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Molecular Detection of Chevon and Mutton Adulteration by Species-specific PCR Assay Targeting Mitochondrial COI Gene

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Abstract

A highly species-specific polymerase chain reaction (PCR) was developed for authentic identification of raw and processed meat products of chevon and mutton. To achieve this, four species-specific primers for mitochondrial cytochrome oxidase I (COI) gene were selected from previous reports. The assay generated PCR products of 157, 157, 268, 251, and 177 bp for chevon, mutton, pork, chicken and duck, respectively. The sensitivity for the detection of adulteration was established to be 0.1% (w/w), while the DNA limit for detection was as low as 0.001 ng. The adulteration was found in all meat product types including raw frozen meat, cold cuts, ground meats and cooked foods. These findings showed that species-specific PCR are potentially reliable method in detection of meat products of chevon and mutton authentication.

Keywords: Chevon, Mutton, Adulteration, Mitochondrial COI gene, Species-specific PCR

1. Introduction

Demand for high-quality meat has led to rapid growth in food industry in the recent years. Meat choice usually reflects the aspects of culture, lifestyle, diet, religion and health concerns. Changes in consumer demand of meat products are causing an unprecedented spur in processing and ingredient system developments within the meat manufacturing sector (Weiss et al., 2010). Many years ago, meat was not extensively associated with adulteration and it could be attributed to the fact that it was sold fresh. Nowadays, the lifestyles have changed greatly and the meat food chain has become too long. This leads to the need to process meat into a variety of meat products (Vandendriessche, 2008). Prices for different meats and meat products differ significantly depending on the species and the current market situation. Usually, the low-priced meat is mixed to costlier meat to gain more benefits. With the process of technology in the meat processing industry, adulteration and fraud have become more prevalent due to economic benefits.

Consumption of goat meat (chevon) and sheep meat (mutton) has increased during the last 10 years in China, due to the nutritional and sensorial features (low fat, cholesterol, flavour, juiciness and tenderness). Consumers rarely have the problem in identification of fresh chevon and mutton when bought it in shops or at markets. The particular color, shape and flavour of chevon and mutton can be distinguished from other animal meats when it is fresh. Processed chevon and mutton pose more of a problem as the product cannot be identified by bare eyes. In such cases, disputes between consumers and sellers may arise. Meat vendors often use other livestock meat to adulterate chevon and mutton to meet the consumers' demands and achieve high gain.

Therefore, the need of methods able to identify meat adulteration has increased. The developed analytical methods are reported by several overviews (Ballin, 2010; Singh & Neelam, 2011; Nakyinsige et al., 2012; Kamruzzaman et al., 2013; Sentandreu and Sentandreu, 2014; Kumar et al., 2015; Prieto et al., 2017) including anatomical and histological identification, microscopical examination, spectroscopic methods, biochemical techniques, immunological approaches and genetic techniques. Among these methods, DNA-based molecular techniques have been used widely for the sensitivity, repeatability and reproducibility when compared with other methods. Moreover, DNA is a relatively stable molecule that can be analyzed for processed and heat treated meat products. Nevertheless, for the detection of meat adulteration, the most common methodology based on DNA detection is unquestionably polymerase chain reaction (PCR).

The aim of this study was to develop a multiplex PCR for simultaneous identification of different livestock meats by using mitochondrial cytochrome oxidase subunit I (COI) gene. The experiment was designed to establish a highly species-specific PCR for identification of chevon and mutton adulteration in raw, heat treated and commercial meat samples.

2. Materials and Methods

2.1 Samples

Species considered for sampling include goat (*Capra hircus*), sheep (*Ovis aries*), pig (*Sus*

scrofa domesticus), chicken (*Gallus gallus*) and duck (*Anas platyrhynchos*). Meat samples were prepared in the laboratory acquired in a local retail market and approximately 100 g each of authentic meat samples were collected. Immediately after purchase, all meat samples were cut into small pieces. Meat samples were then stored at -20 ºC until processing. To evaluate the effect of thermal treatment, raw meat samples were submitted to heat treatment by autoclaving during 30 min at 121 ºC.

2.2 DNA Isolation

The extraction of DNA from collected meat samples were performed according to the manufacturer's instruction provided using the Tissue Genome DNA purification kit (Promega, Madison, Wisconsin, USA). The DNA extracted from meat samples was electrophoresed in 1% agarose gel for 30 min at 100 V and stained DNA bands were visualized by UV trans-illuminator and documented over a gel documentation system.

2.3 Optimization of PCR

Four sets of oligonucleotide primer used for PCR amplification are presented in Table 1. Species-specific primers were designed for mitochondrial COI gene. The primers were synthesized by Nanjing GenScript Biotechnology Co., Ltd (Nanjing, China).

Table 1. Oligonucleotide primers for different livestock species

Species	Oligonucleotide primers	Amplicons
Capra hircus	5'-CACGACGATACTCTGATTAC-3'	157 bp
Ovis aries	5'-GTGGTTAGGTCTACAGTTAG-3'	157 bp
Sus scrofa domesticus	5'-CGGGTACACACTCAACCAAG-3'	268 bp
	5'-TGTGCTTGTCAGTTCTACTGC-3'	
Gallus gallus	5'-ACCCATCATGAACCAAGGC-3'	251 bp
	5'-GGCAGTTAATTCGGGTTGG-3'	
Anas platyrhynchos	5'-TAATTGGCACAGCACTCAGC-3'	177 bp
	5'-TTATCAGGGGGACCAATCAG-3'	

The PCR conditions were optimized to obtain specific amplicons. The reaction mixture was made up of 25 µl volume consisting of 10×PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 1 unit of DNA polymerase, and 50 ng of template DNA.

PCR amplification was carried out in a Thermal Cycler C1000 (Bio-Rad, USA). The PCR cycling conditions involved an initial denaturation at 95 ºC for 3 min, followed by 34 cycles of three successive steps of denaturation at 95 ºC for 30 s, primer annealing at 60 ºC for 30 s and extension at 72 ºC for 30 s. The final extension was done at 72 ºC for 5 min and the PCR

products were held at 4 ºC until electrophoresis. The amplified products were analyzed by electrophoresis in a 2.0% agarose gel for 40 min at 100 V. The relative molecular weight of PCR products was calculated by a DL2000 DNA Marker (fragments range from 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp to 100 bp, vertically).

2.4 Specificity Test

The consumption meats, namely mutton, pork, chicken and duck were adopted for species-specificity detection. The aim was to study the cross-reactions between different combinations of DNA and primers. In a basic part of this study, simplex PCR was carried out to verify the specificity of the primers. Further determination of primers' specificity was performed by mixing four primer pairs with an equivalence ratio.

2.5 Sensitivity Test

Two methods were used to evaluate the sensitivity of the developed PCR assay. Firstly, 10-fold serial diluted DNA templates of meats were used. Four concentrations (0.1, 0.01, 0.001 and 0.0001 ng) were prepared by dilution and amplified by the assay to determine the minimum amount that can be detected. In the second approach, the mimic counterfeiting test was assessed by mixing other meats with different proportions. The samples were minced separately and reference mixtures containing 100%, 99.9%, 99%, 95%, 90%, 75.0%, 50.0%, 25.0%, 10.0%, 5.0%, 1.0%, 0.1% and 0% (w/w) of other meats were prepared by successive stepwise additions of minced chevon and mutton.

2.6 Commercial Meat Products Detection

For robustness and real-world performance testing, the developed assay was used to test meat products of chevon or mutton, including raw frozen meats, cold cuts, ground meats and cooked meats. For each meat products, 30 samples were collected from different local markets and detected.

3. Results

3.1 Specificity and Repeatability

The direct simplex amplifications of all four species-specific primers used in this study were first performed using voucher meat samples. This was done to test the specificity of primers and evaluate the possibility by using direct PCR for meat authentication. The results showed that direct PCR was successfully identified meat species from raw meat samples. In these experiments, PCR products produced by each specific primers came from their target species and produced the expected PCR products of 157, 157, 268, 251, and 177 bp for chevon (*Capra hircus*), mutton (*Ovis aries*), pork (*Sus scrofa domesticus*), chicken (*Gallus gallus*) and duck (*Anas platyrhynchos*), respectively (Figure 1).

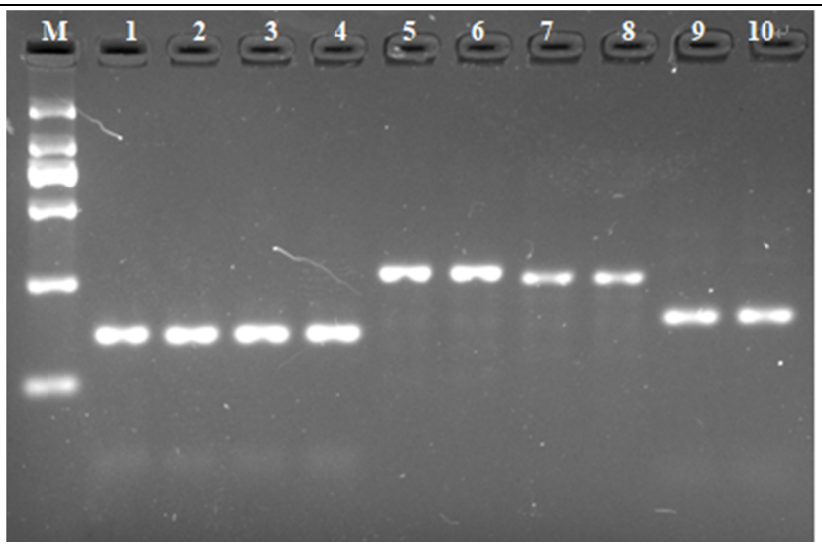

Figure 1. Agarose gel electrophoresis of PCR products. M: DL2000 DNA marker; lanes 1-2: chevon; lanes 3-4: mutton; lanes 5-6: pork; lanes 7-8: chicken; lanes 9-10: duck

When multiplex PCR was performed on the same samples, the set of primers maintained the same specificity. The electrophoresis pattern clearly showed that there was no cross contamination and only the species-specific bands appeared.

3.2 Sensitivity of Species-specific PCR

To determine the sensitivity of the species-specific PCR assay, the target DNA was subjected to 10-fold serial dilution starting from 0.1 ng downwards and the PCR amplification was attempted. The last dilution giving the detectable amplification upon 34 PCR cycles (25 µl reaction volume) was considered as the limit of detection. The limit of detection of the species-specific PCR in this study was 0.001 ng (Figure 2).

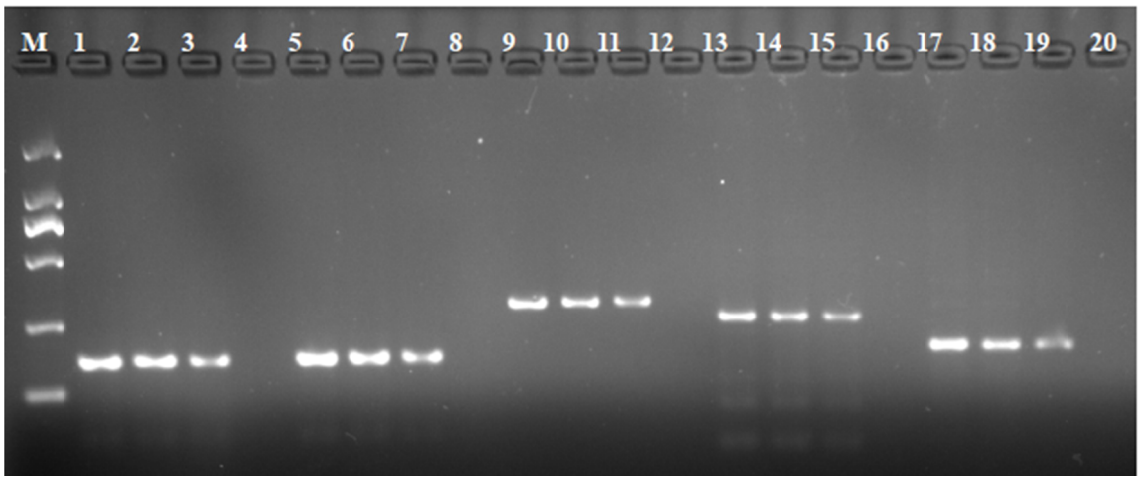

Figure 2. PCR results of serial tenfold diluted DNA template (0.1, 0.01, 0.001 and 0.0001 ng). M: DL2000 DNA marker; lanes 1-4: chevon; lanes 5-8: mutton; lanes 9-12: pork; lanes 13-16: chicken; lanes 17-20: duck

An attempt was made to detection of species from deliberately adulterated meat samples. The meats from different animal species were mixed in different combinations and proportions for this purpose. A total of seven combinations and eight proportions were made in the study. Each combination and proportion was tested in triplicates. All the samples yielded specific amplification product suggesting that mixing meat from different animal species would not affect PCR amplification. The results of detecting the animal species from adulterated meat samples are presented such as chicken in chevon and mutton (Figure 3). This method using base adulteration meat mixture was preferred since it simulates the practical conditions. The species-specific PCR developed in this study was sensitive enough to identify meat adulteration up to the extent of 0.1%.

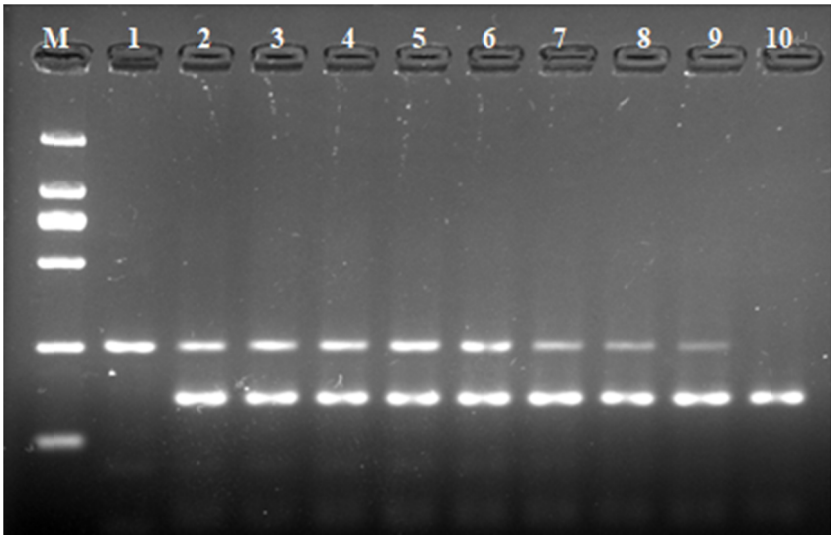

Figure 3. PCR products obtained from binary mixtures of raw meat from chicken in chevon and mutton. M: DL2000 DNA marker; lanes 1-10 are samples of binary mixtures containing 100%, 90%, 75%, 50%, 25%, 10%, 5%, 1%, 0.1% and 0% of the target meats, respectively

To determine the influence of processing treatments on the suitability of the PCR method developed, DNA was extracted from experimentally sterilized (121 ºC for 30 min) meats and tested under the amplification conditions described previously. DNA extracted from sterilized meats shows a typical trailing pattern of nucleic acid degradation, whereas DNA from raw meat samples appears more intact and possesses of high molecular weight. However, the PCR results indicate effective amplification of the expected DNA fragments in all raw and heat-treated meat samples, confirming the ability of the developed PCR to amplify relatively short fragments in highly damaged DNA.

3.3 Application to Commercial Products Detection

The real-world use of the developed method with commercial meats and meat products was demonstrated. The result showed that the multiplex PCR assay was efficient and could be successfully amplified (Figure 4). The adulteration was found in all meat product types

including raw frozen meat, cold cuts, ground meats and cooked meats (Table 2). The highest number of adulteration (66.7%) was found in cold cuts while raw frozen meats had the lowest percentage (6.7%).

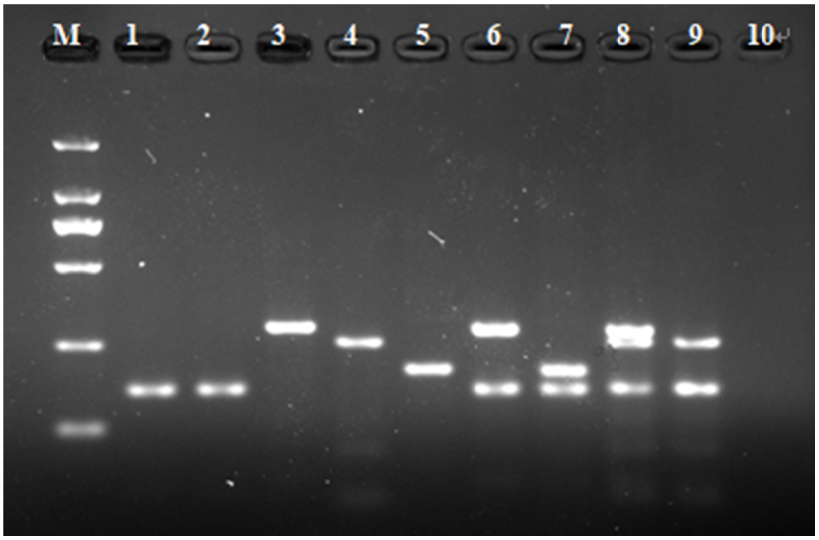

Figure 4. Detection of commercial products of chevon and mutton. M: DL2000 DNA marker; lane 1: chevon; lane 2: mutton; lane 3: pork; lane 4: chicken; lane 5: duck; lane 6: raw frozen meat; lane 7: cold cut; lane 8: ground meat; lane 9: cooked food; lane 10: negative control

Table 2. Detection of adulteration in commercial food products.

Food products	Adulteration ingredients			Fraud ratio
	Pork	Chicken	Duck	(%)
Raw frozen meats	+	-	-	6.7 (2/30)
Cold cuts	+	+	+	66.7 (20/30)
Ground meats	+	+	+	53.3 (16/30)
Cooked meats	+	+	+	63.3 (19/30)

"+" stands for presence and "-" denotes absence.

4. Discussion

Nowadays, replacement of the costlier meat with the cheaper one, is one of the most common ways of economic fraud in the meat industry. It is necessary to identification of the composition of meat products considering the consumer preferences and regulatory purposes. The price of chevon and mutton is similar, but it is obvious higher than pork, chicken and duck in China. Usually, a certain proportion of cheap meats (such as pork, chicken and duck)

were added to chevon and mutton products. This kind of event is widely reported in recent years.

In the preliminary phase of this study, a simplex PCR assays were used to identify individual species and assess the sensitivity of each reaction. PCR amplification results indicated that the size of the amplicons obtained from the four species-specific primers was as expected from sequence analysis. The specific DNA fragments of 157, 157, 268, 251, and 177 bp were successfully amplified with chevon, mutton, pork, chicken and duck primer sets, respectively, showing no amplification from the possible cross-reactions. According to multiplex PCR results, the four sets of primers showed no cross-reactions and unexpected products in any combination, which indicated the mitochondrial COI gene possesses adequate specificity for species identification.

Two methods were attempted for sensitivity assessment in the present work. Firstly, the DNA concentration was determined by spectrophotometry and then diluted into different proportions with nuclease free water for PCR amplification. The limit of detection for target DNA was 0.001 ng and no amplification was obtained up to a dilution of 0.0001 ng. It has been reported that the primers usually give high sensitivity when a short fragment is amplified from a target DNA and the detection limit is enhanced to 0.001 ng (Frezza et al., 2003; Amaral et al., 2014; Song et al., 2017). Secondly, the meat sample was adulterated with meat mixture consisting of other animal species in different proportions. The detection limit (the lowest percentage producing detectable DNA amplifications) of the assay was set on 0.1% (wt/wt) for the species-specific primers, either on raw or sterilized meat mixtures. It was found that species-specific PCR developed was highly sensitive to identification of chevon and mutton adulteration up to the extent of 0.1%. For all animal species, it was observed that the lower percentage of the target meat in the admixture, the fainter band obtained by PCR with the corresponding specific primers. Similarly, a minimum detection limit of 0.1-0.01% for meat products was found in reported literatures (Ghovvati et al., 2009; Karabasanavar et al., 2013; Karabasanavar et al., 2014; Ali et al., 2014; Amaral et al., 2015; Kim and Kim, 2017). Some other workers, especially by real-time PCR and other PCR methods, could even detected <0.01% in the adulterated meat mixtures (Kesmen et al., 2012; Cho et al., 2014; Floren et al., 2015). However, the second method is most preferred because it simulates the actual situation.

Satisfactory PCR results were also achieved when commercial meat products of chevon and mutton were analyzed with the species-specific primer pairs. Results showed that 6.7% raw frozen meats, 66.7% cold cuts, 53.3% ground meats, and 63.3% cooked meats were found to contain other meat. These results indicate that only 52.5% of the total samples were not containing other meat. These findings showed that species-specific PCR assay are potentially reliable technology for detection of meat products in chevon and mutton authentication.

5. Conclusion

Molecular method has been developed in detection of chevon and mutton adulteration by multiplex PCR assay targeting mitochondrial COI Gene. The method could generate species-specific PCR products for chevon, mutton, pork, chicken and duck, respectively. The

sensitivity and detection limit was enough to detect meat adulteration. The adulteration was found in all meat product types including raw frozen meat, cold cuts, ground meats and cooked foods in China.

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References

Ali, M. E., Rahman, M. M., Hamid, S. B. A., Mustafa, S., Bhassu, S., & Hashim, U. (2014). Canine-specific PCR assay targeting cytochrome b gene for the detection of dog meat adulteration in commercial frankfurters. *Food Anal Methods*, *7*, 234-241. https://doi.org/10.1007/s12161-013-9672-y

Amaral, J. S., Santos, C. G., Melo, V. S., Oliveira, M. B. P. P., & Mafra, I. (2014). Authentication of a traditional game meat sausage (*Alheira*) by species-specific PCR assays to detect hare, rabbit, red deer, pork and cow meats. *Food Research International*, *60*, 140-145. https://doi.org/10.1016/j.foodres.2013.11.003

Amaral, J. S., Santosa, C.G., Melo, V. S., Costaa, J., Oliveira, M. B. P. P., & Mafra, I. (2015). Identification of duck, partridge, pheasant, quail, chicken and turkey meats by species-specific PCR assays to assess the authenticity of traditional game meat Alheira sausages. *Food Control*, *47*, 190-195. https://doi.org/10.1016/j.foodcont.2014.07.009

Ballin, N. Z. (2010). Authentication of meat and meat products. *Meat Science*, 3, 577-587. https://doi.org/10.1016/j.meatsci.2010.06.001

Cho, A. R., Dong, H. J., & Cho, S. (2014). Meat species identification using Loop-mediated isothermal amplification assay targeting species-specific mitochondrial DNA. *Korean Journal for Food Science of Animal Resources*, *6*, 799-807. https://doi.org/10.5851/kosfa.2014.34.6.799

Floren, C., Wiedemann, I., Brenig, B., Schütz, E., & Beck, J. (2015). Species identification and quantification in meat and meat products using dropletdigital PCR (ddPCR). *Food Chemistry*, *173*, 1054-1058. https://doi.org/10.1016/j.foodchem.2014.10.138

Frezza, D., Favaro, M., Vaccari, G., Von-Holst, C., Giambra, V., Anklam, E., Bove, D., Battaglia, P. A., Agrimi, V., Brambilla, G., Ajmore-Marsan, P., & Tartaglia, M. (2003). A competitive polymerase chain reaction-based approach for the identification and semi-quantification of mitochondrial DNA in differently heat-treated bovine meat and bone meal. *Journal of Food Protection*, *66*, 103-109. https://doi.org/10.4315/0362-028X-66.1.103

Ghovvati, S., Nassiri, M. R., Mirhoseini, S. Z., Moussavi, A. H., & Javadmanesh, A. (2009). Fraud identification in industrial meat products by multiplex PCR assay. *Food Control*, *8*, 696-699. https://doi.org/10.1016/j.foodcont.2008.09.002

Kamruzzaman, M., Sun, D. W., ElMasry, G., & Allen, P. (2013). Fast detection and visualization of minced lamb meat adulteration using NIRhyperspectral imaging and multivariate image analysis. *Talanta*, *103*, 130-136. https://doi.org/10.1016/j.talanta.2012.10.020

Karabasanavar, N. S., Singh, S. P., Kumar D., & Shebannavar, S. N. (2013). Development and application of highly specific PCR for detection of chicken (*Gallus gallus*) meat adulteration. *European Food Research and Technology*, *1*, 129-134. https://doi.org/10.1007/s00217-012-1868-7

Karabasanavar, N. S., Singh, S. P., Kumar D., & Shebannavar, S. N. (2014). Detection of pork adulteration by highly-specific PCR assay of mitochondrial D-loop. *Food Chemistry*, *145*, 530-534. https://doi.org/10.1016/j.foodchem.2013.08.084

Kesmen, Z., Yetiman, A. E., Sahin, F., & Yetim, H. (2012). Detection of chicken and turkey meat in meat mixtures by using real-time PCR assays. *Journal of Food Science*, *2*, C167-C173. https://doi.org/10.1111/j.1750-3841.2011.02536.x

Kim, M. J., & Kim, H. Y. (2017). Species identification of commercial jerky products in food and feed using direct pentaplex PCR assay. *Food Control*, *78*, 1-6. https://doi.org/10.1016/j.foodcont.2017.02.027

Kumar, A., Kumar, R. R., Sharma, B. D., Gokulakrishnan, P., Mendiratta, S. K., & Sharma, D. (2015). Identification of species origin of meat and meat products on the DNA basis: a review. *Critical Reviews in Food Science and Nutrition*, *10*, 1340-1351. https://doi.org/10.1080/10408398.2012.693978

Nakyinsige, K., Man, Y. B., & Sazili, A. Q. (2012). Halal authenticity issues in meat and meat products. *Meat Science*, *3*, 207-214. https://doi.org/10.1016/j.meatsci.2012.02.015

Prieto, N., Pawluczyk, O., Dugan, M. E. R., & Aalhus, J. L. (2017). A review of the principles and applications of near-infrared spectroscopy to characterize meat, fat, and meat products. *Applied Spectroscopy*, *7*, 1403-1426. https://doi.org/10.1177/0003702817709299

Sentandreu, M. Á., & Sentandreu, E. (2014). Authenticity of meat products: tools against fraud. *Food Research International*, *60*, 19-29. https://doi.org/10.1016/j.foodres.2014.03.030

Singh, V. P., & Neelam, S. (2010). Meat species specifications to ensure the quality of meat-A Review. *International Journal of Meat Science*, *1*, 15-26. https://doi.org/10.3923/ijmeat.2011.15.26

Song, K. Y., Hwang, H. J., & Kim, J. H. (2017). Ultra-fast DNA-based multiplex convection PCR method for meat species identification with possible on-site applications. *Food Chemistry*, 229, 341-346. https://doi.org/10.1016/j.foodchem.2017.02.085

Vandendriessche, F. (2008). Meat products in the past, today and in the future. *Meat Science*, *1-2*, 104-113. https://doi.org/10.1016/j.meatsci.2007.10.003

Weiss, J., Gibis, M., Schuh, V., & Salminen, H. (2010). Advances in ingredient and

processing systems for meat and meat products. *Meat Science*, *1*, 196-213. https://doi.org/10.1016/j.meatsci.2010.05.008