IJHSE
Iranian Journal of Health, Safety & Environment, Vol.1, No.2, pp.83-88

Significance of authenticity in meat and meat products in Iran

***Tahereh Rezazadeh*[*1], *Khosrow Aghaiypour*[1], *Zohreh Heidari*[2]**

1) GMO Detection Lab, Behesht Aein Laboratory Complex, Tehran, Iran.
2) Department of Animal Science, Faculty of Agriculture, University of Tehran, Karaj, Iran
**Author for Correspondence: t.rezazadeh@ag.iut.ac.ir)*

Received: 12 Dec. 2013, Revised: 14 Feb. 2014, Accepted: 18 Mar. 2014

ABSTRACT

The Authenticity of meat products is very important for religious and health reasons in Iran. According to legislation in Iran, the consumption and importation of pork, horse, donkey and cat products should be banned. Therefore, the identification of meat products cannot be judged solely by its appearance. This issue led to the authenticity of bovine, sheep, pig, horse, donkey, chicken and soya (*Glycine max*) in raw and processed meat products.In this study, specific primers were designed for the identification of pig (194 base pair), donkey (325 base pair), chicken (391 base pair), sheep (499 base pair), horse (607 base pair), soya (707 base pair) and bovine (853 base pair) by Polymerase chain reaction. Following PCR, expected194,325, 391, 499,607, 707 and 853 base pair fragments were detectable in pig, donkey, chicken, sheep, horse, soya and bovine, respectively. This protocol can be used for identification of raw and processed meat products in various animal species for replication to regulatory obligations for meat species safety in Iran.

Key words: Authenticity; PCR; Meat products; Meat safety; Iran

INTRODUCTION

In Islamic tradition, meat species and safety are very important for religious and health reasons. In Islam, food containing pig meat is Haram, and horse and donkey sources is Makrooh for Muslims, and Many Muslims will not eat meat that is Haram and or Makrooh [1, 2]. In some cases, misleading labels may be harmful for individuals who have food allergies and the consumption of meat and meat products may create health concerns [3, 4]. Moreover, motivated adulteration has emerged in the whole world, and it can be led to serious threats to the health of consumers, especially for imported products. Therefore, the authenticity of meat products becomes a vital issue because the meat products are not enough for domestic consumption and this country has imported a large portion of animal products and food products from other countries. In fact, Iran imports about 100000-150000 M. tons of meat annually majorly from Brazil and Argentine, Pakistan [5]. According last report, 10 percent of the domestic needs are currently imported into Iran. Also, Iran is currently importing red meat from New Zealand for increasing red meat production in the country and reducing market prices [6]. Based on this fact, the risk associated with single and multiple-choice adulterations in commercial meat products has discouraged many people from consuming meat products. Especially for cooked meat products that the adulteration rates these products are higher than raw meats.

Therefore, Iranian's government should conduct a policy on meat products that indicate the meat products are free of pig, horse and donkey sources. Consequently, these concerns have led to perform strategies to identify meat products. The authenticity of the meat ingredient in food enhances consumer's confidence in a variety of species of meat products.

Generally, the identification of meat and meat products can be conducted by using different methods such as immunosorbent *assay* (ELISA) and PCR method. The ELISA test is widely used in the protein detection [7, 8]. The assay is comparatively easy and has a relatively high sensitivity. But, this assay is not useful for processed meat products due to proteins are denatured during the heat process [9]. Nowadays, DNA based molecular techniques are preferred for several reasons. The identification based on DNA can provide it possible to distinguish meat species of closely related animals. Moreover, DNA is more stable against such factors as high temperature, pressure and chemical compounds [10]. Therefore, these mentioned properties allow identify animal species in cooked meat products. Also, very small portion of adulteration can be identified easily by PCR method. Consequently, PCR method has higher sensitivity than the other two methods. There are many reports that PCR method has been used in order to identify of each species in meat and meat products [11-24]. In this study, specific primers were designed for bovine, sheep, pig, horse, donkey, chicken and soya for the identification of raw and processed meat products in various animal species by Polymerase chain reaction.

MATERIALS AND METHODS

Samples of raw meats from chicken, sheep, and bovine were obtained from slaughterhouses (Tehran), and pig, donkey and horse meats were obtained from Chinese meat company Shuanghui, and processed meat and soya were purchased from the local supermarket in Tehran. The analyzed meat products were as follows: 6 raw meats from chicken, 4 sheep meats, 2 bovine meats, 2 pig meats, 1 donkey meat, 2 horse meats, and 7 processed meats of chicken, sheep, and bovine and 3 soya seeds.

The samples were stored at -20°C prior to DNA extraction in order to prevent the enzymatic degradation of DNA.

DNA extraction

The DNA was isolated using the High Pure PCR Template Preparation Kit (Roche, Germany) following the protocol for DNA isolation from mammalian tissue.

After preparing the lysates, the DNA is purified by using a spin column based centrifugation procedure [25].

The extracted DNA samples were resolved on 0.7 % Agarose gels (0.7 g/100 ml, w/v), in a 1 x TAE [48.4 g Tris base [Tris (hydroxymethyl) aminomethane (pH 8); 11.4 ml glacial acetic acid (17.4 M); 20 ml of 0.5 M EDTA in 1 liter of H_2O].

Sequence alignment and Primer Design

All retrieved sequences from GenBank were aligned and compared with Mega ver. 4 software. PCR primers were designed based on the most conserved regions of known sequences available from DDBJ/GenBank with Oligo ver-5 software (table 1).

Primers were capable of amplifying PCR products based on conserved regions in different animal species. Moreover, the choice of size of target genes is very important. Hence, different sizes of genes were chosen from different animals in this study. In fact, long distances between the sizes of genes can help distinguish one animal from another and PCR products were separated by agarose gel electrophoresis easily. Optimal amplicon size of PCR method usually is between 300-1000 bp. Oligonucleotide primers were synthesized by Metabion Company (Germany).

PCR amplification

DNA amplification reactions were performed in a thermal cycler (Mastercycler, Eppendorf). Reactions were performed in a volume of 15 µL containing of 1X reaction buffer, 2 mM $MgCl_2$, 0.1 mM of each dNTP, 0.5 µM of each primer, 0.5 unit/µL Taq polymerase, and approximately 0.5 ng/ µL genomic DNA (Cinagen Co. Iran). The thermocycler program included 2 minutes for initial strand separation at 94°C, followed by 35 cycles of 1 minute at 94°C, 45 seconds at 56° C, 30 seconds at 72°C, and a final 7 minute step at 72°C. Furthermore, above-mentioned reaction was used for semi-nested PCR, as well.

PCR product confirmation

PCR products were resolved on a 0.7 % Agarose electrophoretic gel and visualized by ethidium bromide staining (0.5mg/ml in deionized water) under a UV-transilluminator.

Table 1: Sequences of primers were used in this study

Primer	Sequence 5′-3′	Specificity	Annealing temperature	Amplicon (bp)
Chicken	GGATCATAAACATAGGTCGG CAAGACTGTCCAATGAACAA	Chromosome z/sense Chromosome z/anti-sense	56° C	391
Soybean	GACAATAATGGAGCGAAGG TGATCCAACAACTTTGCCATG	Lectin/sense Lectin/anti-sense	56° C	707
Donkey	CATCCTACTAACTATAGCCGTG GAATCCTGATAGTGGAGGGA	mitochondrial genome /sense mitochondrial genome /anti-sense	55° C	325
Sheep	TGCTTAGCCCTAAACACAA TCCAGTATGCTTACCTTGTT	mitochondrial genome /sense mitochondrial genome /anti-sense	56° C	499
Horse	TTTATCTGCCTCTTCATTCAC CTAATACGCCGCCTAGTTA	Cytochrome b-like gene /sense Cytochrome b-like gene /anti-sense	56° C	607
Pig	ATCCGACTAGGAACCATGAGG CTCCCGTGGCATATGGAG	Chromosome x Chromosome x	54° C	194
Bovine	GCGAGTCAGGGCTCAAGA AAGTGTGGTGGGCTATAACAAGA	Bola /sense Bola /anti-sense	56° C	853

RESULTS AND DISCUSSION

DNA was extracted by the High Pure PCR Template Preparation Kit from different samples of fresh raw meats and cooked meat with a good quality of DNA.

Fig. 1 is shown the amplification of the pig, donkey, chicken, sheep, horse, soya, bovine, cooked meat of bovine, cooked meat of chicken and cooked meat of sheep genome by using primers. The result shows a band of 391 bp in raw and cooked meat. In fact, BLAST sequence analysis services showed that specific primers hybridized with chicken chromosome z at positions 1606 and 1996 (20 of 20 nucleotides coincide) at accession number: AC197511.4 that resulted in a 391 bp band. These primers did not produce any amplified fragment in the sequence of other animals. It showed that a 391 bp band was obtained from chicken, but not from bovine, donkey, horse, pig and sheep.

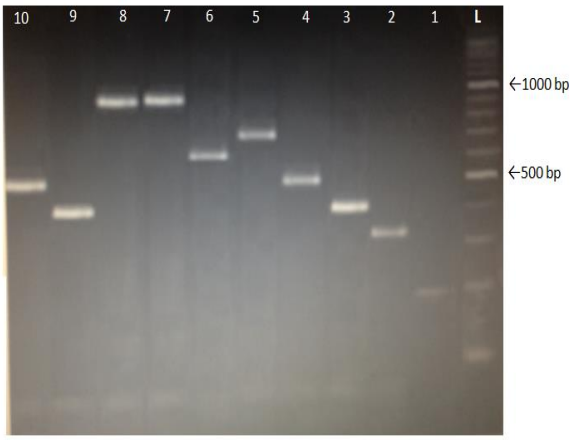

Fig. 1: Electrophoretic Agarose gel, stained with ethidium bromide, of the PCR products of 1-pig (194 bp), 2-donkey (325 bp), 3- chicken (391bp),4- sheep (499bp),5-horse (607bp),6-soya (707bp), 7-bovine (853bp), 8-cooked meat of bovine, 9-cooked meat of chicken, 10- cooked meat of sheep, L: Molecular marker (100 bp ladder)

The mtDNA fragment of donkey was amplified by PCR, with the total DNA that contained mtDNA as a template that produced 325 bp bands (Figure 1). BLAST sequence analysis services showed that Specific primers hybridized with donkey mtDNA with accession number: X97337.1 at positions 4390 and 4714 that produced a 325 bp band. This band is absent in PCR-amplified products from bovine, chicken, horse, pig and sheep.

PCR amplification of a conserved gene fragment (*BoLA gene*) from bovine by using specific primers was performed for the identification of bovine meat that produced 853 bp bands in raw and cooked meat (Figure 1). BLAST sequence analysis services showed that Specific primers hybridized with bovine DNA sequence with accession number: XM_002702852.2 in GeneBank at positions 1459 and 2275 that produced an 853 bp band, whereas no amplification products were obtained with DNA from sheep, chicken, pig, donkey, horse and soya. Also, Fig. 1 is shown the amplification of horse cytochrome b (cytb) gene by using primers that resulted in a band of 607bp. BLAST sequence analysis services showed that these primers hybridized with cytochrome b (cytb) gene with accession number: JF511459.1 at positions 271 and 877 that resulted in a 607 bp band. These primers did not produce any amplified fragment in the sequence of other animals. Also, *lectin gene* in soya was chosen as a PCR target. As a result, specific primers hybridized with soya *lectin gene* at positions 1603 and 2308 that produced a 705 bp band (Figure 1). Besides, oligonucleotide primers were designed that allowed an amplification of specific regions of pig chromosome x and sheep mitochondrial gene and produced a size 194 and 499 bp band, respectively (Figure 1). These partial sequences were used for the design of specific primers that were published in GeneBank under the accession numbers FP015865.8 and KF312238.1 for pig and sheep, respectively. BLAST sequence analysis services showed that specific primers of sheep hybridized with sheep DNA sequence at positions 509 and 1008 that produced a 499 bp band in raw and cooked meat of sheep. Moreover, specific primers of pig were hybridized with pig chromosome x at positions 205126 and 205319 that produced a 194 bp band. Besides, the negative control containing distilled water instead of a DNA template was used that any DNA did not amplify in negative controls. Consequently, 27 samples of raw meat, processed meat and soya were identified by PCR method. It showed that PCR method is suitable for the identification of cooked meats, as well.

Each primer set was tested for its ability to specifically detect its target species DNA in pig, donkey, chicken, sheep, horse and bovine. Pig specific primers were able to identify their target in pig but not in donkey, chicken, sheep, horse, soya and bovine. Specific primers of bovine were highly specific in bovine samples, and these primers not able to amplify any product in other animals. Also, specific primers of sheep, donkey, chicken, horse and soya were not able to amplify any product in other animals.

Furthermore, designed primers in semi-nested PCR technique were capable of amplifying PCR results for final confirmation (Table 2). Following a semi-nested PCR, expected 96, 153, 204, 116, 456, 170 and 515 base pair fragments were detectable in pig, sheep, horse, donkey, bovine, chicken and soya, respectively (Fig. 2).

Table 2: Sequences of Primers are used for semi-nested PCR

Primer	Sequence 5′-3′	specificity	Annaling temperature°C	Amplicon (bp)
Pig	ctgaacctacaccacagctca	Anti-sense	54	96
Sheep	gattggtgaggtttatcgg	Anti-sense	56	153
Horse	agtaccgatgtagggaatt	Anti-sense	56	204
Donkey	ctattcatcctatatgggc	Anti-sense	55	116
Bovine	actcctgcctggaaaatcc	Anti-sense	56	456
Chicken	ttcagtaggagagaagacag	Anti-sense	56	170
Soya	ctgcatttgtcacaaatcatgaa	Anti-sense	56	515

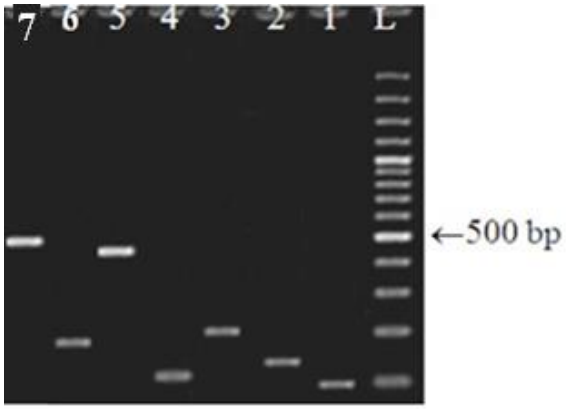

Fig. 2: Electrophoretic Agarose gel, stained with ethidium bromide, of the semi-nested PCR products. ı- pig (96 bp), 2-sheep (153 bp), 3- horse (204 bp), 4-donkey (116 bp), 5- bovine (456 bp), 6- chicken (170 bp), 7- soya (515 bp) L: Molecular marker (100 bp ladder)

The objectives of this study were to use the PCR method as a potential molecular tool for sensitive and rapid identification of meat species in Iran. Primers were suitable for specific amplification target sequence from different animals. In fact, many surveys showed that chicken chromosome z, mtDNA fragment of donkey, bovine *BoLA gene*, horse cytochrome b, soya *lectin gene*, pig chromosome x and sheep mitochondrial gene can be used as specific genes for identification of meat products [26, 27, 28, 29 and 30].

As a whole, PCR assay is an appropriate method for food inspection services for the detection of meat and meat products against food product adulteration and misrepresentations. However, there are several advantages for using PCR method for this purpose. PCR method is rapid, sensitive and highly specific for the identification of a given specific target DNA. Numerous researchers have previously reported PCR assay can be used for the identification of meat and meat products [11 - 24]

Generally, food fraud occurs almost every day in the whole world. Hence, the authenticity of meat products is very vital to consumer health. For example in 2013, horse and pig DNA were identified in beef products sold in several supermarket chains in UK, and horse meat discovered in burgers sold in the UK and Ireland [31, 32]. While the consumption of horse meat is very hazardous for human health due to certain antibiotic drug residues from antibiotic use in horses such as phenylbutazone are highly toxic for humans [31, 32]. With regard to the growth in meat trade is anticipated to high for the next decade due to increase in meat demand and higher meat consumption in Iran. Based on this fact, the importation of meat products is increasing year by year, and most concerns about meat products refer to for health human and religious reasons. But, Iran's food safety should not damage by a dramatic surge in meat product imports. Therefore, there should be a lot of quality control laboratories for the control of imported meat products.

A survey showed that some meat products were not contained halal meat in Iran [1]. Hence, it increases the necessity of the identification of fraud and adulteration in industrial meat products. As a result, the identification of species fraud in meat products should be vital for consumer protection and food industries in Iran. Also, the authenticity of meat products is important in traceability systems for identifying frauds in some of meat samples that soya is used instead of meat in processed meat

products. In some cases, a series of companies in Iran may mislabel meat products as halal imports from Islamic countries or may use cheaper meat instead of expensive meat in order to improve their market. Therefore, PCR method could be used for the detection of frauds in importing samples into Iran.

The results showed that an optimized PCR reaction is suitable for the identification of meat products and primers were specific for every species (table1). In fact, PCR method makes it possible to distinguish meat species in different animals that are close to each other genetically. Moreover, this method is useful for processed meat products due to proteins are denatured during the heat process.

Therefore, PCR test could be used by researchers and quality control laboratories for the control of meat products, and PCR assay is the most widely used technique for the identification of different kind of food products.

CONCLUSION

Molecular biological methods have become an everyday tool to resolve a series of problems and questions in the realm of species identification, fraud and traceability. Therefore, this protocol can be used for the identification of meat products and for the labeling meat samples in order to ensure human safety and religious issue in the Iranian's culture.

ACKNOWLEDGMENTS

We are appreciative of anonymous reviewers for helpful comments. The authors declare no conflict of interest.

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