RESEARCH ARTICLE

Detection of Animal Species in Some Meat and Meat Products by Comparatively Using DNA Microarray and Real Time PCR Methods

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Summary

Determination of animal species in the meat and meat products is one of the interest of food science, it is also important for consumer rights and food safety. Increasing world population has also remarkably impacted the demand for meat and meat products. Based on this fact, issues related to safety and quality in the meat products have brought up in that manner as well. Through DNA based molecular methods are improved in food analysis, it is preferred increasingly in the control of food safety. In this study, a total of 73 samples of the meat and meat products sold in stores, meat selling markets and public bazaars located in different districts of İstanbul province were analyzed for the detection of animal species notified on the label by using Chipron LCD Array Analysis System. The results showed that 39 samples (53.4%) were labelled incorrectly. Randomly selected eleven samples were corrected by Iontek Fluorion Meat Species Identification Kit and FDS Detection System (Real Time PCR). Hence, it was found that the results obtained by DNA Microarray and Real Time PCR methods were identical (100%) with each other, and both methods should extensively be promoted for the detection of animal species in the meat and meat products.

Keywords: *DNA Microarray, Animal Species Detection, Food Safety, Real Time PCR*

Karşılaştırmalı DNA Mikroarray ve Real Time PCR Yöntemi Kullanılarak Bazı Et ve Et Ürünlerinde Tür Tayini Saptanması

Özet

Et ve et ürünlerinde tür beyanı uygunluğunun tespiti gıda biliminin ilgi alanlarından birisi olup; tüketici hakları ve güvenli gıda temini bakımından önem taşımaktadır. Dünya nüfusunda sürekli artış nedeniyle et ve et ürünlerine talep artırmaktadır. Artışın bu hızda devam etmesi gıda güvenliği ve kaliteyle ilgili sorunları gündeme getirmektedir. Gıda analizlerinde DNA tabanlı moleküler yöntemlerin hızla gelişmesiyle, bu yöntemler gıda güvenliği denetiminde artan oranda tercih edilmektedir. Bu çalışmada İstanbul' un farklı semtlerinde yerleşik marketler, et şarküterileri ve semt pazarlarında satışa sunulan 73 adet et ve et ürünü örneğinde DNA Mikroarray Teknolojisi tabanlı Chipron LCD-Array Analiz Sistemi kullanılarak tür beyan uygunluğu incelenmiştir. İncelenen örneklerin 39'unda (%53.4) tür beyanı uygunsuzluğu tespit edilmiştir. Rastgele seçilen 11 örnek için İontek Multipleks Fluorion Et Tür İdentifikasyon Kiti ve İontek FDS Tespit Sistemi (Real Time PCR) kullanılarak doğrulama yapılmıştır. Ayrıca, DNA Mikroarray ve Real Time PCR yöntemleri ile elde edilen sonuçlar arasında %100 uygunluk olduğu; bu iki yöntemin et ve et ürünlerinde tür tayini için yaygınlaştırılarak kullanılabileceği görülmüştür.

Anahtar sözcükler: *DNA Mikroarray, Et Tür Tayini, Gıda Güvenliği, Real Time PCR*

INTRODUCTION

Protein as a macro nutrient and energy source received from meat and meat products has important building and regulatory functions in the body. It is recommended that at least 1/3 of the daily protein requirement in a well and balanced diet should be taken from foods of animal origin. Protein synthesises hormons, enzymes and immune-related species as well as it protects homeostatic balance [1,2]. WHO reports that there is a positive correlation between the level in terms of development and the nutritional fact of protein rich of animal-origin in the report of the Global and

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Regional Food Consumption Patterns and Trends in 2011 [3]. According to this report it is estimated that the annual production of meat and meat products will increase up to 376 millions of tonne in 2030 whereas it was 218 millions of tonne between 1997 and 1999. The statitistics give the consumption rate of meat in Turkey to be about 28 kg per capita [4].

Hygiene and right labelling notified on the label of any food stuff are very important criterias especially for public health. Food safety covers all the preventive measures for the delivery of food in healthy and hygienic conditions to the consumer by protecting it from denaturation, microbiological and chemical contamination and adulteration. Inadequate management of food safety causes serious health problems [5]. There are many infectious diseases called zoonosis transmitted from foods of animal origin such as cyst hydatid, toxoplasma, leptospirosis and brucellosis [6-10]. According to the existing acts the suitable meat for human consumption is defined as the parts from carcasses of animal fitting for slaughter, especially blended mixtures of meat with no adulteration, and the processed ones [11]. The inedible parts of a meat animal are skin, glands, reproductive organs excluding, testicular parts, eyes and eyelids, urinary organs excluding kidney, larynx, trachea, cornea, ears, nails, horn, head, esophagus, craw, intestines, genitals and offals, respectively [12]. The studies have shown that inedible parts of carcases may be infected with some pathogens such as *Brucella melitensis*, *Brucella abortus*, Hepatitis E and coliforms which are potentially risk of zoonosis [13,14]. For instance, bovine spongiform encephalopathy (BSE) known as mad cow disease affected the United Kingdom in 1986. The animals being fed with meat and bone meal as feed additive and the human consuming offals and infected carcasses in the food chain caused this infection to spread out fast [15,16].

In meat and meat products the variety of animal species on the label should be inspected regularly. These Species are pig, chicken, turkey, goat, buffalo, deer, ruminants, sheep, duck, goose, camel, ostrich, kangaroo, quaill, pheasant and equines. The regulations to protect the public health against adulteration and zoonoses strictly prohibit the inedible and lower quality meat either to be directly launched or to be processed in the food chain [17-21]. Hence, detection of meat species by fast and accurate methods should routinely be carried out for the quality control as well as a public task to secure the food safety all over the world [22-28]. A study conducted in İzmir province showed that meat and meat products were mixed with meat belonging to various animal species by 15.5% and detected pork and equine species that were different from the notifications on the label [22]. Another study carried out in Bursa and İstanbul provinces indicated that 22.0% of the samples of fermented sausage, cooked salami, frankfurter, raw meat, ground meat, meat ball, pastramis, ham, bacon, cooked meat and canned product were not in compliance with the Turkish Food Codex violating consumer rights and presenting a potential

public health risk whereas another study in the same region showed that the adulteration in meat ball, sausage and was 19.2% [24]. The results arising from the studies in USA also indicated the adulteration in meat and meat products so that 62% had one species, 36% had two species and 2% had three species, respectively [25,26]. A similar outcome in the States reported that adulteration was 46.4% in this category of food [29]. On the other hand, commercial samples of swine hamburgers marketed in Brasil showed no adulteration with bovine, chicken or horse meats, and expectation of hamburger adulteration was not confirmed [28].

Detection of adulteration has become a challenging area in the food science [30-32]. Recent developments in food additives as well as novel foods have remarkably changed the food matrix lowering the reliability of analytical methods based on sensorical, anatomical, morphological and histological differences in detection of adulteration [33-35]. Some testing characteristics like becoming fast, accurate, sensitive, selective, user friendly and capable of simultaneously detection of more than one species in only one reaction are commonly requested for acceptance of a new analytical method [36]. The detection of adulteration in meat and meat products can be done by using different methods such as HPLC, ELISA and PCR [29,36-43].

A new technique called DNA Microarray has been increasingly used to express the impacting mechanisms of neutraceuticals and functional foods in metabolism, to determine the microorganisms related to food safety studies [44-47]. It has also opened up new challenges for food analysis of adulteration in seafoods, meat and meat products [48-51]. Nowadays, DNA based molecular techniques are preferred in many disciplines like taxonomy, epidemiology, forensic, archeology, environmental sciences and food science [30,52-55]. The conventional methods of molecular biology including PCR and RNA blotting do usually examine only one gene in a reaction resulting in poor understanding of the whole of the picture of gene functions [56] whereas DNA Microarray makes possible the whole genome to be displayed on a chip and to express the interaction of thousands of genes with each other simultaneously [56-60].

In this study, 73 samples of the meat and meat products sold in stores, meat selling markets and public bazaars located in different districts of İstanbul province were analyzed to detect the existing animal species as notified on the label by using Chipron LCD Array Analysis System; and randomly selected 11 samples were controled for the verification of the previously found results by Iontek Fluorion Meat Species Identification Kit and FDS Detection System.

MATERIAL and METHODS

Material

In this study 33 of fermented sausages, 16 of grilled meatball, 11 of ground meat, 7 of salami and 6 of sausages sold

in stores, meat selling markets and public bazaars located in different districts of İstanbul province were collected. All the samples were examined for notification on the label and asessement of adulteration by DNA Microarray method

Method

The collected samples were placed in sterile sampling bags, and transported inside a refrigerated container kept at 4°C for sample preparation and DNA isolation. The pieces taken by means of lancet and spatula were homogenized in a blender. 0.20 gram of the homogenized sample was put into Eppendorf tubes.

DNA was extracted by following up the procedure given in Eurofins GeneScan GENE*Spin* DNA Isolation Kit (Catalog no: 5224400605) as outlined in *Fig. 1*. The extracted samples of DNA were stored at -20°C [61].
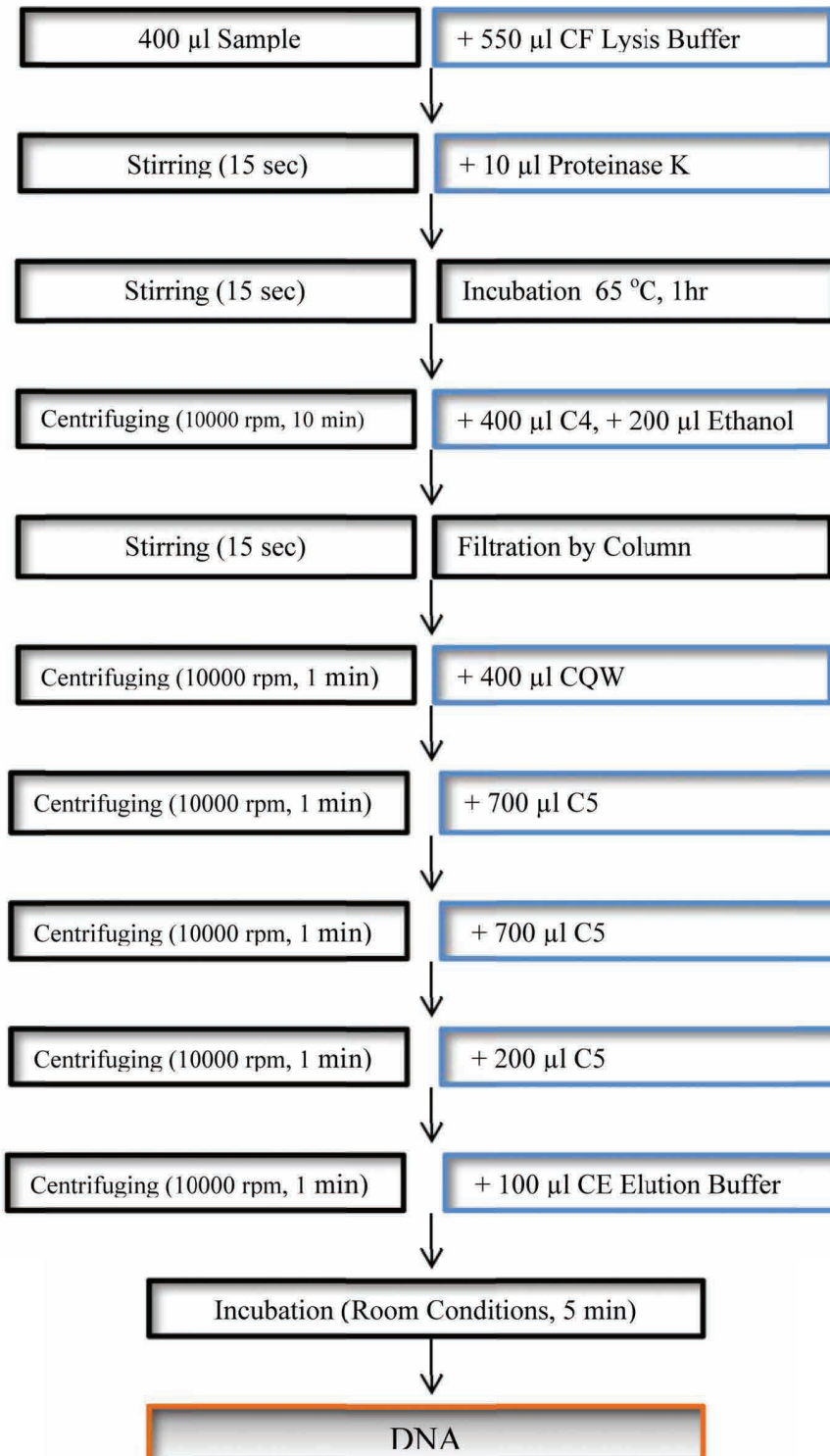

Fig 1. DNA isolation procedure

Şekil 1. DNA izolasyonu prosedürü

The extracted DNA samples were amplified by Real Time PCR (Agilent Stratagene Mx3000P) using the procedure given in Chipron LCD Array Meat Species 1.6 Kit (Chipron GmbH, Germany). Since the kit is ready to use, 12.5 µl of Chipron 2x all in one master mix, 1.5 µl of primer mix and 8 µl of sterile water were put into an Eppendorf tube, respectively. This prepared solution of 22 µl was pipetted to each of the plate wells following addition of 3 µl of DNA template. The plate was closed and was installed in Real Time PCR [62]. Thermal processing was given as 1 cycle at 96°C for 3 min, then 30 cycles at 94°C for 30 sec, 57°C for 45 sec and 72°C for 45 sec, and finally 72°C for 3 min [63].

Twenty two microliter of hybridization solution and 2 µl of modulator solution were added into an Eppendorf tube. This 24 µl of mix was pipetted to each of the plate wells following the addition of 10 µl of extracted DNA samples. Chip in the kit was placed in the chip box. 30 µl of each of the plate wells was pipetted onto the lower left hand corner of each of the eight patterns *(Fig. 2)*. Chip box was closed, allocated to standby at 35°C for 30 min, washed, dried, and then placed in the box again.

Putting the dilution solution into the Eppendorf tube 30 µl of annealing solution was pipetted into each of the patterns of the chip and allowed to standby for 5 min. After the incubation completed washing procedure was done, and chip was centrifuged for 15 sec, allowed to dry, and placed in the box again.

Thirty microliter of staining solution was put into each of the patterns of the chip, and the chip was allowed to standby for 3 min in room conditions. Following staining procedure, it was kept in washing box for 15 sec, and then centrifuged for 15 sec for drying.

Evaluation of the Results

Chipron LCD Array System can detect cattle, buffalo, pig, sheep, goat, horse, donkey, rabbit, hare, chicken, turkey, goose, and two duck varieties in food sample. The detection in this system is based on specific sites within 16S rRNA mitochondrial locus of all meat species in the analyzed food sample. A dark precipitate is formed by the enzyme substrate provided in the test kit, and it indicates a positive hybridization reaction. After staining procedure completed the chip was read with the scanner, and analysis was done by the software from the "Analysis-Package" provided by Chipron. Three different spots on the chip are called the control points (C) to detect a positive reaction which are located in upper-left, upper right and lower right corners, respectively. If no darker visualization occurs, the test should be repeated. The animal species was identified according to *Fig. 2* and *Table 1*.

Verification by FDS Detection System (Real Time PCR)

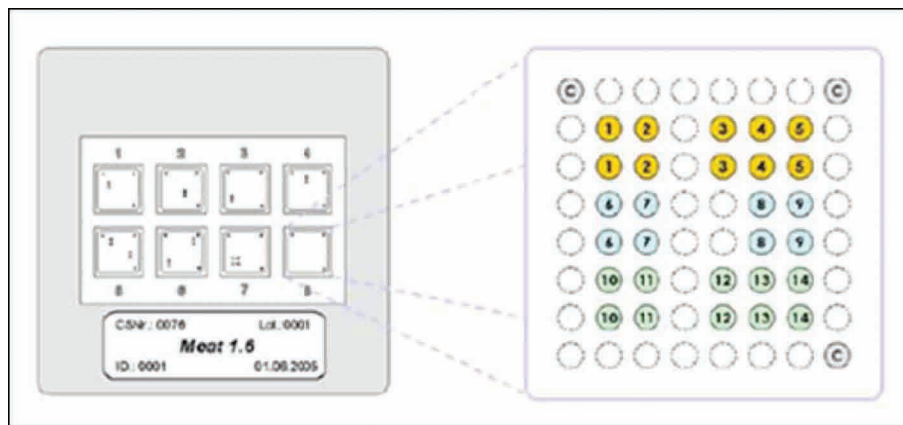Randomly selected 11 samples which analysed by DNA Microarray method were verified by Iontek FDS

Fig 2. Spotting points of LCD-array meat 1.6

Şekil 2. Çip noktaları ve et tür eşlemeleri

Well No	Probe	Specificity	Well No	Probe	Specificity
01	Beef	*Bos taurus*	**08**	Rabbit	*Oryctolagus cuniculus*
02	Buffalo	*Bubalus bubalis*	**09**	Hare	*Lepus europaeus*
03	Pork	*Sus scrofa*	**10**	Chicken	*Gallus gallus*
04	Sheep	*Ovis aries*	**11**	Turkey	*Meleagris gallopavo*
05	Goat	*Capta hircus*	**12**	Goose	*Ansa albifrons*
06	Horse	*Equus caballus*	**13**	Mall. Duck	*Anas platyrhyncos*
07	Donkey	*Equus asinus*	**14**	Musc. Duck	*Cairina moschata*
			C	Hyb-Cont.	Functional controls (Hybridisation + Stain)

Table 1. *Capture probes*
Tablo 1. *Çip noktalarına karşılık gelen et türleri*

Detection System (Real Time PCR) method. The DNA which previously isolated by using Eurofins GeneScan DNA Isolation Kit (Catalog No: 5224400605) stored at -20°C were used. The procedure given by Iontek Fluorion Meat Species Identification QLP 1.0 Kit (Catalog No: F0560102) was followed up. Positive and negative controls were run in duplicate whereas DNA samples were run in triple. All the solutions and materials in the kit were dissolved before use. 23 µl of PCR master mix including 12.5 µl of PCR mix, 4 µl of detection mix and 6 µl of sterile water was pipetted into each of the plate wells. Two microliter of previously extracted DNA was added onto each. The tubes were closed off tightly and placed in Iontek FDS Real Time PCR System. The thermal processing was given as one cycle at 95°C for 15 min, then 40 cycles at 95°C for 25 sec and 62°C for 20 sec. The analysis was done by the FDS software from the "Analysis-Package" provided by Iontek [63].

RESULTS

The results obtained by DNA Microarray indicated that 39 out of 73 samples (53.4%) were labelled incorrectly, and adulteration was made in contrary to the notifications on the label. The adulteration was detected mostly in meat balls (87.5%), ground meat (72.7%), salami (57.1%), sausages (50%) and fermented sausages (30.3%), respectively. The results are presented in *Table 2*. It was mostly seen that meat balls and ground meat have significantly potential risk for adulteration. Following them fermented sausage samples showed incorrect labelling with the range of 30%. On the other hand, these three types of food were having a claim of 100% beef on the labels. Hence, mostly detected meat species in meat ball, ground meat and fermented sausage samples were chicken, turkey and sheep species. No pig and equine species were detected in 79 samples. Randomly selected 11 samples out of 79 were verified by

Iontek FDS Detection System (Real Time PCR). The results obtained by both of DNA Microarray and Real Time PCR were identical to each other with the range of 100%. The verified results are also given in *Table 3*.

DISCUSSION

The inspection of the declared composition of food stuff as notified on its label is officially an obligatory task order to protect the public benefits and health against adulteration and infectious diseases caused by zoonoses. In this study, we compared DNA Microarray (Chipron, Germany) method to Iontek FDS (Real Time PCR) System for routine use.

A variety of animal species present in the meat and meat products were examined in the past. In Turkey many studies related to the animal species detection were carried out by official authorities and academic research institutes. In Izmir province totally 116 samples of joint meat, ground meat, sausage pulp, meat ball, hamburger, canned meat, Turkish Doner, fermented sausage, salami, roasted meat and sausage were reviewed for the notifications declared on the label. The results showed that 18 samples (15.5%)

Table 2. *Results of DNA microarray*
Tablo 2. *DNA mikroarray sonuçları*

Description of Sample	No of Sample	No of Samples Eligible	No of Samples İneligible
Meat ball	16	2	14 (87.5%)
Ground meat	11	3	8 (72.7%)
Salami	7	3	4 (57.1%)
Sausage	6	3	3 (50.0%)
Fermented sausage	33	23	10 (30.3%)
Total	73	34	39 (53.4%)

Table 3. *Compared results between DNA microarray and real time PCR*
Tablo 3. *Real time PCR ile karşılaştırmalı sonuçlar*

No	Description of Sample	Chipron LCD Array Meat Species Kit		Iontek Fluorion Meat Species QLP Kit	
		Beef	**Chicken**	**Beef**	**Chicken**
1	Fermented Sausage, 100% Beef	+	-	+	-
2	Fermented Sausage, 100% Beef	+	-	+	-
3	Fermented Sausage, 100% Beef	+	-	+	-
4	Fermented Sausage, 100% Beef	+	-	+	-
5	Fermented Sausage, 100% Beef	+	-	+	-
6	Fermented Sausage, 90% Chicken + 10% Beef	+	+	+	+
7	Fermented Sausage, 100% Beef	+	-	+	-
8	Fermented Sausage, 100% Beef	+	-	+	-
9	Fermented Sausage, 78% Chicken + 22% Beef	+	+	+	+
10	Salami, 30% Beef + 70% Chicken	+	+	+	+
11	Sausage, 95% Chicken + 5% Beef	-	+	-	+

were labelled incorrectly and containing animal species other than the declared ones like horse flesh (9.5%), pork (9.5%), chicken (23.8%) in the sliced meat; pork and beef (4.8%) whereas chicken and beef mix (9.5%) in ground meat. A hundred percent of sausage pulp samples were containing chicken and beef together. It was reported that 4.8% of meat balls had pork meat, and chicken was also detected in Tas Kebap, Turkish Döner, salami, roasted grill and sausages in contrary to the notification on the label. Consequently, meat and meat products produced in Izmir were mixed with meat belonging to various animal species [22]. Some other studies carried out in Istanbul and Bursa provinces totally 100 samples composed of 28 fermented sausages, 25 salami, 9 raw meat, 16 ground meat, 3 pastrami, 7 ham, 7 grilled meat, 5 canned meat were tested. The obtained results indicated that 11 fermented sausages (39.2%), 8 sausages (62.9%), 2 raw meat (22.2%) and 1 ground meat were contrary to the declarations on the label, and 22% of all collected samples were carrying potentially high risk for health [23]. Another study reported that 65 of ground meat, 35 of meat ball pulp, 50 of fermented sausage pulp, 125 of fermented sausage, 75 of salami and 60 of sausage totally making 410 samples the adulteration ratio was determined to be 19.2% (79 samples) [24]. A study done in USA indicated that 62% of the meat and meat products had only one foreign species, 36% had two, and 2% had three. A similar study in the States also showed that the adulteration ratio has increased up to 46.4% [25,26,29]. In Brasil commercial samples of swine hamburgers showed no adulteration with bovine, chicken, swine or horse meats, and expectation of hamburger adulteration was not confirmed [28]. It was found that our study and those carried out in Turkey and at abroad have delivered identical results. It is understood that the adulteration is a key tool in reducing the costing in the production of meat and meat products, preferably tried in contrary to the notifications on the label poultry, and especially encountered in processed meat products. This fact could somewhat explain the risk of zoonosis harmful to public health.

The detection of animal species in meat and meat products have been done by a variety of analytical methods. Each method has relatively advantages and disadvantages as compared to each others. Recently, Real Time PCR, a DNA based molecular technique, has been very popular in food analyses as a futher step of the conventional PCR. It brings away the demand for immunological and electrophoretic methods, and minimizes the risk of contamination during the testing [63]. Real Time PCR has a sensitivity in detection of meat species by 0.1% whereas ELISA can do it less sensitive by 2% [21,32,35,64-71].

DNA Microarray and Real Time PCR methods differentiate from each other in simultaneously detection of animal species in one reaction. The only common similarity between them is the step of DNA isolation. Microarray Analysis can enable us for detecting more than one species in one reaction only whereas Real Time PCR requires specially designed primers and probes needed to simultaneously amplify the specially selected regions of DNAs belonging to different species. This difference means longer time needed in the optimization step of primers and probes [30]. DNA Microarray can deliver the results faster and more sensitive using amplified DNA by conventional PCR technique [62]. Therefore, DNA Microarray method has been widely preferred for understanding mechanisms, detection of foodborne microbial pathogens and food safety studies, nutreaceuticals and functional foods as well as following up the different expression levels of DNA in bacteria, yeasts, plants and human; genetic and mutation analyses; environmental studies; identification of antimicrobial genes, proteomics, protein-nucleic acids, protein-protein interactions, biochemical analysis of protein functions and drug development [44-47,52,72-75]. In the recent years studies in the literature related to DNA Microarray have focused on the detection of adulteration in seafoods and meat and meat products [48-51]. In our study, DNA Microarray was used to determine adulteration in some selected meat products by making verification by Real Time PCR method. It was found that both of the methods delivered the identical results. Therefore, it was seen that DNA Microarray method is fast, accurate and safe by introducing this technique firstly to Turkey for detection of foreign animal species. DNA Microarray was preferred for higher capacity of data analysis, suitability for species detection, re-usability of the results, higher analysis throughput and becoming user-friendly.

In conclusion, adulteration is a serious food safety and quality issue with an increasing prevalance in meat and meat products all over the world. Regular controls for adulteration in meat and meat products should be frequently and intensively done due to the significant increasing demand for the meat. It was found that the results obtained by DNA Microarray and Real Time PCR assays were identical with each other, and both methods should extensively be promoted for the detection of animal species in the meat and meat products.

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