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Comparison of Two Multiplex PCR Systems for Meat Species Authentication

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HIGHLIGHTS

- Two previously published multiplex PCR methods for meat species authentication were compared.
- The first multiplex PCR was accompanied with cross reactivity, whereas the second one was specific.
- The second multiplex PCR method could be recommended for species authentication.

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Acronyms and abbreviations
PCR=Polymerase Chain Reaction

ABSTRACT

Background: Meat species adulteration has become a problem of concern. This study aimed to compare two previously published multiplex Polymerase Chain Reaction (PCR) methods for meat species authentication.

Methods: The primers used in the first multiplex PCR involved species-specific reverse primer for sheep, goat, cattle, pig, and donkey with universal forward primer. In the second multiplex PCR, the primers included species-specific forward and reverse primer for pork, lamb, ostrich, horse, and cow. The extracted DNA was then amplified with species-specific primers and with mix primers separately in the respective multiplex PCR.

Results: The first multiplex PCR was accompanied with cross reactivity, whereas the second multiplex PCR was specific as expected for pork, lamb, ostrich, horse, and cow. The first set of multiplex PCR showed not always amplification of all species-specific DNAs with a mixture of DNA from mentioned animals. Regarding the second set of primers, the extracted DNA of different meat species was amplified with corresponding species primers as simplex PCR resulting in specific amplicons for species DNA prepared from sheep, ostrich, horse, pig, and cattle with the specific PCR products of 119, 155, 253, 100, and 311 bp, respectively.

Conclusion: Based on the present investigation, we recommend the multiplex PCR with the second set of primers included species-specific forward and reverse primers for species authentication of five meat types, including pork, lamb, ostrich, horse, as well as cow.

Introduction

Adulteration of high-cost meat with a cheaper one is a serious concern for researchers and has prompted to find an appropriate strategy for perfect meat authenticity (Jain et al., 2007). Adulteration in the meat products like sau-

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sage and hamburger has been a widespread problem in markets of different countries. Identification of the species-specific origin in meat samples is very important and necessary for consumers and health sectors for several reasons as economic, religious, and possible health problems (Rodríguez et al., 2004). In this respect, there are some previous published reports indicating fraud in processed meat products distributed in Iran (Doosti et al., 2014; Eslami et al., 2014; Mehdizadeh et al., 2014).

In the most countries in the world, the highest possibility of fraud in meat products is using cheaper meats such as old donkey and chicken instead of beef and sheep. Beef and lamb are generally more expensive than the poultry meat and even goat meat. So, meat species adulteration may be done by some fraudulent producers. Since many people use processed meat products instead of fresh red meat, it is important to have quality control of the ready-to-eat meat products as well (Mahajan et al., 2011).

Authentication of food products involves many procedures capable of confirming the origin of food products that the products agree with the label statements and that they establish the provisions of health applicable laws and regulations (Reid et al., 2006). There is an important association between the authenticity of meat products and its quality because of increased consumer awareness regarding food ingredients and quality. Nowadays some consumers are hesitating of consuming meat products due to repeated food fraud incidence. The most important types of food cheating are the violation of the product components of the brand as known as fraud mislabeling. Given the increasing consumption of processed meat products, therefore, there is considerable need for developing appropriate methods for detection of meat species adulteration. There are some methods based on the protein and genome analysis that were previously reported (Cai et al., 2017; Partis et al., 2000; Ruiz et al., 2015). Beside other methods, the multiplex Polymerase Chain Reaction (PCR) technique was reported as a suitable method for the meat species authentication. Since the primers used for designing of the multiplex PCR are critical for successful detection of specific results, it is important to evaluate the respective assays in the corresponding laboratories.

This study aims to evaluate the effectiveness of two already elsewhere published multiplex PCR methods for meat species authentication. In the used multiplex PCR methods, the amplification of various mitochondrial genes was basic for meat species identification to differentiate meat species samples.

Materials and methods

Samples

The raw meat samples were selected from different species, including sheep, goat, cattle, pig, donkey, horse, and ostrich. The samples stored at -20 °C until used for DNA extraction.

DNA extraction

DNA was extracted from different raw meat species using DNA extraction kit (MBST, Tehran, Iran) and extracted DNA was stored at -20 ºC until used (Shayan et al., 2018). Briefly, 50 mg meat was lysed by 180 µl lysis buffer, mixed thoroughly, and incubated for 10 min at 55 ºC. Twenty µl proteinase K (20 mg/ml; fermentas) was added to the solution and incubated for 20 min at 55 ºC to degrade the proteins. A volume of 360 µl binding buffer was added before incubating for 10 min at 70 ˚C. A volume of 270 µl ethanol (100%) was added to the solution and after vortexing, the complete volume was transferred into the MBST column (DNA extraction kit column). The column was first centrifuged and then washed twice with 500 µl washing buffer. Finally, DNA was eluted from the carrier with elution buffer. Genomic DNA purity and quantity were assessed with spectrophotometer. The quality of extracted DNA was also analyzed by electrophoresis pattern on 0.8% agarose gel visualized using ethidium bromide under ultraviolet light.

PCR analysis with the first set of oligonucleotide primers

According to Edris et al. (2012), species-specific primers were selected for amplification of comparable regions of the mitochondrial genome. The primer sequences had target of the *cytochrome b* (*cytb*) gene sequences of various species. The primer pairs amplify partial-length of *cytb* gene. Forward primer designed by Matsunaga et al. (1999) was used as universal forward primer known as SIM and five different reverse primers (R) for amplifying species-specific mtDNA segments from goat, cattle, sheep, pig, and donkey (Table 1).

Original conventional PCRs for different species were performed in reaction volumes of 50 µl, including 100 ng genomic DNA of each species (gathered from five species), 25 pmol of each primer, 1x Taq DNA polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 U Taq DNA polymerase. The PCR reaction was carried out by initial denaturation at 94 °C for 4 min, followed by 35 cycles each at 94 °C for 60 s, annealing temperature at 58 °C to 65 °C for 60 s, polymerization temperature at 72 °C for 60 s. The final extension was done at 72 °C for 10

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min. The amplifications were performed using a T100™ Bio-Rad thermal cycler (USA).

The amplified mtDNA fragments were separated by 2% agarose gel electrophoresis, stained with ethidium bromide, visualized using UV transilluminator (Kiagen, Iran). The multiplex PCR of animal species was conducted using five species-specific primer mixtures. For this purpose, all primers were mixed in the ratio of 5:1:1:1:1:1 for SIM-F, goat-R, cattle-R, sheep-R, pig-R, and donkey-R, respectively (Edris et al., 2012). Two µl of this mixture were incorporated in the PCR reaction to give 10 pmol of each primer except for SIM-F that was represented by 50 pmol. This multiplex PCR comprised 100 ng DNA/reaction for each species. The expected amplicon sizes were 157, 274, 331, 398, and 439 bp for goat, cattle, sheep, pig, and donkey, respectively (Edris et al., 2012).

Based on another protocol according to Matsunaga et al. (1999), the primers were mixed in the ratio of 1:3:0.6:0.2:3:2 for the forward primer (SIM) and reverse primers for sheep, goat, cattle, pig, and donkey, respectively. The primers used together for the multiplex PCRs as 20 pmol per reaction. The protocol that used for amplification was as follow: initiation denaturation 94 °C for 10 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and final extension at 72 °C for 30 s.

PCR analysis with the second set of oligonucleotide primers

According to Kitpipit et al. (2014), the second set of primers used in the current study were derived from different gene sequences listed in Table 2. The expected PCR products were 100, 119, 155, 253, and 311 bp for pork, lamb, ostrich meat, horsemeat, and beef, respectively. Regarding this type of multiplex PCR, it was performed in reaction volumes of 50 µl containing 100 ng genomic DNA of each species (gathered from five species), 25 pmol of each primer, 1x Taq DNA polymerase buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, and 1.25 U Taq DNA polymerase. The PCR reaction was carried out by initial denaturation at 94 °C for 5 min, followed by 35 cycles each at 94 °C for 30 s, annealing temperature at 60 °C for 60 s, polymerization temperature at 72 °C for 30 s, and the final extension at 72 °C for 10 min. The amplifications were performed using aT100™ Bio-Rad thermal cycler (Bio-Rad, USA). After that, the amplified fragments were separated on 2% agarose gel electrophoresis, stained with ethidium bromide, and then visualized by UV transilluminator (Kiagen Company, Iran).

In the elementary phase of this study, simplex PCRs were performed on DNA extracted from different meat species to verify the specificity of the primers reported by Edris et al. (2012) and Matsunaga et al. (1999). Each set of primers was checked to detect its specificity. For this aim, the primer pair of each species was analyzed with the DNA from the corresponding species and also with the extracted DNA from other species in separate simplex PCRs.

Results

DNA from different samples was extracted and analyzed on the 0.8% agarose gel (Figure 1A). The ratio of OD260/OD280 was determined in range of 1.7 and 1.9 using spectrophotometer. The quantification of the extracted DNA measured by spectrophotometer showed the concentration of 90, 121, 224, 575, 317, 505, and 293 µg/ml, for sheep, cattle, goat, pig, horse, donkey, and ostrich, respectively.

Regarding the first set of primers, each primer was examined with its specific DNA type resulting in expected DNA fragment for each species of donkey, pig, cattle, goat, and sheep with specific PCR product of 439, 398, 274, 157, and 331 bp, respectively (Figure 1B, lanes 1-5).

To control the specificity of primers, different above mentioned extracted DNAs were amplified with each primer pair. Unfortunately, the DNA from other animal species had cross reactivity and was amplified with the used primer pairs. The change in annealing temperature or primer concentration had no effect on the specificity of the PCR reactions.

Regarding the second set of primers, the extracted DNA of different meat species was amplified with corresponding species-specific primers as simplex PCR resulting in specific amplicons for species of cattle, horse, pig, ostrich, and sheep with the specific PCR products of 311, 253, 100, 155, and 119 bp, respectively (Figure 2). To control the specificity of the last primers, mentioned extracted DNAs were amplified with each primer pair. Figures 3 A, B, C, D, and E shows the specific amplification with species-specific primers for sheep, ostrich, pig, cattle, and horse without any mismatch. Additionally, to control the specificity of primers, different above mentioned DNA was amplified with mix primer pairs in multiplex PCR. The results showed that the extracted DNA of the five different meat species was amplified without any mismatch (Figure 4). The extracted DNA from all five species was mixed and analyzed in multiplex PCR. The results showed that often only three species-specific DNA bands (ostrich 155 bp, horse 253 bp, and cattle 311 bp) could be detected (Figure 4).

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Table 1: Nucleotide sequence of primers used for multiplex PCR to amplify partial-length of *cytb* gene from mitochondrial DNA of different animal species (Edris et al., 2012)

Meat species	Primer name	Sequences (5'–3')
SIM	F-universal	GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA
Sheep	R-sheep	CTA TGA ATG CTG TGG CTA TTG TCG CA
Goat	R-goat	CTC GAC AAA TGT GAG TTA CAG AGG GA
Cattle	R-cattle	CTA GAA AAG TGT AAG ACC CGT AAT ATA AG
Pig	R-pig	GCT GAT AGT AGA TTT GTG ATG ACC GTA
Donkey	R-donkey	CTC AGA TTC ACT CGA CGA GGG TAG TA

Table 2: Nucleotide sequence of primer pairs specific for different animal species (pork, sheep, ostrich, horse, and cattle) derived from different genes used as second primer set for second multiplex PCR (Kitpipit et al., 2014)

Meat species	Primer name	Sequences (5` - 3`)	Target gene
Pork	F-pork R-pork	GAAAAATCATCG TTGTACTTCAACTACA GGT CAA TGA ATG CGT TGT TGA T	*Cytb*
Sheep	F-sheep R-sheep	GAA AAA CCA TCG TTG TCA TTC AAC T AAA TAT TTG ATG GAG CTG GGA GA	*t-Glu* – *cytb*
Ostrich	F-ostrich R-ostrich	CCC TTT AAA GAC ATC TGG TAT TGT GAG TAA ATT GTA GGC TCT CTG GGG TTC	12S rRNA
Horse	F-horse R-horse	CGT TTG ATC TGT CCT TAT TAC GGC A CCG AAT GGT TCY TTT TTY CCY GAG TAG TA	*COI*
Cattle	F-cattle R-cattle	CAT CAA CTT CAT TAC AAC AAT TAT CAA CAT AAA G CCG AAT GGT TCY TTT TTY CCY GAG TAG TA	*COI*

Table 3: Comparative nucleotide sequence of used primers as primer set for first multiplex PCR

Name of primer	Nucleotide sequence of primer and respective species sequences	TM
Universal forward primer	Primer 5`GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA 3` Sheep GATCTCCCAGCTCCATCAAATATTTCATCATGATGAAA Goat GACCTCCCAACCCCATCAAACATCTCATCATGATGAAA Cattle GACCTTCCAGCCCCATCAAACATTTCATCATGATGAAA Pork GACCTCCCAGCCCCCTCAAACATCTCATCATGATGAAA Donkey GACCTACCAGCCCCCTCAAACATTTCATCATGATGAAA	70.54
Sheep nucleotide sequence for designing primer	Primer 5`TGCGACAATAGCCACAGCATTCATAG 3` Sheep TGCGACAATAGCCACAGCATTCATAG Goat CGCGACAATGGCCACAGCATTCATAG Cattle CACAGTAATAGCCACAGCATTTATAG Pork TACCGTTATAGCAACAGCCTTCATAG Donkey CACAGTTATAGCTACAGCATTCATGG	63.22
Goat nucleotide sequence for designing primer	Primer 5`TCCCTCTGTAACTCACATTTGTCAAG 3` Sheep CTCCTCTGTAACCCACATTTGCCGAG Goat TTCCTCTGTAACTCACATTTGTCGAG Cattle CTCCTCTGTTACCCATATCTGCCGAG Pork CTCATCAGTTACACACATCTGTCGAG Donkey CTCATCCGTCACTCACATCTGCCGAG	61.65
Cattle nucleotide sequence for designing primer	Primer 5`CTTATATTACGGGTCTTACACTTTTCTAG 3` Sheep CCTATACTATGGATCATATACCTTCCTAG Goat TCTATATTATGGATCATATACCTTTCTAG Cattle CTTATATTACGGGTCTTACACTTTTCTAG Pork CCTATACTACGGATCCTATATATTCCTAG Donkey CCTCTACTACGGCTCTTACACATTCCTAG	61.02
Pork nucleotide sequence for designing primer	Primer 5`TACGGTCATCACAAATCTACTATCAGC 3` Sheep AACAGTTATTACCAACCTCCTTTCAGC Goat AACAGTCATCACTAATCTTCTTTCAGC Cattle AACAGTCATCACCAACCTCTTATCAGC Pork TACGGTCATCACAAATCTACTATCAGC Donkey AACAGTCATCACAAACCTCCTATCAGC	61.93
Donkey nucleotide sequence for designing primer	Primer 5`TACTACCCTCGTCGAGTGAATCTGAG 3` Sheep CACAAACCTAGTCGAATGAATCTGGG Goat CACAAACCTAGTCGAATGAATCTGAG Cattle CACAAATTTAGTCGAATGAATCTGAG Pork AACAGACCTCGTAGAATGAATCTGAG Donkey TACTACCCTCGTCGAGTGAATCTGAG	64.80

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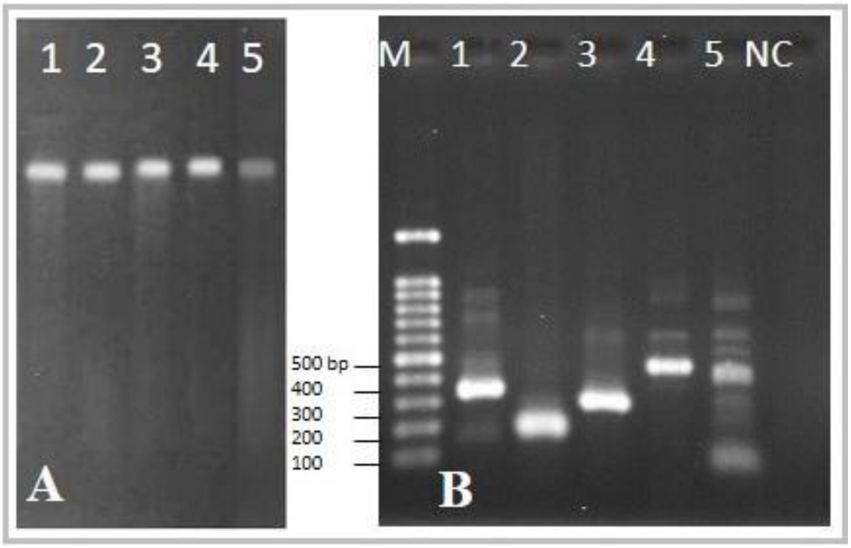

Figure 1: A: DNA was extracted from different meats prepared from sheep (lane 1), cattle (lane 2), pig (lane 3), donkey (lane 4), and goat (lane 5). DNA was analyzed on 0.8% agarose gel. B: The extracted DNA of different meat species was amplified with corresponding species primers of first multiplex PCR system. Lane 1: sheep with fragment size of 331 bp; lane 2: goat with fragment size of 157 bp; lane 3: cattle with fragment size of 274 bp; lane 4: donkey with fragment size of 439 bp; lane 5: pig with fragment size of 398 bp; NC: negative control. M: is 100 bp DNA ladder

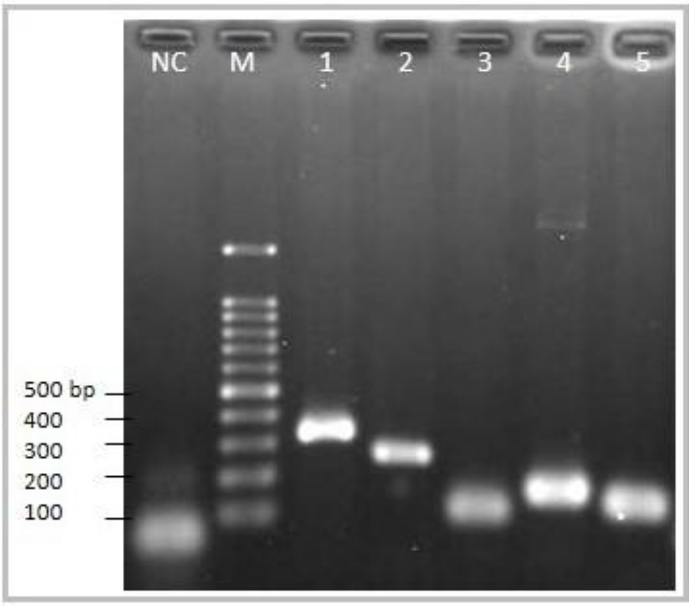

Figure 2: Agarose gel electrophoresis of the amplification of extracted DNA from different meats, including cattle, horse, pig, ostrich, and sheep with species primers as simplex PCR (with primers from the second multiplex PCR system) and analyzed on 2% agarose gel. Lane 1: cattle (311 bp); lane 2: horse (253 bp); lane 3: pig (100 bp); lane 4: ostrich (155 bp); lane 5: sheep (119 bp); M: 100 bp DNA ladder; NC: negative control

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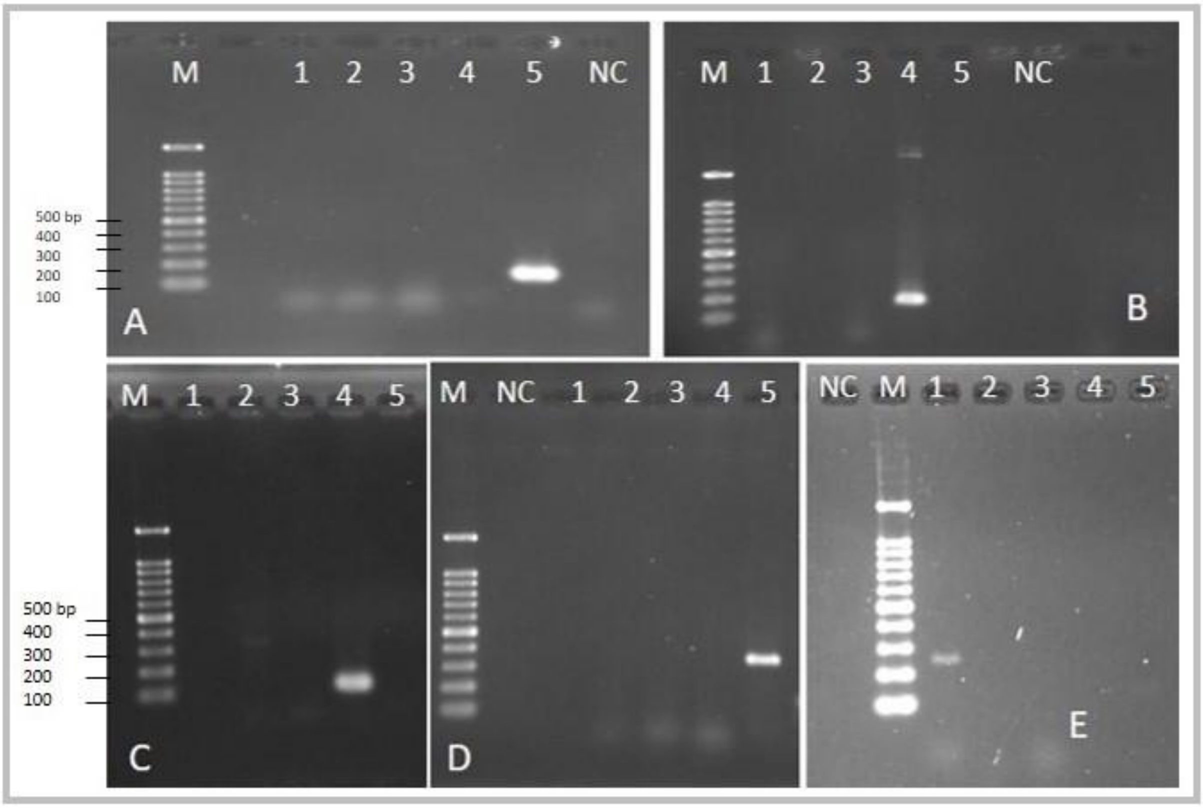

Figure 3: Agarose gel electrophoresis for analyzing the amplification of DNA from sheep, cattle, ostrich, pig, and horse using species-specific primers used in the second multiplex PCR. A: amplification with sheep specific primers with the PCR product of 119 bp (lane 1: ostrich; lane 2: horse; lane 3: cattle; lane 4: pig and lane 5: sheep; M: 100 bp DNA ladder; NC: negative control). B: amplification with ostrich specific primers with the PCR product of 155 bp (lane 1: pig; lane 2: horse: lane 3: cattle; lane 4: ostrich; lane 5: sheep; M: 100 bp DNA ladder; NC: negative control). C: The amplification with pig specific primers with PCR product of 100 bp (lane 1: cattle; lane 2: ostrich; lane 3: horse; lane 4: pig; lane 5: sheep; M: 100 bp DNA ladder; NC: negative control). D: The amplification with cattle specific primers with the PCR products of 311 bp (lane 1: sheep; lane 2: ostrich; lane 3: horse; lane 4: pig; lane 5: cattle; M: 100 bp DNA ladder; NC: negative control). E: The amplification with horse specific primers with PCR product of 253 bp (lane1: horse; lane 2: ostrich; lane 3: sheep; lane 4: pig; lane 5: cattle; M: 100 bp DNA ladder; NC: negative control)

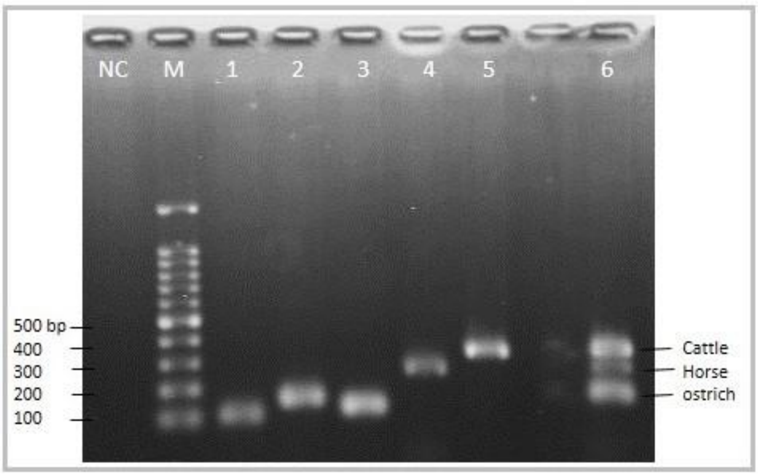

Figure 4: Agarose gel electrophoresis for analyzing the amplification with mix primers of the second multiplex PCR system. Lane 1: pig (100 bp); lane 2: ostrich (155 bp); lane 3: sheep (119 bp); lane 4: horse (253 bp); lane 5: cattle (311 bp); and lane 6: amplicon of all five species simultaneously; M: 100 bp DNA ladder; NC: negative control

Discussion

Meat species authentication is a major concern to prevent the adulteration to maintain the health and to achieve the safety and good quality of food (Di Pinto et al., 2015; Mafra et al., 2008; Ortea et al., 2012). Protein-based techniques, including immunological methods like western blot and chromatography (Armstrong et al., 1992; Hsieh et al., 1998) have been used for detection of food fraud. Unfortunately, these methods are often time consuming and expensive (Saez et al., 2004). Therefore, some DNA based methods like PCR were developed with high sensitivity and specificity (Gil, 2007; Mehdizadeh et al., 2014). In addition, the later mentioned methods are more applicable especially for heat treated meat products. In this regards, the proteins can be denatured by heating and may not be suitable at least for immunological methods, but the DNA remain unchanged (Kesmen et al., 2007). Currently, the multiplex PCR was used as a method capable of detecting different species simultaneously in a single reaction using specific primers under accurate condition as noted previously by Girish et al. (2004) and Lin and Hwang (2008).

In the present study, two multiplex PCR techniques were used and compared with each other. Regarding the first multiplex system (Matsunaga et al., 1999), cross reactivity was observed that could not confirm the results achieved by Matsunaga et al. (1999) and Edris et al. (2012). In this method forward primer was universal primer and used for all species, whereas the reverse primers were species-specific. Therefore, the difference in nucleotide sequence in reverse primers must be decisive. It should be noted that the reverse primers designed in the first set of primers could be observed by nucleotide sequences for the donkey, the difference in one nucleotide at the 5` end and between 3 to 6 nucleotides through the whole nucleotide sequences of the used species-specific reverse primers (Table 3). Furthermore, the difference in the nucleotide sequence of the corresponding reverse primer specific for sheep compared to the same sequence in the goat was only in two nucleotides from which only one nucleotide was at the 5` end. Although these differences theoretically have to be enough for avoiding the mismatching of primers, but we could not have achieved the results which were described by Edris et al. (2012).

Regarding the second set of primers, the extracted DNA from five different meat species, including sheep, ostrich, horse, pig, and cattle was amplified with corresponding species-specific primers as simplex PCR and the results showed specific fragment for each species without any mismatches. The primer pairs showed also no cross reactivity with DNA prepared from other species. The amplification of the extracted DNA from the meat of each animal in multiplex PCR also showed that reactions were species-specific where no cross reactivity was observed.

Interestingly, the multiplex PCR with the mixture of the different five species DNA showed sometimes not all expected amplicons. The amplification failure was in agreement with the results of another study that showed the occurrence of amplification failure in the case of direct amplification from a large sample size (Kitpipit et al., 2014).

In general, in multiplex PCR assay, the specificity and melting temperatures of primers are more important than in single PCR because of the possibility of cross reaction. These conditions are very important and critical in the development of multiplex PCR. The specificity of PCR technique is dependent on the ability of the primers to be selectively annealed with their particular targets. The PCR conditions, such as reaction volume, cycling, as well as annealing temperature play an important role in producing accurate and specific results (Ali et al., 2014; Nejad et al., 2014). The studies of many investigators showed that the multiplex PCR method is highly reliable, accurate, and sensitive for detecting meat species in products of food industries (Nejad et al., 2014; Zha et al., 2010). Recently, another method based on hybridization for simultaneous detection of 13 animals was described. In this method, all extracted DNA from six species in the mixture could be detected simultaneously without any cross-reactivity (Shayan et al., 2018). The disadvantage of the second multiplex PCR appeared inability for simultaneous amplifying of all five DNA species.

Conclusion

Based on this study, we recommend the multiplex PCR with the second set of primers included species-specific forward and reverse primers for species authentication of five meat types, including pork, lamb, ostrich, horse, and cow.

Author contributions

P.S., A.M., and D.A. were responsible for the experiment design and data analysis and carried out all of the experiments and drafted the manuscript; D.A. and D.S. participated in the evaluation of each experiment; P.S., D.A., A.A.B., and H.G. revised the paper and provided technical support and the final edition of the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declared no conflict of interest in this research.

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