Food Forensics: Using DNA-Based Technology for the Detection of Animal Species in Meat Products

Yosef, T.A.[1, 2*]; Al- Julaifi, M.Z.[2] and AL-Rizqi, A.M.[2]

[1]Dept. of Forensic Med. and Toxicology, Fac. of Vet. Med., Kafrelshiekh Univ., 33516, Egypt.
[2]Toxicology lab. Management of Vet. Laboratories, Min. of Agric, Riyadh, 11418, KSA.
tarekyosef70@yahoo.com

Abstract: Meat species specification is an area which needs specialized attention in food forensics. It is a vital field to ensure the food safety to the consumers and conserves the laws related to meat and meat products. The adulteration of inferior quality meat into superior quality meat is a common practice all over the world. DNA-Based techniques can easily solve the problems of vetro-legal or forensic cases and related laws existing worldwide. In this study, DNA Microarray and Real-Time polymerase chain reaction (RT-PCR) techniques were applied for the detection of meat adulteration in processed meat samples. Seventy seven samples of processed beef meat products (Hamburger, luncheon, sausages, hot dog, corned loaves, meat balls, shish kebabs) 11 samples each, were subjected to DNA Microarray analysis (LCD array kit, Meat 4.0)while fourteen samples, 2 of each meat product, including the two positive samples for pig were reanalyzed by RT-PCR (7500 Fast Applied BioSystems, ABI,). The results obtained by both of DNA Microarray and RT-PCR were identical to each other with the range of 100%. The results showed that 51 out of 77 samples (66.2%) were labeled incorrectly, and adulteration was made in contrary to the notifications on the label. The adulteration was detected mostly in meat balls (90.9%), shish kebabs (81.8%), luncheon (72.7%), corned loaves (72.7%), hot dog (54.5), sausage (54.5%) and hamburger (36.4%). It was mostly seen that meat balls, shish kebabs, luncheon and corned loaves have significantly potential jeopardy for adulteration. Hot dog; sausage and finally hamburger samples were lower profile than the others. On the other hand, all these types of food were having a claim of 100% beef on the labels. Hence, detected meat species were chicken, turkey, goat, sheep and pig. Only two samples (one luncheon sample and one sausage sample) were adulterated with pig. No equine species were detected in any of the samples.
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1. Introduction

In the last few decades, adulteration of meat products has become a considerable problem in many countries over and above Arabian countries. Meat species specification is an utmost important field of food forensics. It is more challenging and revolutionary task to ensure the quality of meat and help in conservation of law existing in different countries **(Singh &Sachan, 2009)**.According to the food law, the species' names of meats used to prepare the meat products have to be presented on the label of the product. Moreover, selling other meat species with false labels to get more profit is held as imitation and prohibition according to the foodstuff laws.

An important fraud in the meat industry is the substitution of meat of another species, i.e., horse for beef especially in Britain and Ireland, beef in kangaroo meat in Australia, cat for chicken or rabbit, goat meat for mutton, mutton for venison, dog meat and cat meat for chevon in other countries **(Kang'ehte *et al.,* 1986)**. The ability to detect less desirable or objectionable species in meat products is important not only for economic, health, religious and ethical reasons, but also to ensure fair trade and compliance with legislation **(Nakyinsige *et al.,* 2012)**.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 **(İlhak & Arslan, 2007).**

Most analytical methods utilized to date for meat authentication have relied on the detection of species-specific proteins or DNA **(Ballin *et al.,* 2009).** Today, however, DNA is considered to be the most appropriate molecule for species detection and identification in foods **(Singh & Neelam, 2011).** Unlike proteins, DNA is relatively stable at high temperatures, meaning that it can be analyzed not only in fresh and frozen food products, but also in processed, degraded and mixed commodities **(Lenstra, 2003).** Additionally, while the presence and characteristics of proteins depend on the tissue type being analyzed, DNA exists and is identical in almost all cells, and the unique variability and diversity afforded by the genetic code permits the

discrimination of even closely-related species **(Ballin, 2010).**

Many techniques based on DNA analysis were adopted for the needs of the food industry and allow carrying out investigations aiming at species identification, but the polymerase chain reaction (PCR) method deserves special attention because it is characterized by high sensitivity and specificity as well as relatively short period of time necessary to perform the analysis. From among the methods based on the PCR technique, the most frequent ones employed to check food falsifications include: PCR with species-specific primers, as well as PCR-RFLP (Restriction Fragments Length Polymorphism), PCR-RAPD (Randomly Amplified Polymorphic DNA), PCR-SSCP (Single Strand Conformation Polymorphism), and RT-PCR (Real Time Polymerase Chain Reaction).

Real-Time PCR is a method used for quantitative measurements of gene copies or the level of DNA expression. By measuring the intensity of signals derived from fluorescent dyes, the quantity of the PCR product created in each reaction cycle is monitored. The continuous measurement of fluorescence allows skipping post-PCR processing, i.e. electrophoresis and gel staining as the results are obtained throughout the reaction **(Zeitler *et al.,* 2002 and Huang & Pan, 2004, 2005).** In addition, RT-PCR, as well as meeting the need for quantitative determination in meat species, this technique also has other advantages like a larger dynamic range of detection and less carry-over contamination risk **(Rojas *et al.*,2011)**besides**,** RT-PCR-techniques are especially suitable for heated products because small fragments of DNA can still be amplified and identified**.** Such RT-PCR reactions have been successfully applied in the simultaneous detection of up to seven animal species in a meat sample **(Köppel *et al.,* 2008, 2009).**

A new technique called DNA Microarray has been increasingly used to simultaneously detect various events occurring in plant and animal tissues as well as in bacteria. This article looks at the use of DNA biochip technology in simultaneous detection of various animal species present in food samples **(Bottero & Dalmasso 2010, Hellberg & Morrissey 2011 and Budak & Dönmez 2012)**. As with the RT-PCR for the detection of animal species mentioned above, the use of a DNA chip for the detection of animal constituents in food is well suited for rapid screening of up to twenty four animal species in meat samples in a routine analytical laboratory. Both methods offer a simple, robust, and fast platform for the simultaneous detection of animal species in meat samples. The DNA chip however offers the additional advantage, that undeclared and unknown animal species present in a meat sample, resulting perhaps from inadvertent contamination or deliberate adulteration, can bedetected.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 **(Pereira *et al.,* 2008and Cansu, 2011).**Nowadays, DNA based molecular techniques are preferred in many disciplines like forensic, taxonomy, epidemiology, archeology, environmental sciences and food science (**Pereira *et al.,* 2008and Myers *et al.,* 2010).**

In the present study, the identification of different meat species in processed meat samples was screened by Chipron LCD Array, Meat 4.0 Analysis System and confirmed by 7500 Fast ABI RT-PCR to detect the existing animal species as notified on the label.

2. Material and Methods

A. Samples and sample collection:

A total of 77 packaged meat products were purchased from nearby hypermarkets at Riyadh province, Saudi Arabia. Seven different categories of processed meat products were collected for analysis, namely beef hamburger, beef luncheon, beef sausages, beef hot dog, corned beef loaves, beef meat balls, beef shish kebabs (11 sample each)that poses more of adulteration problem as these products cannot be identified by bare eyes. All samples were examined for notification on the label and assessment of adulteration by DNA Microarray analysis (LCD array kit, Meat 4.0) and fourteen samples were reanalyzed by RT-PCR (7500 Fast Applied BioSystems, ABI,).

B. DNA extraction

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. The homogenized sample was put into Eppendorf tubes. DNA was extracted from ca. 50 mg homogenized meat samples by following up the procedure given in SureFood® PREP Animal X DNA Isolation Kit (product code S1004, r-Biopharm, Germany supplied by ALHayat Center Est.-KSA).The kit is intended to be used for the isolation of animal-DNA from highly processed food and feed.DNA extracts were stored at -20 °C until further analysis.

C. Animal species screening by LCD Array method

The meat samples were screened for the presence of 24 animal species using LCD array kit, Meat 4.0 DNA-identification of animal species, Chipron GmbH, Berlin, Germany), Code: A-400-12.

PCR amplification

The extracted DNA samples were amplified by 3 Prime thermal cycler (TechneBibby Scientific, Version 1.3, USA) using the procedure given in HotStarTaq plus Master Kit (Qiagen, Code: 203645). 12.5 µl of 2x master mix, 1.5 µl of primer mix (Meat) and 6 µl of PCR grade water were put into a reaction tube, respectively. 5.0 µl of extracted DNA was added as template to each reaction tube. Thermal processing was given as 1 cycle at 95°C for 5 min, then 35 cycles at 94°C for 30 sec, 57°C for 45 sec and 72°C for 45 sec, and finally 72°C for 2 min.

LCD Array hybridization and detection

Twenty two µl of hybridization solution, 2 µl of modulator solution and 10 µl of PCR product were mixed and 28 µl of this solution was applied to the respective array field. The slide was incubated (in humidity chamber) at 35°C for 30 min, washed and dried.28 µl of label mix was applied to each field of the slide and incubated for 5 minutes at room temperature; washing and dryness procedures were performed. Staining of slide was achieved by applying 28 µl of stain solution to each slide field and incubated for 5 minutes at room temperature. Following staining procedure, it was kept in washing box for 10 sec, and then centrifuged for 15 sec for dryness.

Analysis and interpretation of the results

Chipron LCD Array System can detect beef, buffalo, pork, sheep, goat, horse, donkey, rabbit, hare, bison, kangaroo, springbok, five deer races, chicken, turkey, goose, ostrich, pheasant 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. The formation of dark visible precipitates at positions (spots) by the enzyme substrate provided in the test kit indicated a positive hybridization reaction. After staining procedure completed the chip was read by Slide Scanner PF3650u in combination with the Slide Reader Software from the “Analysis-Package” provided by Chipron. Different signal intensities can be observed during the analysis of LCD-Arrays and these intensities are generally correlated with the amount of target copies in the starting material. 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. 1 and Table 1.

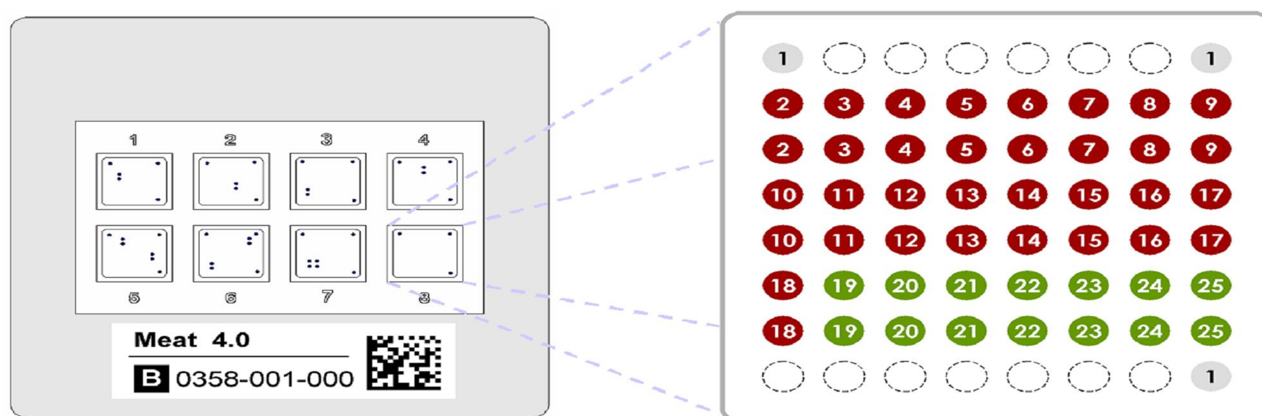

Fig 1. Spotting points of LCD-array meat 4.0

Table 1. DNA of animal species detected in parallel by LCD array kit Meat 4.0 using extracted DNA

Beef	*Bos taurus*	Red Deer	*Cervus elaphus*
Bison	*Bos bison*	Axis Deer	*Cervus axis / Axis axis*
Pork	*Sus scrofa*	Fallow Deer	*Dama dama*
Sheep	*Ovis aries*	Reindeer	*Rangifer tarandus*
Goat	*Capra hircus*	Springbok	*Antidorcas marsupialis*
Buffalo	*Bubalus bubalis*	Chicken	*Gallus gallus*
Horse	*Equus caballus*	Turkey	*Meleagris gallopavo*
Donkey	*Equus asinus*	Goose	*Ansa albifrons*
Hare	*Lepus europaeus*	Mallard Duck	*Anas platyrhyncos*
Rabbit	*Oryctolagus cuniculus*	Muscovy Duck	*Cairina moschata*
Kangaroo	*M. giganteus / M. rufus*	Pheasant	*Phasianus cholchicus*
Roe Deer	*Capreolus capreolus*	Ostrich	*Struthio camelus*

D. Animal species identification by Real Time PCR

Randomly selected 14 samples (2 samples from each meat product) which analyzed by DNA Microarray method were verified by 7500 Fast Applied BioSystems Detection System (RT-PCR). The DNA which previously isolated by using SureFood® PREP Animal X DNA Isolation Kit (product code S1004, r-Biopharm, Germany) and stored at -20°C were used. The procedure given by SureFood®ANIMAL QUANT Beef; Pork and Equus Kits (Art-No's: S1010; S1011; S1016) respectively was followed up.

Preparation of master-mix

For each kit type, 5 reactions for the standard curve; 3 reactions for controls (1x no-template control and 2x positive control) were needed for each reference-gene and detection-gene in addition to samples DNA. All the solutions and materials in the kit were dissolved before use. For beef and pork, 18.1 µl of PCR master mix including 18.0 µl of Ref Reaction Mix/ Bos or Sus Reaction Mix and0.1 µl of Taq Polymerase was pipetted into each of the reaction tubes while 20.1 µl of PCR master mix including 20.0 µl of Ref Reaction Mix/ Equ Reaction Mix and0.1 µl of Taq Polymerase was pipetted for equine.

Preparation of the standard DNA dilutions

Five dilutions of the Standard DNA were diluted in 1:10 steps in Dilution buffer to prepare different DNA concentrations for the standard curves of the reference-gene and the detection-gene as shown in table 2.

Table 2. Preparation of the standard DNA dilutions with the final copy numbers per reaction

Standard	Dilutions	Copy number per µl	Final copy numbers per reaction	
			Beef and Pork	Equines
S1	45 µl Dilution buffer+5 µl Standard DNA	100.000 copies	200.000 copies	500.000 copies
S2	45 µl Dilution buffer+5 µl DNA S1	10.000 copies	20.000 copies	50.000 copies
S3	45 µl Dilution buffer+5 µl DNA S2	1000 copies	2000 copies	5000 copies
S4	45 µl Dilution buffer+5 µl DNA S3	100 copies	200 copies	500 copies
S5	45 µl Dilution buffer+5 µl DNA S4	10 copies	20 copies	50 copies

Preparation of the PCR-mix

18 µl (for beef and pork) and 20 µl (for equine) of the master-mix was pipette into reaction tubes. The negative control was ready for PCR without any addition. Positive control; standard dilutions and the previously extracted sample DNA were added onto each reaction tube by volume of 2µl in case of beef and pork and by 5 µl in case of equine. The tubes were closed off tightly, centrifuged and putted in 7500 Fast Applied BioSystems RT-PCR System. The thermal processing for beef and pork was given as one cycle at 95°C for 5 min, then 45 cycles at 95°C for 10 sec, 62°C for 15 sec and 65°C for 30 sec while that for equine was one cycle at 95°C for 5 min, then 45 cycles at 95°C for 15 sec and 60°C for 30 sec. The reporter dye was FAM and the Quencher one was TAMRA in all species.

Interpretation of results

The calculation for both reactions (reference and detection genes) was made separately by using the calculated copy numbers as the relative target species content of the sample DNA and the positive control was determined from the following equation:

Target species/positive control contents=detection gene copy numberX100/reference gene copy number the result was multiplied by K*

NB: K* correction factor (relation from the true percentage value of the positive control "100%" and the measured percentage of the positive control.

Evaluation of species authenticity and mislabeling

For the evaluation of the accuracy of meat product labeling, the species identifications made using the LCD array and confirmed through RT-PCR were compared with the species declared on the product packaging.

3. Results

The results obtained by DNA Microarray indicated that 51 out of 77 samples (66.2%) were labeled incorrectly, and adulteration was made in divergent to the notifications on the label. The adulteration was detected mostly in meat balls (90.9%), shish kebabs (81.8%), luncheon (72.7%), corned loaves (72.7%), hot dog (54.5), sausage (54.5%) and hamburger (36.4%). The results are accessible in Table 3. It was mostly seen that meat balls, shish kebabs, luncheon and corned loaves have significantly potential jeopardy for adulteration, followed by hot dog and sausage samples and finally hamburger one which was evidenced for mistaken labeling with the range of 36.4%. On the other hand, all these types of food were having a claim of 100% beef on the labels. Hence, detected meat species were chicken, turkey goat, sheep and pig species. Only two samples (one luncheon sample and one sausage sample) were adulterated with pig. No equine species were detected in any of the samples. Fourteen samples out of 77, 2 of each meat product, including

the two positive samples for pig that detected by LCD Microarray were verified by 7500 Fast Applied BioSystems (RT-PCR). The results obtained by both of DNA Microarray and Real Time PCR were matching to each other with the range of 100%. The verified results are also given in Table 4.

Table 3. Results of DNA Microarray (N=11)

Sample type	Real samples		False samples		Substituted species
	Number	%	Number	%	
Meat balls	1	9.1	10	90.9	Chicken, turkey and goat
Shish kebabs	2	18.2	9	81.8	Chicken and sheep
Luncheon	3	27.3	8	72.7	Chicken, turkey and pig
Corned loaves	3	27.3	8	72.7	Turkey, sheep and goat
Hot dog	5	45.5	6	54.5	Turkey, sheep and goat
Sausage	5	45.5	6	54.5	Chicken, goat and pig
Hamburger	7	63.6	4	36.4	Chicken and sheep
Total	26	33.8	51	66.2	Chicken, turkey, goat, sheep and pig

Table 4. Analysis of meat samples (100% beef) with the DNA Microarray compared to the RT-PCR

Sample No.	Description of Sample	RT-PCR			LCD Array		
		Beef	Horse	Pork	Beef	Horse	Pork
1	Meat balls	+	-	-	+	-	-
2	Meat balls	+	-	-	+	-	-
3	Shish kebabs	+	-	-	+	-	-
4	Shish kebabs	+	-	-	+	-	-
5	Luncheon	+	-	-	+	-	-
6	Luncheon	+	-	+	+	-	+
7	Corned loaves	+	-	-	+	-	-
8	Corned loaves	+	-	-	+	-	-
9	Hot dog	+	-	-	+	-	-
10	Hot dog	+	-	-	+	-	-
11	Sausage	+	-	-	+	-	-
12	Sausage	+	-	+	+	-	+
13	Hamburger	+	-	-	+	-	-
14	Hamburger	+	-	-	+	-	-

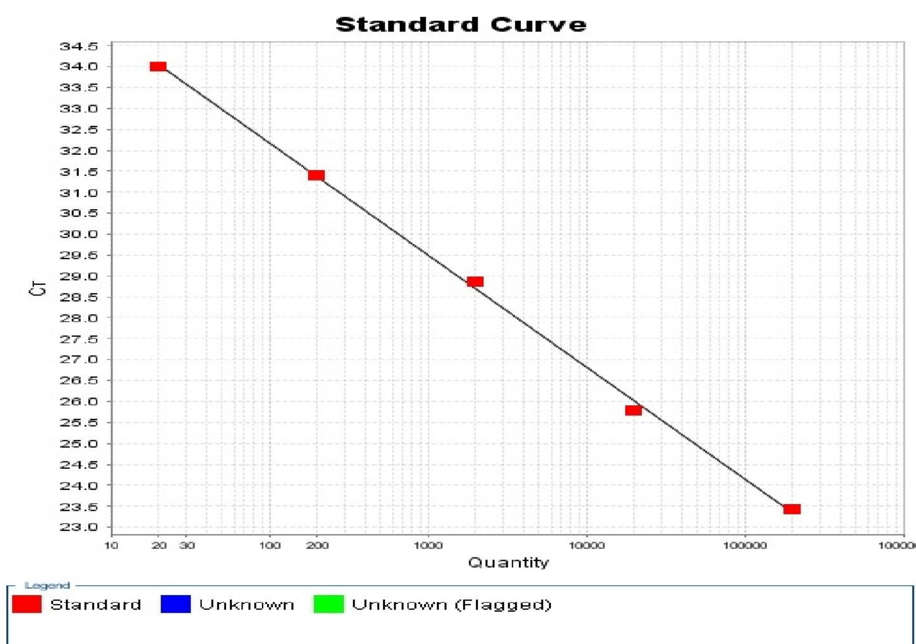

Fig 2. Chromatogram of pork standard curve

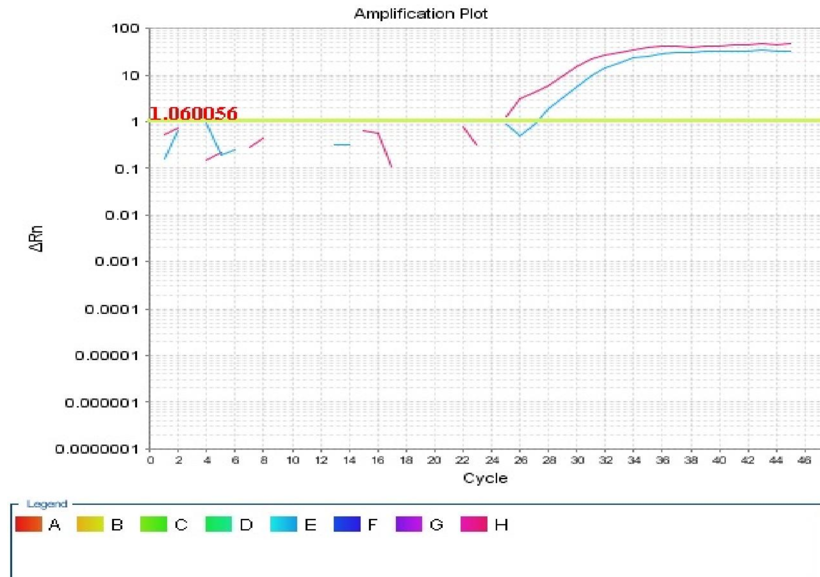

Fig 3. Real-Time PCR amplification plot of pork animal species.

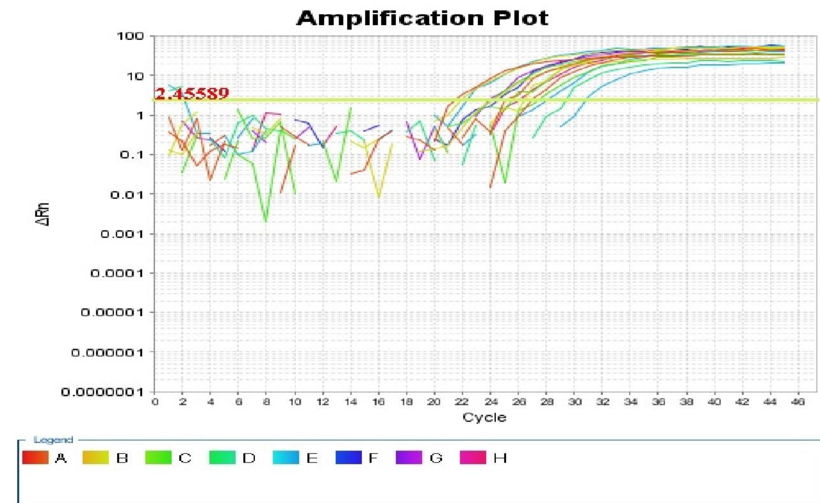

Fig 4. Real-time PCR amplification plot of beef animal species

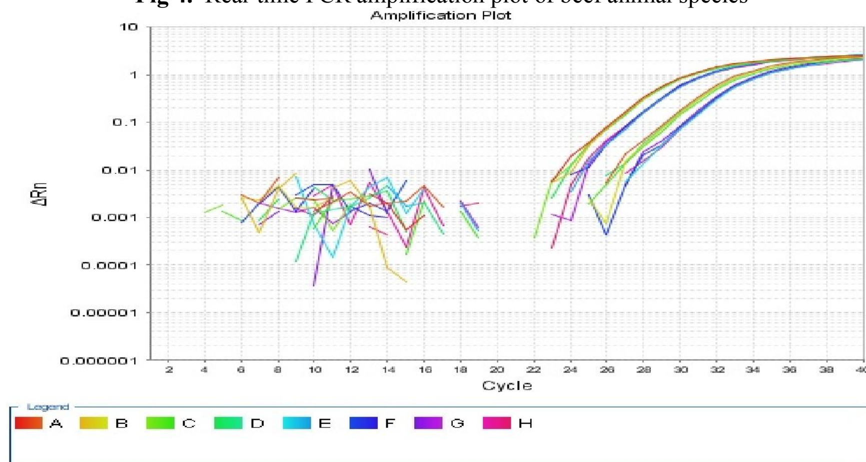

Fig 5. Real-time PCR amplification plot of beef animal species

4. Discussion

Meat species adulteration is a worldwide problem, which infringed food labeling laws, constitutes economic fraud, and raises ethical, religious and food safety concern **(Abd El-Nasser *et al.,* 2010)**.The inspection of the affirmed composition of meat products as notified on its label is officially a compulsory task order to protect the public benefits and health against adulteration. 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 **(Unajak *et al.,* 2011)**.

A multiplicity of meat adulteration particularly in processed meat products were recorded in past studies from different countries such as Australia, Canada, Egypt, Saudi Arabia, Turkey, United Kingdom and USA**(CCWA, 1999; MAFF, 1999; Odumeru, 2003;Ayaz *et al.,* 2006; El-Sangary & Gabrail, 2006;Ibrahim, 2008; Türkyılmaz & Irmak, 2008; Abd El-Nasser *et al.,* 2010 and D'Amato *et al.,* 2013).**It is unspoken that the adulteration is a key device in reducing the production costing of meat and meat products. This fact could fairly explain the risk to public health.

Techniques for authenticating the origin of meat products have advanced recently and have been done by a variety of analytical methods **(Ballin *et al.,* 2009)**. Each method has relatively advantages and disadvantages as compared to each other's. However, methods based on DNA amplification are still preferred, as they are less affected by industrial processing **(Pascoal *et al.,* 2005).**The target genes and DNA fragments used as markers for identifying meat species mainly come from the mitochondrial genome, including 12S rRNA gene **(Wang *et al.,* 2010)**, 16S rRNA gene **(Mitani *et al.,* 2009),**18S rRNA gene **(Kesmen *et al.,* 2007),**cytochrome *b* gene **(Murugaiah *et al.,* 2009),**actin gene, cytochrome oxidase-II gene **(Singh & Neelam, 2011)**, NADH dehydrogenase5/6 **(Unajak *et al.,* 2011)** and the mtDNA control region **(Dooley *et al.,* 2004)**.

This study aimed to evaluate the use of DNA Microarray and RT-PCR for routine identification of meat species in processed meat products. DNA Microarray and RT-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.

Real Time PCR, a DNA based molecular technique, has been very popular in food analyses as a further step of the conventional PCR. It brings away the demand for immunological and electrophoretic methods, and minimizes the risk of contamination during the testing **(Chipron, 2013)**. Real Time PCR has a sensitivity in detection of meat species by 0.1%whereas ELISA can do it less sensitive by 2 %(**Weiss*et al.,* 2010)**.Factors responsible for the popularity of RT-PCR detection assays include rapidity, specificity and enhanced sensitivity of the assays. With regard to the latter, often highly denatured food samples and ingredients can still be processed for RT-PCR detection assays because the DNA may still be reliably amplified, as opposed to loss of processing material in detection methods relying on protein analytical tools **(Santo Domingo & Sadowsky, 2007).**Real Time PCR to our opinion is a very sensitive technique for the identification of species in heat processed meat products.

DNA Microarray offer the distinct advantage of detecting more than one species, twenty four, in one reaction, thus saving time and reducing concomitantly **(Myers *et al.,* 2010)**. DNA Microarray can deliver the results faster and more sensitive using amplified DNA by conventional PCR technique **(Azuka *et al.,* 2011)**.This systems based on the amplification of consensus DNA regions for animal species and the differentiation of the species by species-specific probes that are covalently bound to the surface of the microarrays. The biochip exploits differences within the mitochondrial DNA(mtDNA) of the respective animal species. The high copy number of mtDNA compared to genomic or nuclear DNA makes this locus ideally suited for analysis of highly degraded DNA, arising for example, through denaturing procedures in highly processed food samples. With the Chipron LCD array exploits intra specific differences within the 16S rRNA of mtDNA. This genetic locus has been extensively used in species identification and conservation studies **(Kocher *et al.,* 1989)**. The LCD Array demonstrated a higher sensitivity, with at least 0.1% of all meat species in the analyzed meats detectable. This confirms manufacturer's claims which put detection limits at <0.5% depending on grade of processing. Because of the smaller amplicon sizes generated by the LCD Array (100–125 bp), a bias for more amplification products with the LCD-Array test system could result in the enhanced sensitivity and lower detection limits observed. This suggestion has to be however viewed with some caution as the detection of animal species present in infinitely small concentrations resulting, for example, from contamination during the production process and not from deliberate adulteration of the meat product might not be advantageous in the final analysis.

In addition, DNA Microarray characterized by higher capacity of data analysis, suitability for species detection, re-usability of the results, higher analysis throughput and become user-friendly. DNA Microarray has been widely preferred for understanding mechanisms, detection of food borne

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 inter-actions, biochemical analysis of protein functions and drug development **(Al-Khaldi *et al.,* 2002, Kostrzynska & Bachand, 2006,Pereira *et al.,* 2008, Rasooly & Herold 2008 andBottero & Dalmasso, 2010)**. In the recent years studies in the literature related to DNA Microarray have focused on the detection of adulteration in sea foods and meat and meat products **(Hellberg &Morrissey 2011 and Budak & Dönmez 2012).**

In our study, DNA Microarray was used to determine adulteration in some selected meat products followed by verification by RT-PCR method. It was found that both of the two methods delivered the identical results. Therefore, it was seen that DNA Microarray method is fast, reliable, accurate and safe to introduce as a routine method for identification of meat species in food.

In conclusion, adulteration is a serious food safety and quality issue with an increasing prevalence 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 RT-PCR 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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