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MITOCHONDRIAL CYTOCHROME B GENE AS A TOOL FOR DETECTION OF COMMERCIAL CHEATING OF EGYPTIAN MEAT PRODUCTS WITH PORK

(With 2 Tables and 3 Figures)

By

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(Received at 25/8/2010)

استخدام جين السيتوكروم ب للكشف عن الغش التجارى لمنتجات اللحوم بلحوم الخنزير

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اجريت هذه الدراسة للكشف عن غش منتجات اللحوم الموجودة بالاسواق المصرية بلحوم الخنازير باستخدام تفاعل البلمرة المتسلسل للكشف عن الحمض النووى الخاص بالخنازير باسخدام جين السيتوكروم ب. فقد تم تجميع 42 عينة من منتجات اللحوم من الاسواق المصرية من المفترض خلوها من لحم الخنزير وتم فحصها باستخدام تفاعل البلمرة المتسلسل ولقد اسفرت النتائج عن وجود لحم الخنزير فى 16 عينة بنسبة (38%) من اجمالى 42 عينة وهى خمس عينات لانشون واربع عينات سجق وثلاث عينات فرانكفورتر وعينتان كفتة وعينتان بولوبيف فى حين كانت باقى العينات المختبرة خالية من لحم الخنازيربنسبة (62%). من خلال هذه النتائج نستطيع ان نخلص الى ان اختبار تفاعل البلمرة المتسلسل يعتبر من ادق الاختبارات المستخدمة للكشف عن الغش التجارى لمنتجات اللحوم بلحم الخنازير كما انه اختبار سريع ورخيص نسبيا بالاضافه الى انه يمكن استخدامه فى كل من المنتجات المطهية والغير مطهية. هذا ولقد اوصت الدراسة باستخدام تفاعل البلمرة المتسلسل للكشف عن غش منتجات اللحوم بلحم الخنازير واتخاذ كافة الاجراءات الوقائية للتاكد من ان تكون جميع منتجات اللحوم المعروضة فى الاسواق المصرية حلال (خالية من لحم الخنازير ومشتقاته).

SUMMARY

Pork is a potential source for adulteration of higher value meat such as beef and veal. This is objectionable for economic, religious or health reasons. Hence this study was undertaken for detection of commercial adulteration of meat products with pork meat. Forty two samples of meat products were randomly collected from the Egyptian markets. A procine detection methodology based on polymerase chain reaction (PCR) amplification of specific procine fragment (277-bp procine mitochondrial Cytochrome b gene fragment) was used. The obtained results revealed that 16 samples (38 %) out of 42 samples of processed meat products (5 luncheon, 4 sausage, 3 frankfurter, 2 kofta and 2 corned beef samples) were adulterated with pork meat. From the achieved results in this study it could be concluded that a high percentage (38 %) of processed meat products sold in the Egyptian markets adulterated with pork meat so it is necessary to establish a systematic food screening methodology for detection of pork meat adulteration in the Egyptian meat products to safeguard the consumer rights. PCR method as a rapid, specific, and sensitive, method has been recommended.

***Key words**: Meat products, adulteration, pork, PCR, Cytochrome b gene.*

INTRODUCTION

In food industry, quality control and consumer satisfaction require that the origin of materials used in processed food be labeled in the products. Preparation of meat products by mixing meats and fat of different origin is illegal; this kind of adulteration is common in most countries (Alaraidh, 2008).

Adulteration with foreign meats or improper labeling of meat products as Halal while it contained pork or pork fat clearly interfere with the religious prohibitions for Muslims. This is of particular importance not only for religious reasons but also may impose a potential health risk to peoples with allergies to certain proteins. In addition there are consumers who are intolerant to mutton or sensitive to chicken meat and vegetarians (Ong *et al.,* 2007). Therefore, species differentiation of raw materials used for preparation of meat products and detection of animal species in the final products becomes more important as the society progress in step with science.

Consumers rarely have a problem identifying fresh meat when bought from markets. The characteristic color and shape of beef can be distinguished from pork meat whereas processed meat like sausage,

luncheon and canned foods, poses a problem as these products can not be identified by naked eyes.

After killing of pigs bred in Egypt, consumers in Egypt are afraid from addition of pork meat to the rest of the meat products sold in stores, particularly after the announcement of the slaughtering huge numbers of pigs who arrived so far to nearly 250 thousand pigs.

Currently species identification had been achieved through different available methods rely mainly on protein analysis (Morales *et al.,* 1994) developed an indirect enzyme–linked immunosorbent assay (ELISA) to quantitavily determine raw pork adulteration in beef and chicken. In the same way, Martin *et al.* (1998) developed a radial immunodiffusion test and ELISA to quantify pork adulteration in raw ground beef.

However proteins loose their biological activity after animal death, and most of them are heat labile and get denaturated by cooking. Thus for species identification and quantification in processed food a DNA method rather than protein analysis would be preferable (Calvo *et al.,* 2001). This method have been proved to be more accurate, fast, easy to use and could work with cooked products because even after subjecting to heat during cooking, the DNA would still be amenable to PCR amplification (Gurdeep.Rastogi *et al.,* 2004).

Some techniques for DNA testing have been developed to identify the species in meat products, such as CAPS (Cleavable Amplified Polymorphic Sequences) (Meyer *et al.,* 1994), Southern blotting by using species-specific satellite DNA as probes (Hunt *et al.,* 1997), RAPD (Random Amplification Polymorphic DNA) fingerprinting (Martinez and Yman 1998; Saez *et al.,* 2004), multiplex PCR (Matsunaga *et al.,* 1999), quantitative intra-short interspersed element PCR (Walker *et al.,* 2003); sequencing the specific gene such as the mitochondrial 12S rRNA (Girish *et al.,* 2004).

Processed meats are likely to contain degraded DNA, therefore mitochondrial DNA (mtDNA) was considered more suitable than nuclear DNA for this analysis. The cytochrome b gene on the mitochondria has been used successfully in species identification and in taxonomic and phylogenetic (Hsing-Mei Hsieh *et al.,* 2005).

In this study, a PCR based methodology was used for detection of pork meat in processed meat products bought from commercial markets in Egypt.

MATERIALS and METHODS

Collection of Samples:

Forty two samples of processed meat products, seven samples from each product, were collected from Egyptian markets, these samples were labeled as beef luncheon, sausage, kofta, frankfurter, corned beef, and minced meat. In addition to 3 control samples labeled as pork sausage and luncheon (control positive) obtained from a supermarket selling pork products in Cairo and beef minced meat control negative was obtained from the abattoir of the Faculty of Veterinary Medicine Suez Canal University, Ismailia, Egypt. Sampling was performed monthly over 6 months period from September 2009 to February 2010. All samples were wrapped in polyethylene bags and transported in an icebox to the Animal Wealth Development Laboratory at Suez Canal University, Ismailia, Egypt where they were analyzed.

DNA extraction:

DNA was extracted using high salt method (http://sciencepark.mdanderson.org/mbcore/protocols.html). Two grams of the frozen tissue were homogenized in 600 µl of TNES buffer for 10–15 s. Then 35 µl of 20 mg/ml proteinase K were added and mixed well. The samples were incubated at 50°C overnight, after which 166.7 µl of 6 M NaCl (NaCl saturated H2O) was added to each sample. Samples were vortexed for 30 s at maximum speed, and then the samples were Microfuged at full speed (12-14,000 rpm) for 5-10 minutes at room temperature. The supernatant was transferred to fresh tubes. An equal volume of 100% ethanol was added to each sample, mixed well, and samples were incubated at –20°C for 1 h. Samples were then centrifuged at full speed (12-14,000 rpm) for 10-20 minutes at 4ºC. The supernatant was poured off and the pellet was washed with 70% ethanol, dried and finally resuspended in 100-200 µl of sterile distilled water or Tris-EDTA. The DNA concentration was measured by absorbance at 260 nm.

Oligonucleotide primers:

Two primer sequences, sense (F) and antisense (R), were designed from the published DNA sequence of pork mitochondrial Cytochrome b (accession no. AY830188). The primers sequences are shown in Table 1.

PCR and agarose gel electrophoresis:

DNA (200 ng) was amplified using 2X PCR master mix (Fermentas, Egypt) composed of 0.05 units/µl Taq DNA Polymerase in reaction buffer, 4 mM MgCl2, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP. PCR was carried out in a final reaction volume of 25 µl containing the DNA sample (1µl), forward and reverse primers (1µl each), PCR master mix (12.5 µl) and distilled water (9.5 µl). PCR done using TC-25/H thermal cycler, The cycle conditions for PCR were as

follows: initial denaturation at 95°C for 10 minutes followed by 40 cycles of 95°C for 45 sec, 50° C for 45 sec and 72° C for 90 sec, then 72° C for 10 minutes for further extension (Hsing-Mei Hsieh *et al.,* 2005). In order to confirm the amplification of the target sequence and the pork specifity of the oligonucleotide pork, the PCR product was eleectrophoresed on 1% agarose gel of 0.5x TBE buffer and made visible by staining with ethidium bromide at a constant voltage of 100 for 1 hour. The resulting fragments were visualized by UV transillumination (Slimeline^{TM} series).

RESULTS

Table 1: Oligonucleotide primers used in PCR amplification of pork mitochondrial cytochrome b.

Primer	Nucleotide Sequence	Nucleotide location
F R	5'- TACTATTCTCACCAGACCTACT-3' 5'- TAGTGTAATGAGGTCTGCTACT-3'	728-749 984-1005

Table 2: Frequency distribution of pork meat in the examined meat products samples.

Meat products(N=49) 7 from each product	Positive samples (adulterated with pork meat)		Negative samples (not adulterated)	
	F	%	F	%
Luncheon	5	11.9	2	4.8
Sausage	4	9.5	3	7
Kofta	2	4.8	5	12
Frankfurter	3	7	4	9.5
Corned beef	2	4.8	5	12
Minced meat	0	0	7	16.7
Total	16	38	26	62

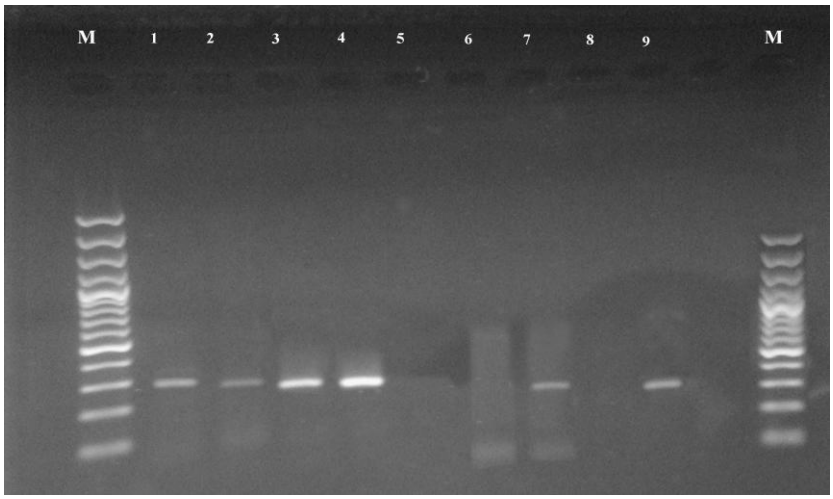

Fig. 1: Gel electrophoresis showing the detection of the specific 277 bp porcine mitochondrial Cytochrome b gene in the examined meat products from the first market. Lane 1: kofta, lane 2: luncheon, lane 3: pork suasage, lane 4: pork launchon, lane 5: luncheon, lane 6: minced meat, lane 7: sausage, lane 8: frankfurter, lane 9: corned beef, lane 10: beef minced meat.

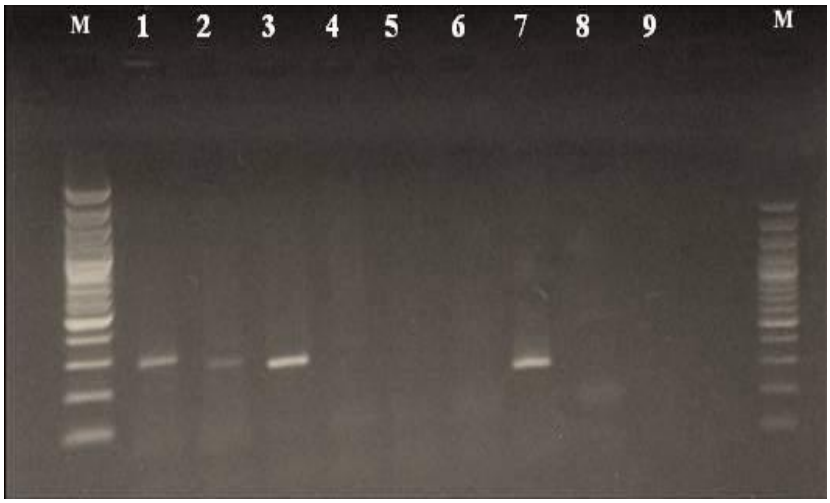

Fig. 2: Gel electrophoresis showing the detection of the specific 277 bp porcine mitochondrial Cytochrome b gene in the examined meat products from the second market. lane 1: corned beef, lane 2: kofta, lane 3: pork suasage, lane 4: luncheon, lane 5: sausage, lane 6: minced meat, lane 7: frankfurter, lane 8: frankfurter and lane 9 beef minced meat.

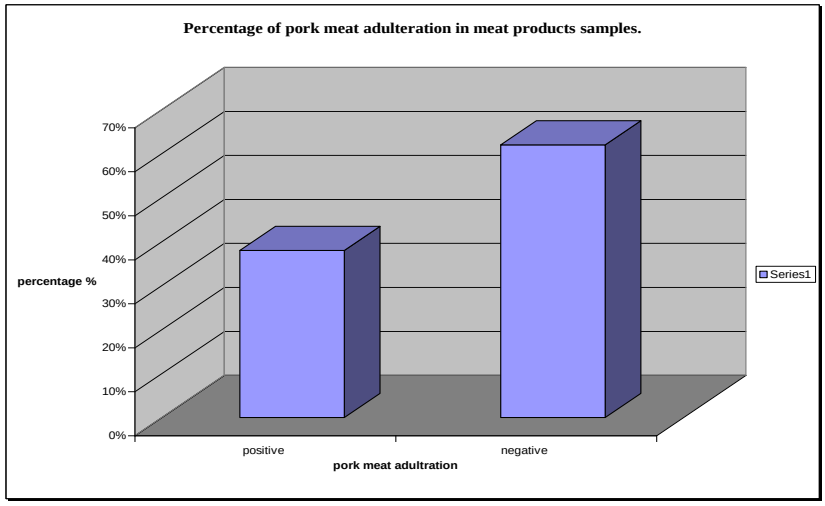

Fig. 3: Percentage of pork meat adulteration in meat products samples

DISCUSSION

Food products have been adulterated since food is sold, species that achieve a good price in the market have been a target for deceivers to substitute or adulterate with similar but cheaper species. In the case of meat products this is objectionable. Identification of pork in meat products has several important applications in the food industry, not only to detect falsely labeled products but also for economic, religious and health reasons.

Egypt's 80-million populations consist mainly of Muslims, whose religion forbids them from eating pork meat, as well as an estimated six to 10 percent Christian Copts who may eat pig meat.

For the identification of animal species, it is preferable to detect DNA; DNA carries an organism's total genetic information and has to be stable **.**It is identical in all cell types of an organism; therefore it does not matter whether the DNA is extracted from blood, muscles, liver or any other tissue. The information content of DNA is greater than that of the protein, for these reasons protein based methods are being replaced by methods based in nucleic acid (Brodman, 2002).

Polymerase chain reaction offers analytical approach based on nucleic acid to identify species. (Kocher *et al.,* 1989) have shown that some highly conserved regions on the mitochondrial Cytochrome b gene are suitable for species identification. PCR technology allows amplification of specific regions of DNA facilitating the detection of genetic differences between species (Brown, 2001; Abdel Rahman and Ahmed, 2007).

In this study, the amplification of the species specific 277-bp procine mitochondrial Cytochrome b gene fragment using specific primers showed specific detection of pork meat in the examined samples.

The two oligonucleotide primers (forward and reverse) were used to amplify the species-specific 277-bp porcine mitochondrial Cytochrome b gene fragment showing that these primers are able to detect porcine adulteration in non-pork food products as established in Figure 1. The fragments are shown in lane 1, beef kofta, lane 2, beef luncheon, (pork suasage sample in lane 3 and pork luncheon sample in lane 4, as positive controls) beef sausage sample in lane 7 and corned beef sample in lane 9. Whereas lanes 5, 6, and 8 containing other meat products (beef luncheon from another company, minced meat, and frankfurter respectively) in addition to minced meat sample lane 10 (negative control) did not show any existence of the porcine mitochondrial Cytochrome b gene fragment.

Figure 2 shows the amplification of the 277- bp porcine mitochondrial Cytochrome b gene fragment in beef samples collected from another market, the fragments are shown in lane 1, corned beef, lane 2, beef kofta, lane 3, "pork suasage", lane 7, beef frankfurter' whereas lanes 4, 5, 6, and 8 containing beef luncheon, beef sausage, minced meat, and frankfurter from different sources respectively did not show any existence of the porcine mitochondrial Cytochrome b gene fragment. Lane 9 minced beef control negative.

The choice of the target gene and the design of the primers have a great impact on the sensitivity and the specifity of a detection system (Soichi Tanabe *et al.*, 2007). It is well known that very sensitive PCR assays can be established when the primer target is a multicopy gene such as a mitochondrial gene (Holzhauser *et al.*, 2006). In this study the porcine Cytochrome b region of mitochondrial DNA as the target to detect pork meat adulteration in commercial meat product has been chosen.

The obtained results as shown in Figure 3 revealed that high percentage (38 %) of the examined meat products samples from Egyptian markets contains pork meat. This is attributed to the decision taken by the Egyptian government to slaughter all swine herds in an effort to prevent swine flu spreading, since the appearance of swine flue in May 2009. Estimates of the number of slaughtered pigs during this period ranged from 250,000 to 400,000. This huge numbers explains the fear of consumers in Egypt form mixing of pig meat with meat products.

The results shown in Table 2 revealed that the positive samples which adulterated with pork meat were luncheon (11.9%), sausage (9.5%), kofta (4.8%), frankfurter (7%) and corned beef (4.8) whereas the examined samples of minced meat were negative (not adulterated by pork meat). The

comminuted meat products could easily mixed with different types of meat and this could not be detected by naked eye.

The existence of pork meat in meat products makes it a potential health threat to consumers due to the microbial and parasitic infections transmitted through pork, non-communicable diseases attributed to the consumption of pork, risk factors in relation to chronic degenerative diseases, in addition to hazards in the pig breeding establishments (http://www.firstchurchoftheinternet.org/pdf/PigMeat.pdf). The most important and definitely proven hazards connected with the consumption of pork are the two zoonotic parasites trichinellosis and systemic cysticercosis. Both infections can be life threatening and their prevention requires difficult measures. (Schenone, *et al.*, 1982). Of the non-communicable diseases attributable to pork consumption (food) allergy and liver cirrhosis have been shown to occur (Nanji *et al.*, 1985).

Also, consumption of pork and lard can give rise to hyperlipidaemi constituting a risk factor in cardiovascular diseases (WHO, 1982). Furthermore, high pork and lard consumption in a low fiber diet would have a correlation with high incidence of cancer of the colon (http://www.firstchurchoftheinternet.org/pdf/PigMeat.pdf).

These facts highlight the need to establish a systematic food screening methodology for detection of pork meat adulteration in the Egyptian meat products. The study also demonstrates the need for more vigilant inspection and stricter enforcement of applicable public health laws by food monitoring authorities to prevent illegal addition of pork meat to other meat products.

It is apparent from the obtained results that the use of simple DNA test is of particular value in identifying the animal species used in processed meat and in investigating the situation about the fraudulent misdescription of food contents on product labels in Egyptian markets.

In summary, a PCR method could be applicable for the specific detection of pork in processed food. Since this method is rapid, specific, and sensitive, and the information from this test could support the law enforcement to decrease the fraudulent misdescription situation.

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