



Original research

TaqMan real-time PCR: a reliable method to detect meat species

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ABSTRACT

The aim of this study was to detect and identify meat species (beef, lamb, pork, chicken, donkey, and horse) in kebab as a halal meat-based food sold in on-road-restaurants of North Khorasan province (Iran) using TaqMan real-time polymerase chain reaction (PCR). Raw kebab samples (150 samples) were obtained from on-road restaurants. The samples were prepared, DNA was extracted and TaqMan real-time PCR using beef, lamb, pork, chicken, horse, and donkey target genes was performed. The results indicated that 100% of samples yielded positive results of beef, but none of them generated donkey and pork amplicons. The samples of kebab indicated 93.33% lamb, 83.33% chicken and 10% horse positive results. The information claimed for the source of meat was not correct for more than 95% of the samples and only one food center labeled the kebab mix correctly. Except for one kebab sample that showed a positive result for the presence of horse meat, no sample contained meat of species considered illegal in Halal foods.

Keywords: Kebab, Polymerase chain reaction, Species detection, TaqMan probe

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1. Introduction

Due to rising prices and reduced availability of meat in some parts of Iran, manufacturers of meat products may use non-meat ingredients or cheaper types of meat instead of beef and it has become a serious issue in the food safety field over the past decades. It is claimed that raw meat products such as hamburgers made from cheap animal species meat are more susceptible to be mixed with other animals' meat than fresh meat. In Iranian food culture, kebab is one of the most popular grilled meat dishes which are made from ground beef or mutton (lamb) and onion. There have been several reports of the counterfeiting of higher value meat (beef and mutton) with low value meat types (chicken, goat, donkey, horse, etc.) or the use of plant protein such as soybeans instead of beef or lamb. The other aspect that should be considered is the authorized and legal meat products in Iran and halal foods. Dog meat is banned in Islam and Buddhism, while pork is banned in Islam and Judaism and donkey and horse is forbidden in Islam (Rahmati et al., 2016). Therefore, reliable and sensitive analytical tools need to be developed to facilitate control the fraudulent use of less valuable species instead of expensive meats for economic, commercial, religious and health purposes (Kesmen et al., 2014).

Conventional PCR techniques are used to qualitatively detect different animal species in a mixture but are not suitable for measuring animal tissue in the product. Recently, reports have focused on the use of real-time PCR to identify and quantify meat species. Real-time PCR has been widely used as a robust test for species identification and measurement of nucleic acid molecules due to its higher sensitivity and specificity and larger dynamic range of detection (Kesmen et al., 2009). It is considered as a highly specific technique among other methods as it is possible to detect even minor amounts target DNA, therefore, useful to identify trace amounts of non-halal meat in halal-products (Farouk et al., 2006).

The aim of this study was to introduce a rapid and reliable method to detect and identify meat species (beef, lamb, pork, chicken, donkey, and horse) in kebab as a halal meat-based food sold in on-road-restaurants of North Khorasan province (Iran) using TaqMan real-time polymerase chain reaction (PCR). Thirty kebab raw mixture samples were obtained from on-road restaurants.

2. Material and Methods

2.1. Collection of meat samples

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Raw meat of beef, lamb, pork, chicken, donkey, and horse were prepared from local suppliers as reference samples. One kilogram of breast, shoulder, or thigh of all species mentioned above were freshly sampled under hygienic conditions and transported directly to Molecular Laboratory of Shirvan Veterinary Office of North Khorasan Province (Iran) under cold chain condition and stored at -20 °C until DNA extraction in order to prevent the enzymatic degradation of DNA.

2.2. Collection of kebab samples

A total of 150 raw kebab samples (5 samples from each restaurant) were collected. The samples (150 grams of fresh ground meat mix prepared for Kebab) were collected under sterile condition, placed in plastic bags, vacuum packaged, kept in insulated refrigerated boxes (5-8 °C), transferred to the Molecular Lab by refrigerated condition, and kept at -20 °C until extraction of DNA.

2.3. Extraction of DNA

Prior to DNA extraction, meat and raw kebab samples were prepared according to the method mentioned as follows: 20 mg of the sample was incubated in an homogenization buffer (consisted of 10 mM Tris HCl, 10 mM NaCl, 1 mM EDTA, 0.1% SDS) at 55 °C for 15 min and then homogenized at 11,000 rpm for 45 s. DNA was extracted using Animal Tissue DNA Extraction Kit (Geneall Biotechnology Co., Kirchleingern, Germany) (Ibrahim et al., 2019).

2.4. Primers and probes

Gene sequences of the cytochrome *b* gene for the following species were obtained from the gene bank of NCBI (ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs, 2019). Beef (*Bos taurus*), lamb (*Ovis aries*), pork (*Sus sacrofa*), and chicken (*Gallus gallus*), primers and mammal probe are published by Dooley et al. (2004), horse (*Equus caballus*) and donkey (*Equus asinus*) primers are published by Kesmen et al. (2009; 2014).

The probes were labeled using reporter dyes FAM (6-carboxyfluorescein, a Fluorophore Reporter), HEX (hexachloro-fluorescein, a fluorescent Reporter) and BHQ (Black Hole

Quencher™). BHQ was used as the quencher on both mammalian and poultry probes. The primer and probe pairs and optimal concentrations used in this work are listed in Table 1.

2.5. Real-time PCR

The real-time PCR amplification was carried out using PCR Master Mix (2x qPCRBIO probe mix no-ROX, UK) on a Rotor-Gene® Q Software 2.3.1.49, Real-Time PCR Detection System (Qiagen, Maryland, United States). The initial heat denaturation step was run at 95 °C for 3 min, and then 40 cycles were set up as follows: 95 °C for 15 s, 60 °C for 60 s (Azizkhani et al., 2013).

2.6. Gel electrophoresis

Electrophoresis on agarose gel (1%) with ethidium bromide (0.25 mg/ml) was used to analyze PCR products and confirm product amplification. It was run in TBE buffer (0.5X) for 30 min at 110 V. Images were documented with a digital imaging system under UV light (Azizkhani et al., 2013).

3. Results

The results obtained from spectrophotometer revealed that DNA extract had appropriate quality and quantity for PCR amplification. The threshold cycle or Ct value is the cycle number at which a detectable quantity of amplicon product makes the generated fluorescence cross the fluorescence threshold; therefore, a fluorescent signal significantly above the background limit will be observed. A TaqMan real-time PCR was applied to detect meat of different species in 150 Kebab samples, in duplicate. The results (Fig. 1) indicated that 100% of samples yielded positive results of beef, but none of them generated donkey and pork amplicons. The samples of kebab indicated 93.33% lamb, 83.33% chicken and 10% horse positive results.

In order to crosscheck the PCR products, amplicons were run on a 1% agarose gel which was stained with ethidium bromide. Electrophoresis of agarose gel revealed that clear bands of the expected size of 116 and 133 and 133 bp were produced by beef, lamb, and chicken primers, respectively (Fig. 2).

Table 1. Primers and probes for cytochrome *b* used in this study.

Species	Primer sets	Sequences (5'-3')	T_m (°C)	Amplicon size (bp)	Reference
Beef	Forward	CGG AGT AAT CCT TCT GCT CAC AGT	58.9	116	Dooley et al. (2004)
	Reverse	GGA TTG CTG ATA AGA GGT TGG TG	58.6		
Lamb	Forward	GAG TAA TCC TCC TAT TTT GCG ACA	56.3	133	Dooley et al. (2004)
	Reverse	AGG TTT GTG CCA ATA TAT GGA ATT	56.7		
Pork	Forward	ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C	58.9	149	Dooley et al. (2004)
	Reverse	CTA CGA GGT CTG TTC CGA TAT AAG G	58.4		
Chicken	Forward	AGC AAT TCC CTA CAT TGG ACA CA	59.4	133	Dooley et al. (2004)
	Reverse	GAT GAT AGT AAT ACC TGC GAT TGC A	58.3		
Horse	Forward	GAC ACA CCC AGA AGT AAA GAC A	60	145	Kesmen et al. (2009; 2014)
	Reverse	TGC TGG GAA ATA TGA TGA TCA GA	59		
Donkey	Forward	TGC TAG CCT CAT TAT CAG TAT	55	183	Kesmen et al. (2009; 2014)
	Reverse	GTG ATG AGG ATA CGT GCT	54		
Poultry probe		(HEX) ACA ACC CAA CCC TTA CCC GAT TCT TC(BHQ)	65.8		Dooley et al. (2004)
Mammal probe		(FAM)TGA GGA CAA ATA TCA TC ATT CTG AGG AGC WARGTYA(BHQ)	68<		Dooley et al. (2004)

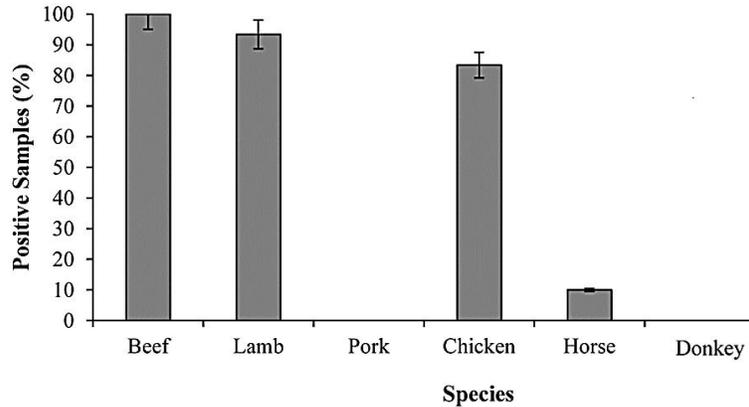


Fig. 1. Presence of meat of different species in kebab samples.

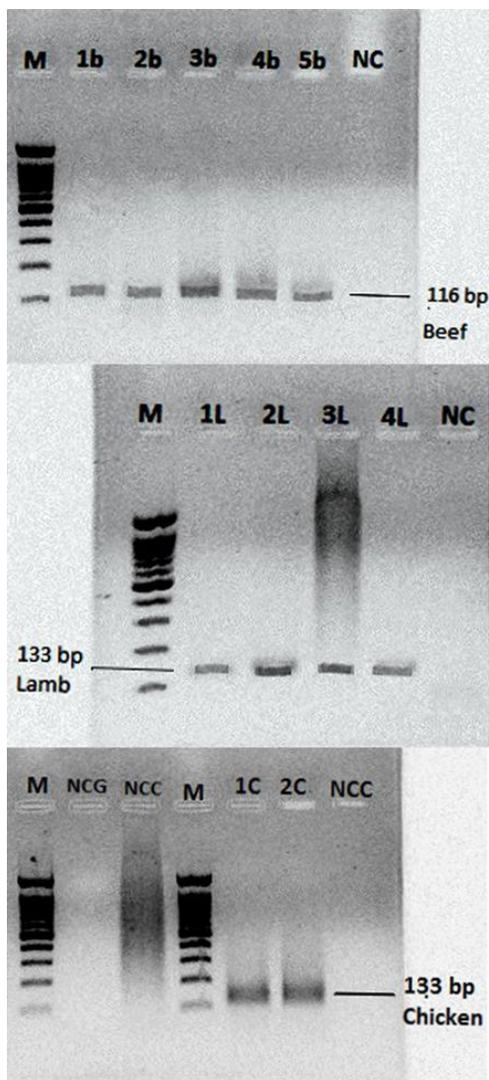


Fig. 2. Agarose gel (1%) electrophoresis of PCR products; M: ladder; NC: negative control for beef and lamb; NCC: negative control for chicken; 1b-5b: beef positive samples, 116 bp; 1L-4L: lamb positive samples, 133 bp; 1C and 2C: chicken positive samples, 133 bp.

4. Discussion

In order to quality assurance and providing food safety, several analytical techniques were introduced to determine the type of meat in meat products. Some of those methods were performed on basis of peptide mass fingerprinting, protein analysis, immunological assays and chromatography which are time- and labor-consuming and expensive (Saez et al., 2004); therefore, recently accurate rapid methods based on DNA detection as different techniques of PCR (multiplex PCR, nested PCR, RFLP-PCR, TaqMan or SYBR GREEN real-time PCR) are used (Abdel-Rahman et al., 2009; Keyvan et al. 2017; Kitpipit et al., 2014).

The technique applied in the present paper, combining the real-time PCR with mammalian and poultry endogenous controls and species-specific primers provide a wide and reliable system for detection of minimal amounts of meat in different meat-based food products which is very important in terms of public health, religious, ethical and economic aspects (Rodríguez et al., 2005). As reported by previous studies, PCR technique for species identification in meat and meat products is performed using a fragment of mitochondrial DNA as target region. Mitochondrial DNA, generally cytochrome *b*, expresses an acceptable range of intra and interspecies variability and is present as high number of copies per cell (Dooley et al., 2004; Kesmen et al., 2009; Montiel-Sosa et al., 2000).

In the present work, assays for the six species were conducted around two probes. As mentioned by Dooley et al. (2004), using two probes is required to prevent sequence variations in the *cytb* gene during the evolutionary heterogeneity of the two classes of vertebrate, mammals (Mammalia) and poultry (Avis). Also, it must be mentioned that using two probes provides a high level of specificity to the assays and minimum cross-reactivity between mammalian assays or poultry assays with the poultry species or the mammalian species, respectively. Specificity of the assays was also obtained using species-specific primer sets which were tested against other meat species.

As DNA in processed foods such as kebab is fragmented extensively, detecting small amplicon size (lower than 150 bp) provides more reliable results than large targets, therefore TaqMan real-time assay is considered as the preferred method to achieve authentic results. Here, this method was applied to investigate the presence of meat of different animal species (beef, lamb, pork, chicken, horse, and donkey) in kebab sold in on-road restaurants.

This method allows detection of trace quantities of meats in mixtures like kebab (only beef and lamb are allowed in this meat product) or of pork, horse or donkey meats in halal foods. Preventing and recognizing adulteration and detection of meat species in meat based products is a critical mission in food safety and quality control systems. Several researches have used the species-specific PCR techniques to detect meat species in different types of food products (Campos et al., 2018; Dooley et al., 2004; Ren et al., 2017; Rodríguez et al., 2005; Spychaj & Mozdziak, 2009).

In the present study 150 kebab samples from 30 on-road restaurants in North Khorasan Province were analyzed. The results of examining kebab samples by running real-time PCR with species-specific oligonucleotide primers and probes were all positive, except for pork and donkey. The results revealed that the information about the source of meat claimed by the producer was not correct for 96.66% of the samples. Except for one kebab sample that showed positive result for the presence of horse meat, no sample contained meat of forbidden species in halal foods. All kebab samples contained some amounts of beef, and also lamb meat was detected in 86.66% of the samples, but 80 and 10% of samples consisted of chicken and horse or mixture of them as cheap meats.

In a study by Al-Qassab et al. (2019), mislabeling was detected in 94.7% (108 samples out of 114) of sausages samples in Tehran (Iran) and the producers have used the cheap chicken tissues like gizzard instead of red meat. According to their analysis, 52.6% of sausage samples were prepared from chicken, 42.1% of beef and chicken and only 5.3% of the examined sausages consisted of beef. In another study in United States, 18.5% of the game meat samples collected from commercial market was determined to be mislabeled and 9.3% of the samples legally contained a vulnerable or near-threatened species and were labeled correctly. The researchers claimed that mislabeling was due to reasons such as product mishandling and economic profit gain (Quinto et al., 2016). Also, Kane and Hellberg (2016) detected animal species in ground meat products in the United States commercial market applying DNA-based techniques. They reported that 38 of the 48 samples (79.16%) analyzed in their study, were labeled correctly and 10 (20.84%) were found to be mislabeled. Real-time PCR results showed that 9 of the mislabeled products contained additional meat species, and one sample was totally mislabeled. Two of the samples contained horse meat (illegal to sell on the commercial food market in the United States). According to authors, the mislabeling observed in their study seemed to be due to intentional mixing of lower-cost meat species in higher cost meat products or unintentional mixing of meat species due to cross-contamination during processing. In the present study, mixing lower-cost species like chicken and horse into Kebab mixture doesn't appear to be unintentional due to species-specific Ct values obtained from analysis of samples.

Results found by Mousavi et al. (2015) showed mislabeling detected by species-specific PCR in 47.2% of raw ground meat commercially sold in Iran. Also, it was reported that 94.4% of raw hamburger samples contained undeclared chicken meat (Mehdizadeh et al., 2014). In another work, poultry DNA was found in sausages labeled with red meat in Tehran, Iran (Nejad et al., 2014).

In most of the researches, mislabeling and adulteration in meat products was due to the substitution of expensive animal meat with cheaper animal tissue. The overall rate of mislabeling observed in this work (> 95%) was much higher than previous studies reported

above, in Iran and other countries. The high rate of mislabeling and fraud found in our study may be due to inefficient inspection and monitoring programs for food products sold in on-road restaurants.

5. Conclusion

Meat of illegal species in halal foods was not detected in kebab samples, except for one sample that contained horse meat. It is apparent that multiple species meat is used in kebab, in spite of regulations of food quality and safety monitoring systems; the detected meats are almost of lower-cost species that were intentionally added into the higher-cost species in kebab mixture for economic profit gain. The results of the present work accentuate the important role of continuous and efficient governmental monitoring of meat-based foods for mislabeling, especially those sold in on-road restaurants.

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Conflict of interest

The authors declare that there is no conflict of interest.

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