biotechnology & Biotechnological Equipment 34(1): 48-57. https://doi.org/10.1080/13102818.2019.1711185