Specific identification of chicken and soybean fraud in premium burgers using multiplex-PCR method

Farzaneh Tafvizi · Masumeh Hashemzadegan

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Abstract The increased consumption of meat products, such as hamburger in large cities such as Tehran, has highlighted the importance of quality control for these products. Due to the escalating cost of red meat, and the difficulty of detecting adulteration in ground meat, the replacement of red meat with cheaper animal and plant proteins in these products is clearly possible. As a result, the aim of this study was to investigate the validity of labeling in premium hamburgers made of beef. In addition, the presence of soybean and chicken meat, which constitutes commercial fraud in premium hamburgers, was detected using a sensitive and quick multiplex-PCR method. In total, 10 specified brands of premium hamburgers purported to consist of beef were collected from markets in Tehran City, Iran. DNA was extracted from the premium hamburgers, then, simplex-PCR and multiplex-PCR were optimized using specific beef, chicken and soybean primers. The 118, 183, and 274 bp fragments, were amplified in all samples from soybean lectin, 12 s rRNA, and mitochondrial cytochrome b genes, respectively. The results indicated the addition of chicken meat and soybean in the premium hamburgers which were not indicated on their labels.

Keywords Premium hamburger · Chicken meat · Soybean · Multiplex-PCR

Introduction

Meat is considered to be an excellent and nutritious source of protein. However, due to the high cost of this animal protein, and unfair competition by producers to gain economic benefits, adulteration frequently occurs in meat products (Soares et al. 2010). This includes; use of meat varieties of commercially lower value, use of sub-standard raw materials, presence of unknown species, replacement of animal or plant proteins, incorrect labeling or not declaring ingredients at all (Pascoal et al. 2004; Sakaridis et al. 2013; Kitpipit et al. 2013; Cheng et al. 2014). Moreover, consumers now pay closer attention to health issues (e.g., absence of allergenic compounds), diet (e.g., level of nutrients and calories), religion (e.g., absence of pork), and lifestyle (e.g., organic and vegetarian foods), and this increases the need to control fraud and protect consumer rights (Sakaridis et al. 2013). Given all of the above, the evaluation and validation of basic ingredients of meat products is one of the major concerns of food industry experts (Meyer et al. 1996). On the one hand, improper labeling of food products is indicative of another form of adulteration which concerns consumers, and is in fact the replacement of an animal species with a commercially cheaper species. On the other hand, improper labeling may not cite allergens and harm consumers that may be sensitive or allergic to them (Pascoal et al. 2004). According to the European Commission Law 178/2002, validation is a term defined by the ability to identify animal species and products at different stages of the food production chain (from production to distribution stages) (Murugaiah et al. 2009). Quality and safety assurance of food

F. Tafvizi (✉)
Department of Biology, Parand Branch, Islamic Azad University,
Parand, Iran
e-mail: farzanehtafvizi54@gmail.com

F. Tafvizi
e-mail: farzaneh.tafvizi@yahoo.com

F. Tafvizi
e-mail: farzanhtafvizi54@gmail.com

M. Hashemzadegan
College of Agriculture & Natural Resource, Science and Research
Branch, Islamic Azad University, Tehran, Iran

products for consumers requires rapid confirmation of the validity and reliability of food products. This requirement is gaining importance due to increased global requirements to know the origins of food products, identification of risks associated with the consumption of improper food products on human health, and increased attention on the effects of genetically modified organisms on the human food chain and the environment (Opara 2003).

According to Appendix standards, all raw materials used in the formulation of meat and poultry products should be declared, and when a product is made from a mixture of red meat and chicken, the name of the meat that has the highest proportion should be cited on the label of raw materials. In products containing red meat, chicken, and plant protein, if the proportion of plant protein to meat species is less than 1:13 or equal to 1:10, the name of the plant protein should be cited on the ingredients label, as well as by the product's name (Hiu 2007).

One of the most widely consumed meat products worldwide are varieties of hamburgers. In Iran, a hamburger is defined as minced red meat from Halal sources, usually beef, to which other ingredients are added, including: plant protein (soybean and gluten), oil, spices, filling and binding materials, salt, and aromatic herbs. According to the National Iranian Standards, factory produced hamburgers are divided into three groups; the first group includes ordinary burgers, and the second and third are premium burgers. Ordinary burgers contain a minimum of 30 % red meat, together with a certain amount of plant proteins and other permitted ingredients. This group of premium burgers contains 60–74 % meat, without soybean protein, along with other permitted ingredients. Another group of premium burgers contains 75–95 % meat, without soybean protein, and with other permitted ingredients (Institute of Standards). According to production permits issued by the Ministry of Health and Medical Education, only the use of beef is permitted in the production of factory-made hamburgers (Hosseini et al. 2009).

According to the National Iranian Standards, quality control of this product is performed by microbial and chemical tests. However, one of the most important problems with hamburgers is the adulteration that is occasionally conducted by profiteering people through the unauthorized addition of cheap materials, which exposes the consumer to low quality products (Rokni et al. 2004). Identifying meat in meat products, compared to bone and carcass meats is harder, since the meat undergoes considerable industrial processes, and thus the structural properties of the meat are no longer recognizable. Hence, it is possible to adulterate meat with other proteins (Pascoal et al. 2004; Kesmen et al. 2013).

One type of adulteration in hamburgers involves the partial replacement of beef with chicken meat, because chicken meat contains less saturated fat and cholesterol than mammalian meat, therefore the opportunity for mixing mechanically

deboned chicken flesh with minced mammalian flesh is very high (Doosti et al. 2011).

Furthermore, in recent years, the addition of soybean protein as a raw material replacing red meat in premium burgers (over 60 % meat) has increased significantly due to its functional characteristics (including: increased water and fat binding capacity, emulsification ability, and improved organoleptic properties, such as: appearance, (smooth texture, and cutability), nutritional value, as well as its low price (Renčová and Tremlová 2009; Taski-Ajdukovic et al. 2009; Soares et al. 2012). Meanwhile, the Codex Alimentarius Commission, World Health Organization, Food and Agriculture Organization, and European Commission have recently declared a list of 12 groups of allergens according to their prevalence and intensity, and food packages must declare their names on the labels. Soybean is also included in this list (Mafra et al. 2008; Gomez Galan et al. 2011) as it can cause various allergies, even at low concentrations (Gomez Galan et al. 2011).

Methods used to identify the various protein species (animal and plant) in meat products in recent years include: chemical methods, sensorial analysis, histological differences, fat tissue characteristics, glycogen level in muscle tissue, electrophoresis, immunological, chromatography, and DNA hybridization. However, each has disadvantages and limitations, including: being non-specific, time consuming and costly (Ilhak and Arslan 2007; Safdar and Abasiyanik 2013; Karabasanavar et al. 2014).

Recently, due to the unique features of DNA, such as its stability in cells over the animals' life span, specific sequences for each species, and its durability in industrial food processes (Hiu 2007), such as: high pressure, temperature and chemical treatments, DNA-based methods have raised interest as a useful tool in the identification of species in animal products and other food materials. Among the DNA-based methods, PCR is considered to be the most advanced molecular technique for identifying the origins of meat species, added non-meat proteins, and all food allergens in meat products, especially in mixed products, due to its simplicity, speed, specificity and sensitivity, repeatability, and low detection range (Cammà et al. 2012; Amaral et al. 2013; Sakaridis et al. 2013; Safdar and Abasiyanik 2013; Chun Chi et al. 2014; Karabasanavar et al. 2014).

DNA-based methods include; species specific PCR, random amplified polymorphic DNA (RAPD), restriction fragment length polymorphic PCR (RFLP), single strand conformation polymorphic PCR (SSCP), real-time PCR, and multiplex-PCR (Ghovvati et al. 2009; Stamoulis et al. 2010; Karabasanavar et al. 2014). In the present study, a sensitive and specific multiplex-PCR method was used with species-specific primers for detection purposes (Ilhak and Arslan 2007). The reasons for using multiplex-PCR in this study were the simultaneous detection of multiple target DNAs in the test

phase, fewer reactions, less time and money, and high detection sensitivity in a mixture of DNAs of various species in a single stage PCR reaction (Forte et al. 2005; Ghovvati et al. 2009; Kim et al. 2013).

Material and methods

Samples

Raw beef and chicken meats which were sourced from a butcher and soybean from a supermarket were used as positive controls. Industrial meat products (hamburger) were collected from 10 different companies in Tehran, Iran (randomly coded as 1–10), and these were stored at 4 °C until used for DNA extraction.

DNA extraction

Total cellular DNA was extracted from the samples according to the cetyltrimethylammonium bromide (CTAB) method with some modifications (Sambrook et al. 1989). In brief, 0.06 g of chopped tissue was mixed with 600 µl 2× CTAB lysis buffer (1 M Tris HCL, 4 M NaCL, 0.5 M EDTA, 2 g CTAB, pH 8.0), and 30 µl proteinase K in 1.5 ml microtubes, then incubated at 65 °C for 3 h. Microtubes were inverted every 15 min to produce a good solution of the crushed tissues with the buffer. A total of 600 µl phenol–chloroform mixture was added, followed by vigorous shaking for 10 min and centrifugation at 12,000 rpm for 5 min. The upper aqueous phase was separated without disturbing the interphase which contained cell debris and proteins, then 600 µl of cold (–20 °C) isopropanol was added. The resultant mixture was centrifuged at 12,000 rpm for 10 min and the upper aqueous phase was removed. The DNA pellet was washed with 200 µl cold 70 % ethanol followed by 15 min mild inversion at room temperature and centrifugation at 12,000 rpm at 4 °C for 10 min. The washed DNA pellet was dried by leaving the tubes at 37 °C for 40 min. The DNA sample was dissolved in 50 µl distilled water.

Genomic DNA purity and quantity was assessed with a NanoDrop™ ND-2000 spectrophotometry. By measuring the A260/230 and A260/280 absorbance ratios, the DNA concentration, purity and protein contamination of the samples were determined. The quality of the extracted DNA was analyzed by electrophoresis pattern of the samples in 0.7 % agarose gel, which was stained with ethidium bromide and visualized under ultraviolet light.

Oligonucleotide primers

Species-specific primers were selected for amplification of different regions of the mitochondrial genome and these are presented in Table 1.

Simplex-PCR

In the preliminary phase of this investigation, primer specificity was assessed with DNA extracted from raw meats. For detection of a cross-reaction, the primer set of each species was analyzed by another DNA species in separate simplex PCRs. Simplex PCRs were carried out in a final volume of 25 µl containing 2.5 µl of 10× PCR buffer (500 mM KCl, 100 mM Tris, pH 8.8), 1 U smart Taq DNA Polymerase (Fermentas, Germany), 200 µM each of dNTPs (Fermentas), 1.5 mM MgCl₂, 0.4 µM of poultry, cattle and lectin primers and 20 ng of DNA template. Amplification was carried out in a thermal cycler (Biorad). After an initial denaturation step at 95 °C for 1 min, 35 cycles were programmed as follows: strand denaturation at 94 °C for 1 min, primer annealing at 60 °C for 1 min, primer extension at 72 °C for 90 s, and final extension at 72 °C for 5 min. PCR products were determined by visualization of amplicons on 2 % agarose gel which were stained with GelRed.

Multiplex-PCR

In order to simultaneously identify individual animal species and soybean, all primer sets were used to develop a one-step reaction. Amplifications were developed in a final volume of 25 µl with the same conditions as described for simplex-PCR. Amplification was carried out in a Biorad thermal cycler. After an initial denaturation step at 95 °C for 2 min, 35 cycles were programmed as follows: 94 °C for 1 min, 60 °C for 1 min, 72 °C for 90 s and a final extension at 72 °C for 5 min.

Specificity and sensitivity of PCR

The specificity of each species-specific primer was confirmed by amplification of 20 ng DNA of other target species used in this study (beef, chicken and soybean genomic DNAs). In order to determine the detection limit of specific primers, serial 1:10 (50, 5, 0.5, 0.05 and 0.005 ng DNA/µl water) dilutions of raw beef, chicken and soybean DNAs were prepared, and each dilution was added separately to the reaction mixtures.

Results and discussion

Specific simplex-PCR

First, simplex-PCR was performed on the extracted DNA from beef, chicken, and soybean (control samples) with specific beef, chicken, and soybean primers. Specific beef cytochrome b gene primer, chicken 12 s rRNA gene, and soybean lectin were amplified; 274, 183, and 118 bp, respectively. To ensure the specific function of each primer and to eliminate

Table 1 Specific primer sequences

Primers	Species	Genes	Sequences	Amplicons	References
Poultry	Gallus gallus	12S rRNA	5' TGAGAACTACGAGCACAAAC 3' 5'GGGCTATTGAGCTCACTGTT 3'	183 bp	Dalmasso et al. 2004
Beef	Bos taurus	cytochrome b	5'GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA3' 5'CTAGAAAAGTGTAAGACCCGTAATATAAG 3'	274 bp	Matsunga et al. 1999
Soybean	Glycine max	Lectin	5' GCCCTCTACTCCACCCCATCC3' 5' GCC CAT CTG CAA GCC TTT TTG TG 3'	118 bp	Greiner et al. 2005

cross-reaction of primers, simplex-PCR for each primer was tested three times with non-target DNA, which showed the specific function of each primer. Amplification results of simplex-PCR on 10 brands of premium burgers with species-specific primers of cattle, chicken, and soybean on 2 % agarose gel are presented in Fig. 1. Results of all samples indicated the presence of beef, chicken and soybean in all premium burger samples.

Specific multiplex-PCR with control and burger meat DNA

First, multiplex-PCR was performed with control species DNA (beef, chicken, and soybean), which means that three control DNAs, with their own specific primers, were simultaneously amplified in a PCR. Then, multiplex-PCR was performed on the DNA of each burger with three specific primers. In total, 10 premium burgers were tested without

cross-reactions; specific beef cytochrome b gene primer, chicken 12 s rRNA gene primer, and soybean lectin primer, amplified 274, 183, and 118 bp, were found, respectively. Amplification results of multiplex-PCR on 10 brands of premium burgers with species-specific primers of beef, chicken, and soybean on 2 % agarose gel are presented in Fig. 2. Results indicated simultaneous amplification of the above products in all samples, which confirmed the presence of beef, chicken, and soybean in all premium burger samples. Results of simplex-PCR and multiplex-PCR agreed 100 %.

Sensitivity of the species-specific primers

The detection limits of the specific PCR assay were determined by PCR amplification of DNA extracted from each species. The sensitivity of the method was determined as 0.05 ng DNA for each species (Fig. 3a-c).

Fig. 1 Specificity of simplex-PCR of DNA from 10 hamburger brands with cattle primer (**a**), poultry primer (**b**), soybean primer (**c**). **a** Lane 1–10: 10 hamburger brands, lane 11, 12: Specificity of simplex PCR with DNA from chicken meat and soybean with cattle primer respectively, **b** Lane 1–10: 10 hamburger brands, lane 11, 12: Specificity of simplex PCR

with DNA from cattle meat and soybean with poultry primer respectively, **c** Lane 1–10: 10 hamburger brands, lane 11, 12: Specificity of simplex PCR with DNA from chicken meat and cattle with soybean primer respectively, c+: positive control with target meat DNA in each figure, C-: Negative control; M: 100 bp DNA ladder

Fig. 2 Multiplex PCR on 10 hamburger brands on 2 % agarose gel, lane 1–3: positive control with soybean DNA, chicken meat DNA and cattle meat DNA respectively, lane M: 100 bp DNA ladder, lane C+: Multiplex PCR of cattle, chicken and soybean DNAs, lane 4–13: 10 hamburger brands, C⁻: Negative control

Evaluation of extracted DNA

In the PCR test, the first step was to extract the appropriate DNA. For quality assessment of the DNA, extracted DNA from control samples of beef, chicken, and soybean were isolated and made visible with agarose gel 1 % electrophoresis. As well as the quality of the extracted DNA, its quantity and purity are also important (Ilhak and Arslan 2007). The purity of the control and premium burgers DNA, according to quantitative tests, ranged from 1.8–2, which indicated a high quantity of extracted DNA, high efficiency of the extraction method, and its suitability for PCR amplification (Greiner et al. 2005; Kesmen et al. 2013).

Test specificity and sensitivity

In identifying species in mixed meat products, such as burgers, multiplex-PCR uses a number of target specific primers of different species (animal, plant, or both), and these are used for the amplification of a few short pieces of target DNA. The design of the multiplex-PCR primer is very important, because in this technique the specificity of the primer and T_m temperature are more important than in regular PCR, as it determines the sequence of primers, product length, and melting temperature (Safdar and Abasiyanik 2013). To determine multiple detections of beef, chicken, and soybean in premium burgers, specific primers of these three species, designed by

Fig. 3 The results of PCR sensitivity test for cattle primer (a), poultry primer (b), soybean primer (c) with control DNAs. lane1: 50 ng, lane 2: 5 ng, lane 3: 0.5 ng, lane 4: 0.05 ng, lane 5: 0.005 ng, lane 6: 0.0005 ng, lane7: 0.00005 ng, C⁻: Negative control; M: 100 bp DNA ladd.

Matsunaga et al. (1999); Dalmaso et al. (2004); Abd El-Nasser et al. (2010), were used, respectively. To evaluate the specific function of the designed primers for each species compared to other species, PCR amplification was performed with each pair of primers and extracted DNA from other species. Specificity of function and amplification of the intended pieces were proven by primers for the detection of beef, chicken, and soybean, without cross-reaction of primers with non-target species. In this study, reliable mitochondrial DNA genes were selected as primers to identify animal species because mitochondrial DNAs are simple, and have a short sequence (Ghovvati et al. 2009), in addition, several copies are present in each cell, whereas there is only one copy of nuclear DNA in each cell (Cammà et al. 2012). Since mutation of mitochondrial genes in vertebrates is 10 times higher than in nuclear genes, mutation points have accumulated in sufficient quantities to allow the detection of closely related species. Moreover, mitochondrial markers are more plentiful than nuclear markers, and thus they are more suited to the detection of target species and adulterations (Dalmaso et al. 2004; Mane et al. 2009). Primers' binding sites with band lengths less than 300 bp were considered for amplification of specific pieces, because the samples' DNAs may have been severely damaged in the high temperature process, and this damage may lead to complications in the amplification of PCR (Dalmaso et al. 2004; Ghovvati et al. 2009). Furthermore, the primer used to identify soybeans must be well-known. The primer used in this study has been used in various previous articles (Meyer et al. 1996; Burns et al. 2003; Greiner et al. 2005; Xu et al. 2005; Abd El-Nasser et al. 2010).

Application of the test for premium hamburger samples

Considering the increasing levels of adulteration in meat products, including premium hamburgers with their high meat content and costs, we attempted to use an accurate and reliable method to identify the addition of chicken meat and soybean protein in a variety of premium burger brands. Simplex-PCR and multiplex-PCR were performed on the samples, and the results obtained were identical in both methods, while control species-specific simplex-PCR confirmed the multiplex-PCR method.

Multiplex-PCR was performed on the DNA of the control species and 10 premium hamburger brands. The results obtained showed amplification of specific beef cytochrome b gene primer, 274 bp fragment, specific chicken 12 s rRNA gene primer, 183 bp fragment, and specific soybean lectin primer, 118 bp fragment. According to the declared percentages of red meat on the burger labels of; 60, 75, 85, 90, and 95 %, the results obtained indicated fraud in all samples, because red meat means beef, and the addition of soybean in over 60 % of the burgers is not permitted.

In this study, three types of specific primers (beef, chicken, and soybean) were used to detect high potential adulteration levels in premium burgers produced in Iran. In previous studies, the presence of beef, chicken, and soybean in meat products has been investigated separately (Jahed Khaniki and Rokni 2004; Hosseini et al. 2009; Doosti et al. 2011). In a recent study conducted in 2013, soybean and beef were detected in commercially produced sausages. No study has yet been conducted to simultaneously identify two species of meat (beef and chicken) and one plant (soybean), in burgers produced in Iran (including ordinary and premium), or in other countries around the world. To the best of our knowledge, this is the first time that multiplex-PCR has been optimized for concurrent assessment and detection of soybean with other meat species in burgers.

In 2004, Jahid Khaniki and Rokney investigated histological detection of soybean in frozen raw Iranian burgers by staining with hematoxylin and eosin, and observed soybean tissue in red and muscle cells using a special shiny color in the prepared micrograph. This was a laborious method, and preparation of the samples took a week, in addition, it was unable to detect concentrated and isolated soybean materials (JJahed Khaniki and Rokni 2004).

A relatively simple and enzymatic qualitative method used for the detection of soybean proteins in meat and soybean mixtures by Morrissey et al. comprised of measuring the resulting glucose from acid-enzyme hydrolysis of the mixture, which should contain 66 $\mu\text{M/g}$ soybeans and 1 $\mu\text{M/g}$ meat. In this method, heating at 100 °C for a period of over 60 min, leads to a drop in the release of galactose and arabinose, while, multiplex-PCR is efficient in heated products, as well (Morrissey et al. 1982).

Zilio et al. conducted a qualitative and quantitative investigation of genetically modified soybean RRS (Roundup Ready Soybean) in processed meat products using nested-PCR and real-time PCR methods respectively, but they did not study natural soybeans (Zilio Dinon et al. 2010).

In 2007, Hosseini et al. studied a variety of meats (beef, lamb, and chicken) used in the production of burgers marketed in Tehran, using a sandwich-ELISA method with ready kits. In this method, the sample consisted of a whole burger which was examined, and its juices cooled overnight, whereas multiplex-PCR requires less time (Hosseini et al. 2009).

Flores-Munguia et al. investigated the fraud of replacing meat species of beef, pork, and horse in Mexican burgers and sausages using an immunodiffusion method. Disadvantages of this method included the length of time required for protein extraction, and inconsistent availability of anti-serum (Flores-Munguia et al. 2000).

In 2009, the multiplex-PCR method was used to detect adulterations in industrial meat products, using two mitochondrial genes: 12 s rRNA and 16 s rRNA and three specific primers of poultry, ruminants, and pig. Results showed the

effectiveness of molecular techniques such as; PCR, RAPD, PCR-RFLP, and DNA hybridization, in detecting the meat origins of food products, however, none is without limitations. Multiplex-PCR, when compared to other methods is a highly reproducible, fast, and specific method with a very low detection range (Ghovvati et al. 2009).

In another study in 2011, meat species adulteration in Iranian meat products were investigated, this included hamburgers using the PCR method, and the distinction between horse and donkey species was conducted using PCR-RFLP with digestive enzymes. Results showed that molecular methods like PCR and PCR-RFLP are potentially reliable techniques for the detection of meat species in meat products, and for validation of Halal labeling. However, PCR-RFLP requires more time and it is more expensive than multiplex-PCR (Doosti et al. 2011).

In 2013, Safdar and Abasiyanik studied multiplex detection of beef and soybean in commercial sausages using specific beef and soybean primers, without any cross-reactions. They considered this improved test an innovation due to its ability to amplify two target genes, which could be applied in the detection and distinction of species like; deer, sheep, goat, donkey, horse, and fish, through optimization of the melting temperature. Tests used in this study are molecular tools for use in quality control tests to confirm and control contaminated food products, as well as to authenticate the origins of raw materials (Safdar and Abasiyanik 2013). Multiplex-PCR features, including the simultaneous detection of animal and plant species adulterations in a single stage test, have high sensitivity and specificity, they are economical in terms of costs and time, and repeatability, as a result this makes it a suitable method for the rapid analysis of adulterations in hamburgers (Forte et al. 2005; Kim et al. 2013).

Conclusion

The aim of this study was to develop a simple method for the detection of meat and non-meat proteins added to a mixed meat product. Results obtained from simplex-PCR and multiplex-PCR with specific beef, chicken, and soybean primers, indicated that in addition to beef, chicken and soybean were also observed in all burger samples. This is irrefutable evidence of adulteration, because according to the National Iranian Standards, premium burgers (over 60 % red meat) should only contain beef, and the addition of soybean to these burgers is not permitted. Given the results obtained, it can be concluded that this method has the ability to detect adulteration in all samples, without cross-reactions. Furthermore, the high sensitivity and repeatability features make this method suitable for the rapid analysis of adulterations in hamburgers. Results also showed that the methods used, especially PCR, are applicable in other meat products,

such as: Loghmeh kebab, sausages, salami, nuggets etc., in the detection of the origins of animal and plant proteins, moreover, it can also be used as a control system in the food industry health sector.

Conflict of interest

The authors declare that they have no conflict of interest.

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