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IDENTIFICATION OF DIFFERENT MEAT SPECIES USED IN SUCUK PRODUCTION BY PCR ASSAY

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Abstract
The PCR method has become a crucial tool for the identification of meats of different animal species in recent years and came into prominence as an alternative method that can replace the existing methods. In this study, a specific PCR method has been developed for identification of horse, donkey and porcine meats in a traditional, dry-fermented Turkish sausage, sucuk. For this purpose, sucuk samples were prepared from binary meat mixtures which were obtained by adding different amounts (0.0, 0.1, 0.5, 1.0 and 5.0%) of horse, donkey and porcine meats into either cattle or sheep meats. Oligonucleotide primers (producing fragments at a base pair (bp) length of 153, 145 and 227 respectively) that are specific to horse, donkey and porcine species were designed on the mitochondrial DNA. The results showed that each meat species could be identified at all the levels studied in the range of 0.1% to 5%, in all sucuk samples. In conclusion, it was suggested that the specific PCR method developed within the scope of this study could be used as a routine control method in food control laboratories for the identification of horse, donkey and porcine meats in the meat products.

Keywords: PCR, meat species identification, sucuk, horse, donkey, porcine

SUCUKTA KULLANILAN FARKLI HAYVAN TÜRLERİNE AİT ETLERİN PCR TEKNİĞİ İLE BELİRLENMESİ

Özet
Et ürünlerinde farklı hayvan türlerine ait etleri tespit etmek amacıyla son yıllarda PCR tekniği oldukça önem kazanmış ve mevcut yöntemlerin yerini alabilecek alternatif bir yöntem olarak öne çıkmıştır. Bu çalışmada geleneksel bir et ürünümüz olan sucukta at, eşek ve domuz türlerine ait etlerin tespitinde kullanılmak üzere spesifik bir PCR metodu geliştirilmiştir. Bu amaçla, sığır ve koyun etlerine at, eşek ve domuz etlerinden her birisi, farklı seviyelerde (%0.0, 0.1, 0.5, 1.0 ve 5.0) ilave edilerek hazırlanan ikili et karışımlarından sucuk örnekleri üretilmiştir. Mitokondrial DNA üzerinde at, eşek ve domuz türlerine spesifik (sırasıyla 153, 145 ve 227 baz çifti uzunluğunda fragment üreten) oligonükleotid primerler dizayn edilmiş ve neticede sucuk örneklerinin hepsinde; her bir tür %0.1'den %5'kadar olan tüm seviyelerde tespit edilebilmiştir. Sonuç olarak, bu çalışma kapsamında geliştirilen spesifik PCR yönteminin, et ürünlerinde bulunabilecek at, eşek ve domuz etlerinin tespitinde ülkemiz gıda kontrol laboratuarlarının ihtiyacını giderecek, rutin bir kontrol metodu olabileceği ortaya konulmuştur.

Anahtar kelimeler: PCR, tür tayini, sucuk, at, eşek, domuz

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INTRODUCTION

Sucuk is a traditional meat product which has the highest consumption volume among the meat products in Turkey. It is a potentially risky meat product, as different meat species that are low quality or not consumed by the general public can be used in its production. Identification and prevention of fraudulent practices in a view to protect the consumers and prevent unfair competition, simple and rapid methods of analysis which are suitable for routine applications in such products are required.

In order to identify the meat and meat products coming from different animal species and avoid the fraudulent practices, various methods based on analysis of species-specific components such as protein and DNA have been developed. Species can be identified analyzing the proteins by different methods which employ immunological, chromatographic and electrophoretic techniques (1-5). However, denaturation of meat proteins during heat treatment or any other technological processes and variation of protein compositions, even in the same species, reduce the chance of success of these methods. Furthermore, these methods may be inadequate to discriminate between species which are in close relation and are not suitable for routine use, as the isolation of species-specific proteins is difficult and time consuming (6-8).

All these problems faced during identification of meats of different animals in the products are overcome to a large extent by making use of the Polymerase Chain Reaction (PCR) technique which ensures rapid amplification of DNA sequences. The PCR technique can briefly be described as the amplification of one or more specific fragments of the DNA by an oligonucleotide primer which binds to the flanking regions of the fragments. In PCR applications, specific gene or gene region of a substance is amplified in order to obtain an adequate amount of genetic material, and then the amplicons are further analyzed using secondary methods for identification of the species (9). The DNA fragment to be amplified is selected considering the purpose of the study, taking into account the differences it shows for identification of the individual, the population, species or the family. Sequences of the DNA fragment to be used for identification of the species shall be the ones showing the most difference between species and least difference between individuals and populations (10, 11).

Today the nucleotide sequence information of many species is available, and this enables development of specific PCR primers. These primers produce PCR products under suitable reaction conditions only if the DNA that they are specific to is present, and the nucleotide information of these products allows prediction of size of the product. Thus, observation of the amplicon with the expected size on the gel during electrophoreses validates the identification (9).

Species-specific primers were designed using DNA sequences of many animal species identified in various researches, and these primers were used in species identification studies. Meyer *et al.* (12) reported that pig meat was identified at a ratio of less than 2% in beef products that were subjected to heat treatment at 121 °C for 10 minutes, using a specific primer which produces a fragment of 108 base pairs from pig growth hormone. Behrens *et al.* (13) indicated that species could be precisely identified in heat treated meat products of different species (poultry, cattle, pig, sheep, donkey and horse) by a PCR assay using specific primers, and the detection limit was approximately 1%.

In Turkey, there are a few studies performed to identify the meats of different species in fresh or processed meat products using the PCR technique. One of these studies was conducted by Aslan *et al.* (14) on beef samples that were heat treated at different temperatures and durations, using a specific PCR method which employed species-specific primers. In another study, the same researchers obtained characteristic RADP profiles that enable identification of various species (cattle, goat, sheep, camel, pig, wild boar, donkey, cat, dog, rabbit and bear) using a primer of 10 bases (ACGACCCACG). However, this method was found to be insufficient for identification of binary mixtures of sheep-pig, horse-cattle and cattle-sheep meats (15). In another study, İlhak (16) identified each species at a level of 0.1 % using specific PCR assay in the meat mixture prepared by adding pig, horse, cat and dog meats into cattle, sheep and goat meats at different levels (%0.1, 0.5, 1, 2.5 and 5).

In Turkey, new types of methods for the identification of meats from different animal species in meat products should be investigated, and the most suit-

able and economical method needs to be adapted in practical applications. This will contribute to the performance of precise quality controls of meat products in a routine manner, production of quality and safe products in the meat industry, as well as protection of public health and the environment. In addition, consumer concerns, due to past experiences about fraudulent practices in traditional meat products such as sucuk, pastrami and fried meat (kavurma), will be eliminated. Furthermore, these studies are important in other respects such as regaining consumer confidence, reduction of losses due to unfair competition and production deficiencies, and increasing the contribution of the sector to the national economy. Increased precision of the methods used will allow increased trust in the safety of the products in international trade and will provide reliable evidence for the probable court cases.

From the above discussion, it is concluded that, in this country, a reliable method is necessary for the precise results that will be used for the accurate identification of fraudulent practices in meat control systems. The purpose of this study was to develop a specific PCR method for the identification of meats from different species in sucuk which is a highly popular and vulnerable meat product for fraudulent practices in Turkey.

MATERIAL AND METHOD

Material

In this study, skeletal muscle tissues of 5 different animal species, namely horse (*Equus caballus*), donkey (*Equus asinus*), pig (*Sus scrofa domesticus*), cattle (*Bos taurus*) and sheep (*Ovis aries*) were used. Binary mixtures of meats were prepared mixing each of horse, donkey and pig meats either with cattle or sheep meat at different rates (0, 0.1, 0.5, 1 and 5%). Sucuk was prepared as follows: 90% each binary meat mixture, 10% lamb tail fat, 2% salt, 1% garlic, 0.7% red pepper, 0.5% black pepper, 0.89% cumin, 0.25% allspice, 0.6% sugar and 0.033% $NaNO_3$ and 0.005% $NaNO_2$ (17). Starter cultures (*Pediococcus pentosaceus* and *Staphylococcus carnosus* BactofermTM; Chr. Hansen, Denmark) were added to the sucuk dough and stuffed into collagen casings before fermentation and ripening for 15 days under ambient conditions around 20C, 90-75% relative humidity and 0.1 m/s air velocity.

Method

Specific primers design

Mitochondrial DNA sequences of 5 different animal species (cattle, sheep, porcine, horse and donkey) used in this study were obtained from the database of National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD). Species-specific primers for the detection of horse, donkey and porcine DNA were designed from different regions of the mitochondrial genome, following the alignment of available sequences from GenBank database with version 1.6 of Clustal W (18), (Table 1). A homolog of the specific DNA sequence was determined for each species while the DNA sequences of other species were identified using the "BLAST" algorithm.

Table 1. The primer pairs used in specific PCR identification of horse, donkey and porcine meats

Species	Primer	Oligonucleotid sequences	Gene	Tm (°C)	Amplicon size (bp)	Accession Number
Horse	Forward	5'-CTATCCGACACACCCAGAAGTAAAG-3'	ATP8	65.5	153	NC-001640
	Reverse	5'-GATGCTGGGAAATATGATGATCAGA-3'	ATP6	66.7		
Donkey	Forward	5'-CATCCTACTAACTATAGCCGTGCTA-3'	ND2	61.3	145	NC-001788
	Reverse	5'-CAGTGTTGGGTTGTACACTAAGATG-3'	ND2	63.3		
Porcine	Forward	5'-CATTCGCCTCACTCACATTAACC-3'	ND5	60.6	227	NC-0008
	Reverse	5'-AAGAGAGAGTTCTACGGTCTGTAG-3'	ND5	61.0		

ATP8:(ATP synthase F0 subunit 8), ATP6: (ATP synthase F0 subunit 6), ND2: (NADH dehydrogenase subunit 2), ND5: (NADH dehydrogenase subunit 5)

Specificity and sensitivity test of specific primers

In specificity tests of primers specific to horse, donkey and pig species, it was investigated whether a PCR reaction takes place between primers specific to target species and DNA (100 ng/µl) of 5 animal species tested. On the other hand, in sensitivity tests of these primers, PCR amplification was examined between specific primers and concentrations of DNAs isolated from meats of target species in 100, 10, 1, 0.1 and 0.01 ng/µl water dilutions.

DNA isolation

Two grams of meat samples of each species and sucuk samples were placed in 20 ml tubes; 10 ml of extraction buffer [0.2 M Tris (pH 8), 0.1 M EDTA (pH 8) ve %1 SDS] was added to the tubes and mixed until the mixture becomes homogenous. 40 µl of K proteinase of 20 mg/ml concentration was added to 750 µl of this homogenous mixture and kept at 55 °C over night. Following the incubation, 300 µl of 5 M NaCl was added to each tube and the tubes were centrifuged at a speed of 9.000 x g for 10 minutes. The upper phase was taken to a clean eppendorf tube, and 750µl of phenol:cholorophorm:isoamylalcohol (25:24:1) solution was added and centrifuged at a speed of 10.000 x g for 5 minutes. Following centrifugation, the separated upper phase was taken in another tube and 600µl of 65% ethanol was added in order to ensure DNA precipitation. After drying, 100µl of TE buffer (10 mM Tris, 1mM EDTA, pH 8.0) was added onto the precipitate and waited until the precipitate fully dissociated, then a concentration of the isolated DNA was identified at 260 nm by measuring the absorbance.

PCR reaction

The PCR reaction of each sample was performed in a 50 µl volume consisting of 5 µl of 10xPCR buffer, 3 mM of MgCl2 buffer, 200 µM of dNTP mixture, 0.3 µM of forward and reverse primers, 2.5 U of Taq DNA polymerase and 2 µ of DNA template (500 ng/µl). The reaction tubes, placed in the PCR equipment (Eppendorf Mastercyler Gradient Authorized Thermal Cycle) were applied an initial denaturation step at 94 °C for 3 minutes and then, during 35 cycles; the following thermal cycle conditions were carried out: melting at 94 °C for 50 s, annealing at 61, 57 and 55 °C for horse, donkey and porcine, respectively, for 50 s extension at 72 °C for 1 min. A final elongation phase was applied at 72 °C for 5 minutes.

Agarose gel electrophoreses

The PCR amplification products were loaded on 2% agarose gel containing 0.5 µg/ml of ethidium bromide and were processed in electrophoreses equipment at 70 V for 2 hours. Following the completion of the process, PCR bands were visualized by UV transillumination and then photographed.

RESULTS

Primer specificity and sensitivity test results

Results of the primer specificity and sensitivity tests are presented in Fig. 1, 2 and 3. DNAs of the target species were used at 5 different levels as 0.01, 0.1, 1, 10 ve 100 ng in specificity and sensitivity tests of primers specifically designed for horse, donkey and porcine species. DNAs of other species were used only at a level of 100 ng. The primers generated species specific fragments of 145, 153 and 227 bp for donkey, horse and porcine tissue, respectively in all dilutions, and band intensity increased with increasing DNA concentration. These yielded PCR products only from the DNA extracted species that they were designed for, and showed no cross-reactivity with the DNA from the other species.

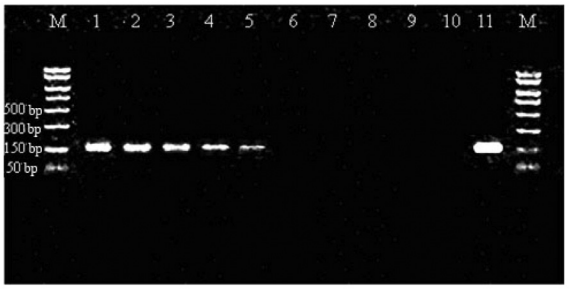

Figure 1. The result of PCR specificity and sensitivity test for the specific horse primers. M: marker, 1: 100 ng horse, 2: 10 ng horse, 3: 1 ng horse, 4: 0.1 ng horse, 5: 0.01 ng horse, 6: donkey, 7: porcine, 8: beef, 9: lamb, 10: negative control and 11: positive control.

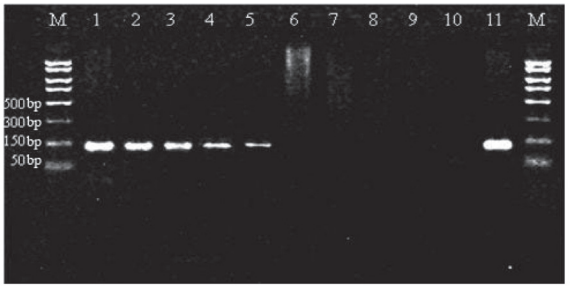

Figure 2. The result of PCR specificity and sensitivity test for the specific donkey primers. M: marker, 1: 100 ng donkey, 2: 10 ng donkey, 3: 1 ng donkey, 4: 0.1 ng donkey, 5: 0.01 ng donkey, 6: horse, 7: porcine, 8: beef, 9: lamb, 10: negative control and 11: positive control.

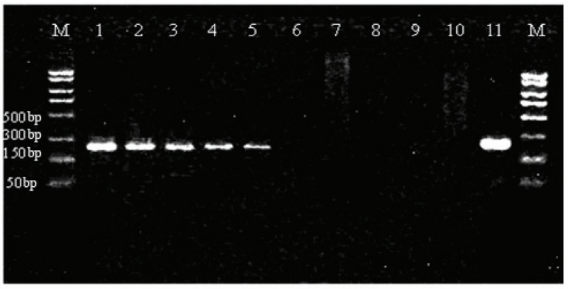

Figure 3. The result of PCR specificity and sensitivity test for the specific porcine primers. M: marker, 1: 100 ng porcine, 2: 10 ng porcine, 3: 1 ng porcine, 4: 0.1 ng porcine, 5: 0.01 ng porcine, 6: donkey, 7: horse, 8: beef, 9: lamb, 10: negative control and 11: positive control.

Identification of species in sucuk samples

Results of the species identification in sucuk samples prepared from binary mixtures was shown in Fig. 4. Horse, donkey and pig meats were identified in all binary samples that were prepared by mixing each of target species in the range of 0.1-5% either with beef or lamb. On the other hand, it was also determined that preparation of meat mixture with beef or lamb, use of additives in production, and the fermentation process did not have any effect on identification of the species (Figure 4).

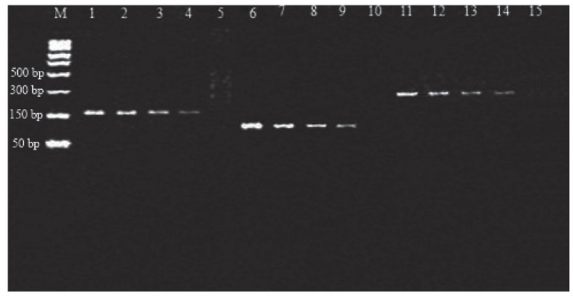

Figure 4. Electrophoretic gel of the sucuk samples prepared from binary mixtures. M- Marker, 1- 5% Horse-95% Beef, 2- 1% Horse - 99% Beef, 3- 0.5% Horse - 99.5% Beef, 4- 0.1% Horse - 99.9 % Beef, 5- 100% Beef, 6- 5% Donkey - 95% Beef, 7- 1% Donkey - 99% Beef, 8- 0.5% Donkey - 99.5% Beef, 9- 0.1% Donkey - 99.9% Beef, 10- 100% Beef, 11- 5% Porcine - 95% Beef, 12- Porcine 1% - %99 Beef, 13- 0.5% Porcine- 99.5% Beef, 14- 0.1% Porcine - 99.9% Beef, 15- 100% Beef

DISCUSSION

The problems faced during identification of animal species that are used in production of meat products were largely overcome by using the PCR technique. Accurate identification of meat species that are not preferred by consumers but are presented for consumption either directly or by incorporating them into different meat products will be an important step forward to prevent fraudulent practices and reduce consumer losses due to these adulterations. In this study, a practical PCR method which enables rapid and precise identification of meats of different animal species such as horse, donkey and porcine in a traditional fermented meat product, sucuk, was developed. The detection limit of the developed PCR method was determined as 0.01 ng DNA (in water), and identified at levels of 0.1% in each sucuk sample. The results of the study largely correspond to the findings of previous studies on this subject. Matsunaga *et al.* (19) showed that the detection limit was 0.25ng DNA in their study which aimed at identification of horse, sheep, cattle, pig and goat species using the multiplex PCR technique. Calvo *et al.* (20) reported that 1.25 pg porcine DNA was identified in beef DNA after 30 amplification cycles, and 250 pg of this DNA was identified following 20 amplification cycles. İlhak (16) showed that each species of horse, sheep, cattle, goat, cat and dog in binary mixtures was identified using species specific primers at 0.5% level at the end of 30 cycles and at 0.1% level at the end of 35 cycles.

In identification of species using the PCR technique, positive results are obtained even at a level of 0.1% and below. The identification study shall be performed quantitatively if the aim is to identify whether the presence of the foreign meat is a result of technically unavoidable contamination or an intentional incorporation. Recently, in order to

perform these quantitative studies, real time PCR technique is used. While detection limits of 0.1% and above are preferred for species identification in order to avoid economic losses, it is clear that more precise methods with detection limits below 0.1% are required when the adverse effects on human health and religious and cultural concerns of the public are considered (21).

Homogenous distribution of the target meat species in the meat mixture is as important as its ratio in the mixture. Therefore, in order to increase the possibility of detection of the meat with low levels in a mixture, accurate homogenization is required (16).

DNA molecules are more stable than proteins during heat treatment and the other processes used in production of meat products such as marinating, fermentation, curing, etc. For this reason, methods relying on genetic material give more accurate results in species identification studies than protein analysis (21). In this study, it was found that additives used in sucuk production and the fermentation process had no adverse effect on the identification of meat species.

Researchers noted that band intensity of PCR products on agarose gel are proportionate to the DNA concentration, and hence a specific PCR technique can be used as a semi-quantitative method in identification of meat species (19, 21). In this study, band intensity of PCR products increased with an increasing concentration of the DNA of target species in the reaction (Figures 1, 2 and 3).

In conclusion, identification of adulterations in meat products is crucial for the protection of consumers, enforcement of labeling legislation and prevention of unfair competition. For this reason, rapid, simple, precise and reliable methods suitable for routine application are required for the identification of a meat's origin. In light of the results of this study, it can be suggested that the specific PCR technique used in identification of meats of horse, donkey and porcine species can be used as a routine control method in food control laboratories in this country.

REFERENCES

1. Kang'ethe EK, Gathuma JM, Landqvist KJ. 1986. Identification of the species of origin of fresh, cooked and canned meat on meat products using antisera to thermostable muscle antigens by Ouctherlony's double diffusion test. *J Sci Food Agric,* 37, 157-162.

2. Berger RG, Mageau RP, Schwap B, Johnston RW. 1988. Detection of poultry and pork in cooked and caned meat foods by enzyme linked immunosorbent assays. *J Assoc Off Anal Chem Int,* 71(2), 406-409.

3. Zerifi A, Labie C, Benard G. 1992. SDS-PAGE Technique fort he species identification of cooked meat. *Fleishwirtschaft,* 1, 54-59.

4. Amstrong SG,. Leach DN. 1992. The use of HPLC protein profiles in fish species identification. *Food Chem,* 44, 147-155.

5. Gallardo JM, Sotelo CG, Pineiro C, Perez-Martin RI. 1995. Use of capillary zone electrophoresis for fish species identification. Differentiation of flatfish species. *J Agric Food Chem,* 43, 1238-1244.

6. Hofmann K. 1987. Fundemental problems in identifying the animal species of muscle meat using electrophoretic methods. *Fleischwirtshaft,* 67, 820-826.

7. Jemmi T, Schlosser H. 1992. Tierartenbestimmung bei erhitztem fleisch von haus-und wildwiederkäuern mittels isoelektrische fokussierung. *Fleischwirtshaft,* 72, 1191-1195.

8. Koh MC, Lim CH, Chua SB, Chew ST, Phang STW. 1998. Random amplified polymorhic DNA (RAPD) fingerprints for identification of red meat animal species. *Meat Sci,* 48, (3/4) 275-2859. Lockley AK, Bardsley RG. 2000. DNA based methods for food authentication. *Trends in Food Sci Technol,* 11, 67-77.

10. Solak M, Bağcı H, Şengil AZ, Öztaş S. 2000. Moleküler Genetik ve Rekombinant DNA teknolojisi. Kocatepe Üniversitesi, Eğitim Sağlık ve Bilimsel Araştırmalar Vakfı. Yayın No:5. Afyon.

11. Rodriguez MA, García T, González I., Asensio L, Hernández P.E, Martín, R. 2004, PCR identification of beef, sheep, goat, and pork in raw and heat-treated meat mixturex. *J Food Protect,* 67(1),172-177.

12. Meyer R, Candrian U, Lüthy J. 1994. Detection of pork in heated meat products by Polymerase Chain Reaction (PCR). *J Assoc Off Anal Chem Int,* 77, 617-622.

13. Behrens M, Unthan M, Brinkmann Y, Buchholz R, Latus N. 1999. Nachweis von tierarten in erhitzten und komplexen fleischerzeugnissen durch tierart-spezifische PCR-reaktionen. *Fleischwirtschaft,* 79, 97-100.

14. Aslan A, İlhak İ, Çalıcıoğlu M. 2004. Isıl işlem uygulanmış sığır etlerinde polimeraz zincir reaksiyonu (PCR) yöntemiyle tür tayininin saptanması. I. Ulusal Veteriner Gıda Hijyeni Kongresi. 29 Eylül-1Ekim 2004. Ankara Üniversitesi Veteriner Fakültesi Dışkapı Kampusu/Ankara.

15. Aslan A, İlhak İ, Çalıcıoğlu M, Karahan M. 2004b. Identification of meats using random amplified polymorphic DNA (RAPD) technique. *J Muscle Foods,* 16, 37-45.

16. İlhak O.İ. 2004. Polimeraz Zincir Raksiyonu (PCR) Yöntemi ile Et Türlerinin Belirlenmesi. Doktora Tezi. Fırat Üniversitesi Sağlık Bilimeri Enstitüsü Besin Hijyeni ve Teknolojisi Ana Bilim Dalı. Elazığ.

17. Gökalp HY, Kaya M, Zorba Ö. 1999. Et Ürünleri İşleme Mühendisliği. Atatürk Üniversitesi Ziraat Fakültesi Ofset Tesisi. Erzurum.

18. Kesmen Z. 2005. Taze ve işlenmiş model et ürünlerinde farklı hayvan türlerine ait etlerin PCR yöntemi kullanılarak belirlenmesi. Atatürk Üniversitesi Fen Bilimleri Enstitüsü Gıda Mühendisliği Anabilim Dalı Doktora Tezi, Erzurum, Türkiye, 79 s.

19. Matsunaga T, Chikuni K, Tanabe R, Muroya S, Shibata K, Yamada J, Shinmura Y. 1999. A quick and simple method for identification of meat species and meat products by PCR assay. *Meat Sci,* 51, 143-148.

20. Calvo JH, Zaragoza P, Osta R. 2001. Technical note: A quick and more sensitive method to identify pork in processed and unprocessed food by PCR amplification of a new specific DNA fragment. *J Anim Sci,* 79, 2108-2112.

21. Meyer R, Höfelein C, Lüthy J, Candrian U. 1995. Polymerase Chain Reaction- Restriction Fragment Length Polymorhyism Analysis: A simple method for species identification in food. *J Assoc Off Anal Chem Int,* 78 (6) 1542-1551..