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ORIGINAL ARTICLE

Simple and fast multiplex PCR method for detection of species origin in meat products

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Abstract Identification of animal species is one of the major concerns in food regulatory control and quality assurance system. Different approaches have been used for species identification in animal origin of feedstuff. This study aimed to develop a multiplex PCR approach to detect the origin of meat and meat products. Specific primers were designed based on the conserved region of mitochondrial Cytochrome C Oxidase subunit I (*COX1*) gene. This method could successfully distinguish the origin of the pig, camel, sheep, donkey, goat, cow, and chicken in one single reaction. Since PCR products derived from each species represent unique molecular weight, the amplified products could be identified by electrophoresis and analyzed based on their size. Due to the synchronized amplification of segments within a single PCR reaction, multiplex PCR is considered to be a simple, fast, and inexpensive technique that can be applied for identification of meat products in food industries. Nowadays, this technique has been considered as a practical method to identify the species origin, which could further applied for animal feedstuffs identification.

Keywords Species identification · Mitochondrial Cytochrome C Oxidase subunit I (*COX1*) · Multiplex PCR

Introduction

Meat products, as one of the main dietary sources, are available in various forms all over the world. Hence, meat quality control is regarded as a critical issue in the food industry (Ballin 2010). Based on a recent report published by the Food and Agriculture Organization of the United Nations (FAO), 308.2 million tons of meats were produced in 2013 and this number is increasing substantially every year. Therefore, meat identification and verification technology is of great importance due to the potential to provide meat supplies to feed 9 billion people by 2050 (Sentandreu and Sentandreu 2014). However, a growing number of consumers are aspiring to "*know which species of animals exist in their food*". Meat authentication and species validation mainly focus on detection of any fraud, such as use of cheaper replacement with an expensive one in meat products. The difference in costs, especially, in large scale can be noticeable for meat consumers and distributors (Anita Spycha 2009). Although, the presence of any original species of meat products must be claimed and labeled accurately, there is still a great need for verification. Also, due to religious laws, recognition of Halal meat such as camel, cow and sheep from Non-permissible, such as pig and dog is compulsory for Muslims and Jews and some meats such as cow are prohibited for Hindu and Buddhist (Ghovvati et al. 2009; Murugaiah et al. 2009). International Standard Organization (ISO) prescribed

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general standardized principles and requirements to design a tracking system for Halal food which apply to all organizations and whole manufacturing process. Also, environmental concerns play a crucial role in the necessity of meat verification. Environmental organizations have banned hunting of endangered species or those which are declining due to irregular hunting in wildlife (Linacre and Tobe 2011).

Other than religious aspects, there are health related aspects to consider regarding meat products consumption which necessitates species identification such as immunological allergens or vegetarian diets. Specific diseases in some species such as Bovine Encephalopathy (BSE) in bovine and Influenza in avian count as essential health related issues that requires consideration of consumption limit in specific animal foods (Ali et al. 2014). So, species identification should be considered as a beneficial and critical method for identification of species used in meat, readymade and processed meat products to confirm their identity.

It is obvious that species identification would be one of the pioneer methods to assess quality of meat. In most cases, it is impossible to identify animal species present in the meat based on their smell and taste. However, various protein- and nucleic acid-based methods including Nested polymerase chain reaction (PCR) (Ono et al. 2007), Random Amplified Polymorphic DNA (RAPD) (Abasiyanik 2011), Real time PCR (Farrokhi and Jafari Joozani 2011), Restriction Fragment Length Polymorphism (RFLP) (Kumar et al. 2014), Multiplex PCR (Ali et al. 2014) and the latest technology, NGS (Next generation sequencing) could be valuable alternatives in species identification (Orfan LHAK 2007; Rogberg-Muñoz et al. 2013). In the last decade, DNA molecules have been used as target compounds for species identification due to their high stability and variability, which allow differentiation of closely related species (Mane et al. 2009). However, to select the proper exact, fast, and simple method, it is important to consider availability of equipment and total costs of the procedure. Multiplex PCR based on specific primers for animal genome target is preferred not only because of simultaneous detection of multiple animal species in one reaction, but also due to ease of use and its rapidity (Matsunaga et al. 1999; Tobe and Linacre 2008). In addition, there are a high copy number of mitochondrial genes (> 5 copies per cell). Such sample extraction contains appropriate amounts of genome, which would leads to more accurate detection. Because of less likely recombination in mitochondria during evolution, the diversity in conserved regions within one species is limited while it could be highly variable between various species. *COX1* (Cytochrome C Oxidase subunit I) gene, as one of mitochondrial genes, has been conserved through evolution with some variation in different species. So, this gene has been introduced as an effective target for novel methods, including Barcoding and NGS, which can be used for species identification through multiplex PCR with specific primers for each species (Barcaccia et al. 2015; Hebert et al. 2003).

In the current study, the genome extracted from meat or meat products, was applied for identification of different meat species origin including chicken (*Gallus gallus*), camel (*Camelus bactrianus*), pig (*Sus scrofa*), sheep (*Ovis aris*), donkey (*Equus asinus*), goat (*Capra hircus*), and cow (*Bos Taurus*) using multiplex PCR. Then, the PCR products were indicated in agarose gel according to different molecular weights of mentioned species.

Materials and methods

Sample collection

As a control group, previously authenticated tissue samples of pig(n:1), camel(n:10), sheep(n:10), goat(n:10), cow(n:10), chicken and donkey(n:1) were obtained from the Iranian Biological Resource Center (IBRC). Then, twenty unknown samples of raw meats were bought randomly from markets, including minced sheep beef and processed products such as sausages, burgers and ham nuggets. In each case, 100 mg of sample were cut into small pieces using a sterile scalpel, kept in a sterile plastic bag and stored at -20 °C to prevent DNA degradation until further use.

Oligonucleotide primers design

Specific primers for pig, sheep, goat and cow were designed for *COX1* as target gene and 18srDNA as an internal control gene using protocol described (Cooper et al. 2007). Primers were designed using Primer3 of National Center for Biotechnology Information (NCBI) software for chicken (NC_001323.1), donkey (NC_001788.1) and camel (NC_009628.2). *COX1* genes for these species were obtained from NCBI Gene Bank. Finally, each primer was analyzed by online NCBI alignment software for specificity to confirm there is no cross reactivity with other species. Primer sequences are shown in Table 1.

Tissue and meat DNA extraction and concentration analysis

DNA was extracted from all samples as described in Aljanabi & Martinez protocol (Aljanabi and Martinez 1997). Briefly, each sample was added into a sterile plate,

Table 1 Details of primer sequence and the relative concentrations in PCR reaction

Size (bp)	Animal species	Final concentration (nM)	Primer sequence
550	*Gallus gallus* (Chicken)	200	Ch F: 5′-AGGTGTCATGAAGGGCAATA-3′
			Ch R: 5′-CTCTCCTTACCCGTCCTAGC-3′
497	*Camelus bactrianus* (Camel)	200	Cm F: 5′-TCCCCTGCCATACTGTGAGCCCTTG-3′
			Cm R: 5′-TGGAGGACATCCGTGCAGTCACTCT-3′
460	*Sus scrofa* (Pig)	200	Pi F: 5′-CTACTATCCCTGCCAGTT-3′
			Pi R: 5′-GAATAGGAAGATGAAGCC-3′
267	*Ovis aris* (Sheep)	200	Sp F: 5′-CGATACACGGGCTTACTTCACG-3′
			Sp R: 5′-AAATACAGCTCCTATTGATAAT-3′
157	*Equus asinus* (Donkey)	200	Dk F: 5′-TTCCAGTCCTAG CAGCAGGT-3′
			Dk R: 5′-AAAGCCTGGCAGAATGAGAA-3′
117	*Capra hircus* (Goat)	200	Go F: 5′-ATA TCAATCGGGTTTCTAGGATTTATT-3′
			Go R: 5′-AGTTGGGATAGCGATAATTATGGTAGT-3′
102	*Bos Taurus* (Cow)	200	Cw F: 5′-GCTATTCCAACCGGGGTAAAAGTC-3′
			Cw R: 5′-GAAAATAAAGCCTAGGGCTCAC-3′
70	Internal control (IC)	40	Ic F: 5′-CGGGGAATYAGGGTTCGATTC-3′
			Ic R: 5′-GCCTGCTGCCTTCCTTKGATG-3′

cut into small pieces and then collected in a micro tube. Minced fragments were suspended in 200 µL of Phosphate Buffer Solution (PBS). 50 µl of Proteinase K (20 mg/ml) was used to lyse the proteins. Samples were then further lysed using lysis buffer. For DNA precipitation, pure isopropanol was added. Pellet was washed three times with ethanol 70% to eliminate chemical contaminations. Finally, the pellet was dissolved in 100 µL Tris–EDTA buffer. Purity and quality of samples were evaluated with NanoDrop™ ND-2000 spectrophotometry. Furthermore, the quality of extracted DNAs was investigated by electrophoresis of samples in a 1% agarose gel. 100 ng of extracted DNA was optimized in dilution buffer and used for single and Multiplex PCR amplification.

PCR amplification

For PCR amplification, in the first step, each species primer was optimized in a single PCR reaction which carried out in 50µL volumes comprising of 4 mM MgCl2, 0.2 mM dNTP, 200 nM of particular species primer, and 1U of Taqplus DNA polymerase (Kawsar Biotech Co, Iran). In the next step, primers were mixed together as 200 nM concentrations of every seven species and multiplex PCR were applied same as single PCR in thermo cycler (Bio Rad, USA). The PCR cycles were comprised of initial denaturation for 5 min at 95 °C, followed by 30 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and a final extension for 5 min at 72 °C. PCR products were run on a 2% agarose gel stained with Gel Red (Biotium, USA).

Validity test

Investigation of the validity was examined for its specificity, sensitivity, and reproducibility with the unknown tissue sample and food products. In this regard, primers were checked with NCBI basic local alignment search tool (BLAST) and also, each set of primers for a particular species was cross tested against all extracted DNA from other species by PCR. Also, for the reliability of the results, primers were checked at the IBRC cell bank by known animal species sample extracted from five hundred cell lines. Furthermore, DNA templates from seven target species were prepared in six serial dilutions which including 100, 50, 25, 12.5, and 6.25, 3.1 ng/µl in the elution buffer. These templates we applied for sensitivity examination to determine the minimum detectable concentration of sample DNA.

Results and discussion

Single and multiplex PCR

Identification of animal species is one of the most important subjects in quality assurance and certification of meat products. It is of great importance from health, ethical, and economical aspects. Until now, various techniques have been applied to meat and meat product authentication (Ballin 2010). Due to high copy number of mitochondrial genome in cells, it has been evidenced that molecular based

approaches are more preferred to detect mitochondrial genome. In the current study, multiplex PCR approach was used to determine seven animal species in meat products in a single reaction. It has been reported previously that Nicotinamide Adenine Dinucleotide Dehydrogenase (NADH) or mitochondrial cytochrome b gene could be targeted to design the specific primers to identify animal species in food products (Bai et al. 2009; Nejad et al. 2014). Furthermore, cytochrome c oxidase seems to be a suitable candidate due to high interspecies variation and low intraspecies variation which was chosen for the current study as well as used in genome Barcoding method in a very broad range of eukaryotic species (Hebert et al. 2003; Ono et al. 2007). Practical application of *COX1* has been reported previously to identify the species origin in cells derived from endangered animals at IBRC (Gorji et al. 2016a, b; Steube et al. 2008).

The *COX1* PCR primers and product size for chicken, camel, pig, sheep, donkey, goat, and cow meats, was introduced in Table 1. In this study, there was no primer cross-reaction with *COX1* genes of other species in known samples, which indicated the specificity of primers for each species. The mixture of primers was evaluated for various samples and indicated that unknown species could be successfully recognized (Fig. 1a, b). In addition, a mixture of unknown DNAs was analyzed as one sample unit in a single multiplex reaction to confirm the ability of this method for detection of mixture of different samples. Internal control primers were also designed based on ribosomal 18 s rDNA which amplified a PCR product of 70 bp. Internal control prevents false negative results in a PCR reaction. Repeatability of this method was confirmed by species identification for known and unknown samples derived from mixture of several species available at IBRC (Fig. 2).

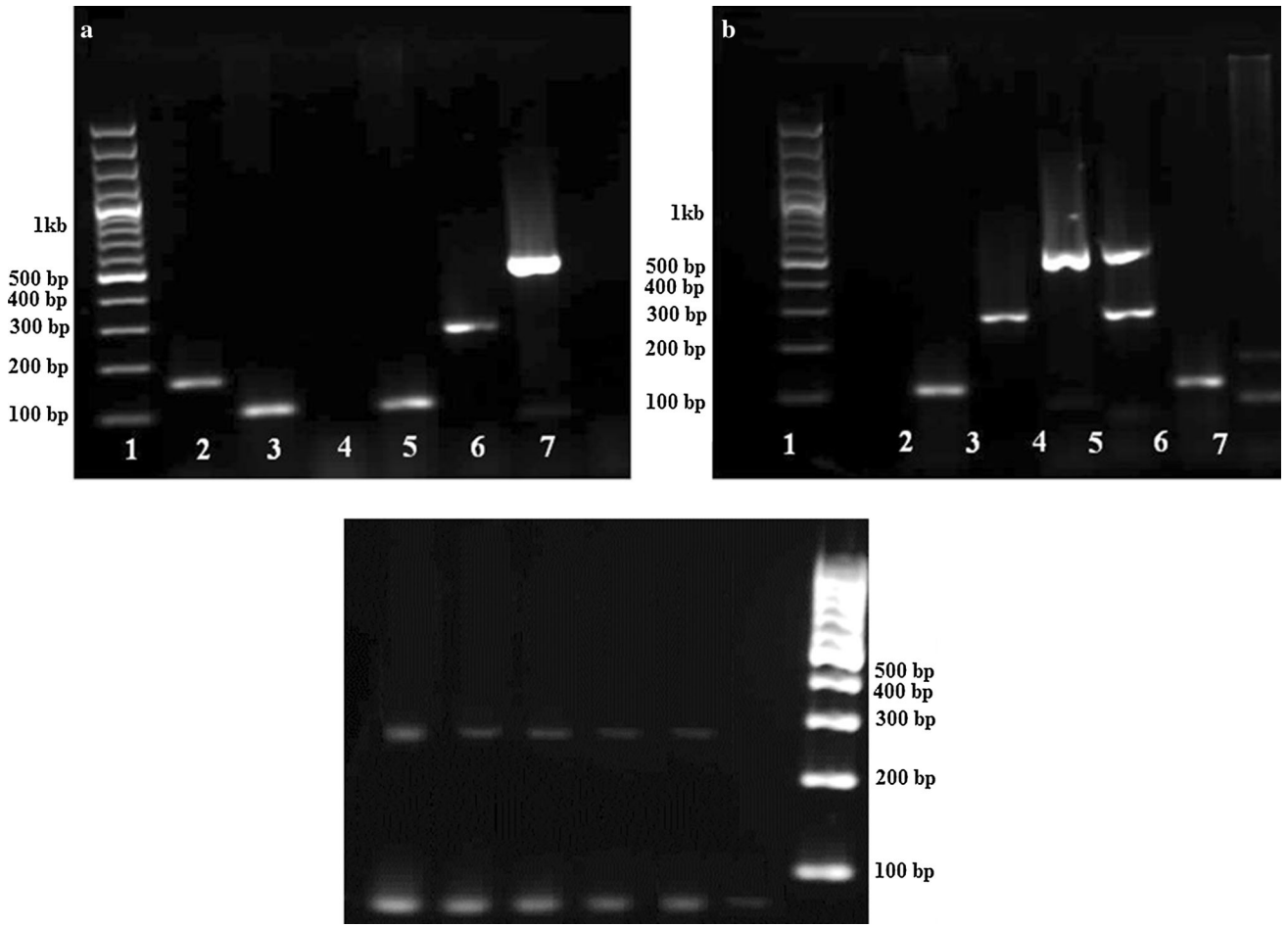

Fig. 1 Agarose gel electrophoresis of COX1 PCR products of animal species. **a** Multiplex PCR products for known species; (1) 100 bp DNA ladder; (2) Donkey; (3) goat; (4) no chicken detected; (5) goat; (6) sheep and (7) cow and chicken. **b** The species identification of unknown samples with two or more animal species origins supplied from IBRC; (1) 100 bp DNA ladder; (2) goat; (3) sheep; (4) chicken; (5) sample mixture of chicken and sheep; (6) goat; (7) Donkey and cow. **c** Determination of minimum concentration of DNA sample. Extracted DNA from sheep meat were prepared in six serial dilutions which including (1) 100, (2) 50, (3) 25, (4) 12.5, and (5) 6.25, (6) 3.1 ng/µl respectively, (7)100 bp DNA ladder

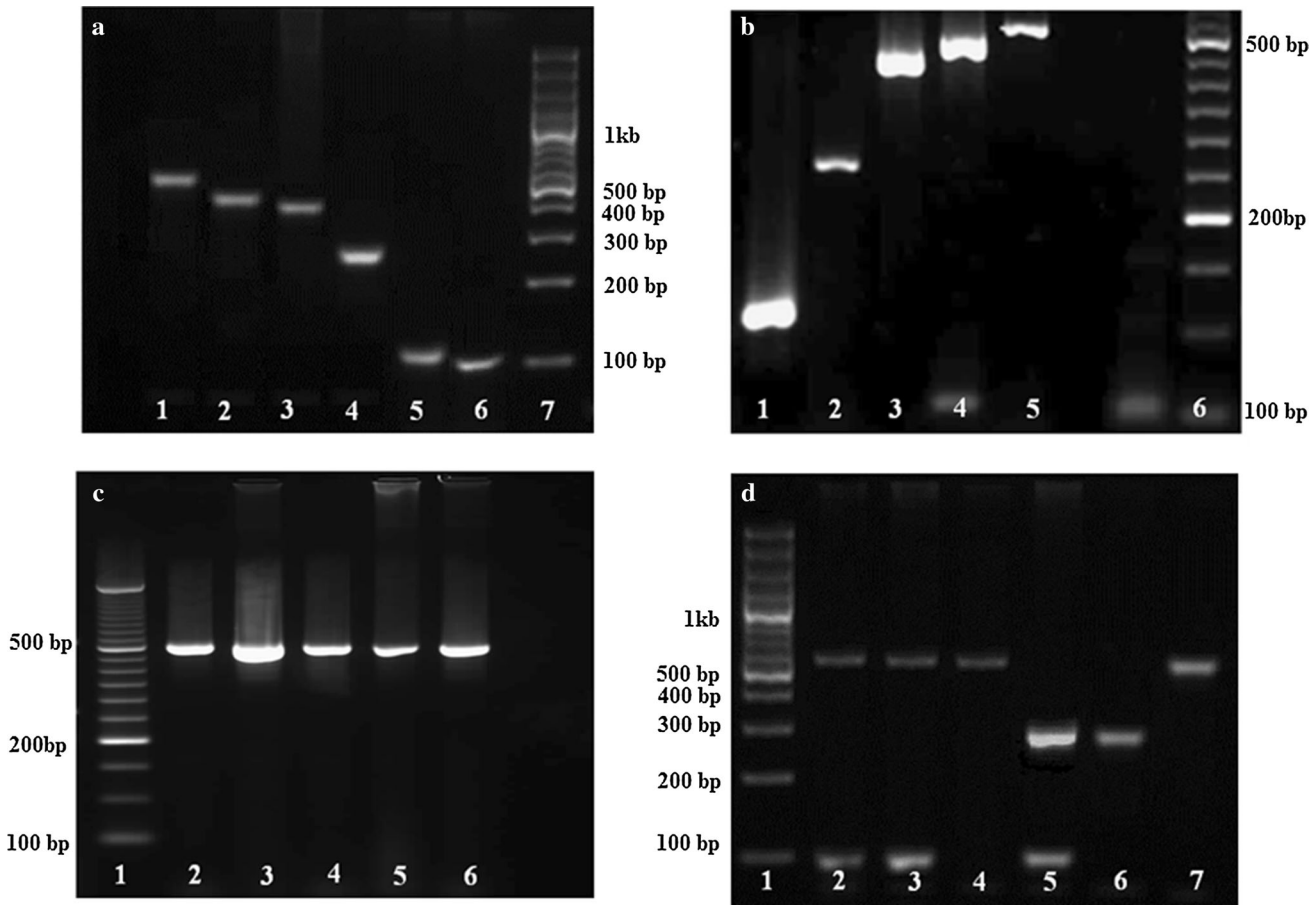

Fig. 2 Agarose gel electrophoresis of COX1 PCR products of animal species. **a** Multiplex PCR products for known species; (1) chicken; (2) camel; (3) pig; (4) sheep; (5) goat; (6) cow and (7) 100 bp DNA ladder. **b** Multiplex PCR products for unknown species; (1) cow; (2) goat; (3) sheep; (4) pig; (5) camel; and (6) 50 bp DNA ladder. **c** Identification of chicken specie in random samples (2-6), (1) 50 bp DNA ladder. **d** Identification of unknown sample in DNA samples of sausages, hams and burgers (2-5), (1) 100 bp DNA ladder; (2,3) sample mixture of chicken and cow in burger; (4) chicken in ham; (4) sheep and cow in sausage; (5) sheep in minced meat; (6) chicken in nugget

In the current study, we illustrated that *COX1* could be effectively applied for quality assurance of meat products. Pig, cow, sheep, dog and goat were correctly identified compared to previously report (Cooper et al. 2007). We were able to develop the method of further identification of chicken, camel and donkey in a single multiplex PCR reaction. The method can be further improved by designing more specific primers for other animal species. Compared to RFLP and Nested PCR, this technique is faster, more reliable and economically preferred because of one reaction amplification has become feasible simultaneously (Cooper et al. 2007; Kumar et al. 2014). The present molecular method is advantageous since there is no need to use restriction enzymes as used in methods such as RFLP to distinguish specific electrophoresis fragment on a gel. Moreover, application of PCR for identification of a variety species meat mixtures was also confirmed in the present study, as represented in past reports (Hopwood et al. 1999; Partis et al. 2000).

Although, there are some reports for meat verification using multiplex PCR, but most of them used Cytochrome b, ribosomal or *COX1* genes in a different combination pattern for animal species (Ghovvati et al. 2009; Kitpipit et al. 2014). To prevent cross-reactivity of the primers for non-specific region of DNA, quality of extracted DNA is important. Furthermore, application of primers with similar melting temperature is also recommended to prevent false negative results. This method is simple and fast and would take only up to four hours for the results to be analyzed and reported. The whole process has been shown schematically in Supplementary Figure 3.

Validity assay

For specification of validity, it was not observed any cross reactivity or false detection for specific primers of each seven species. Also, PCR assay of primers show no cross-species amplification and false detection (Data were not

shown). The species identification results received from five hundred cell lines authentication in IBRC, confirmed with the species origin of the cells even on repetition in blind samples. These finding show the reliability of the assay as well. Minimum detectable concentration of DNA sample for species identification is 6.25 ng/μL in this procedure (Kitpipit et al. 2014) (Fig. 1c). All results reveal that the procedure has validated in the points of specificity, sensitivity and reproducibility.

Conclusion

As represented in previous sections, this procedure can perform in less than four hours from DNA extraction to final analysis, without necessity to advanced equipment and tools such as Real time PCR. Therefore, this test can be considered as a rapid and cost effective technique. Based on reports of novel techniques including barcoding method, *COX1* gene was selected because of its advantages for identification of species, including conservation during development and indication of variations in different species. The outcome of this study revealed that final results were obtained without any false positive which is due to specificity of primers. Primer sensitivity indicated that *COX1* gene can be applied in multiplex PCR approach to identify animal origin species in quality control testing of meat and meat product with the ability to recognize small volume of unknown or targeted sample. With this description, we propose that this procedure can be applied as a complementary standard procedure for a distinct identification of meat products.

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