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Improved DNA Extraction Method for Porcine Contaminants, Detection in Imported Meat to The Saudi Market

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Abstract

A porcine detection methodology based on deoxyribonucleic acid (DNA) extraction and polymerase chain reaction (PCR) amplification of a specific porcine fragment was used in this paper. With the advent of mass globalization and the fast growing and rapidly changing halal industry of the international market it is of vital need that a practical scientific system be applied and established in the Kingdom of Saudi Arabia to detect and monitor the ingredients in food especially porcine contaminants. A simple modified technique was designed to extract DNA from food material. ASL buffer (Qiagen) for lysis and a series of incubation steps were applied prior to PCR and detection limits were established. The optimized method was then used to identify pork in food products obtained from different local hypermarkets. The used method was displayed to be robust and reliable. Out of thirty-three food samples labeled as *halal*, there were two imported samples; one beef steak and one beef sausage that were positive for porcine contaminants. From the results it can be concluded that sufficient PCR ready DNA can be obtained by this single solution methodology. Although the incubation for the lyses is recommended to be overnight the cost of the experiment and the relative simplicity and reliability of the experiment allows a mass food testing ability and a viable commercialization opportunity in the Kingdom. It is also of absolute importance that a systematic scientific precaution be taken to ensure that imported food products in Saudi Arabia are actually *halal*, as Allah says in the Holy Qur'an: "Forbidden to you (for food) are: *Al-Maitah* (the dead animals - cattle - beast not slaughtered), blood, the flesh of swine" (Sûrat Al-Mâ'idah, Verse 3).

Key Words: Porcine, DNA, PCR, Primer, Processed meat, Detection, *halal.*

Introduction

Preparation of meat products by mixing meats and fats of different origin is illegal. This kind of adulteration is a common procedure in most countries. Adulteration with cheaper ambiguous meats or improper labeling of meat products as "*Halal*" while it contained pork or pork fat clearly interferes with the religious prohibitions for Muslims around the world. Permissible nature (*Halal*) of any product including food, accessories and relationships is of particular importance for Muslims, whether in moral or physical life, particularly in the current era of globalization, where a single processed food product could be subject to elements sourced from several nations with its origin doubted or neglected due to ignorance or lack of technology. Therefore, in the current era where free trade and mass market globalization is dominating, certain food producers take advantage of every opportunity to increase profits and neglect proper food safety protocols with respect to religious or cultural beliefs. Hence, several studies are currently being carried out in the field of food detection systems.

Especially with regard to pork in processed or non-processed food products, various methodologies that are equally effective and reliable in their relevant approaches are currently used for detection systems.

For instance the detection of pork and lard as adulterants in processed beef and mutton mixtures is carried out where the mixture of derivative and saturated triglycerides is analyzed by liquid chromatography using a reverse-phase column and a UV detector. Pork fat has larger amounts of triglyceride containing saturated fatty acid at the C-2 position than does the fat of other meat. The ratio of triglyceride containing saturated fatty acid vs. triglyceride containing unsaturated fatty acid at the same (C-2) position

(SSU/SUS) in a sample is compared with those of pure meats. The presence of pork in the sample causes the ratio to increase compared with ratios for pure beef or mutton. The increase in the SSU/SUS ratio is significant for the addition of 1% pork in beef. In the case of mutton, the addition of 3% pork causes a noticeable change (Saeed*, et al* ., 1989).

Detection of pork by PCR amplification of nuclear 18S ribosomal RNA and growth hormone gene or Y chromosome has been previously described (Meyer *et al*., 1995 and Meyer *et al*., 1994). In other methods, the use of fluorescence sensor capillary electrophoresis allows identification of specific DNA fingerprint species of pork, goat, and beef generated by restriction enzyme digestion using fluorescence–labeling PCR amplification (Al-Rashood *et al*., 1995). Specific detection of processed pig and cattle materials treated at 134 degrees Celcius in various feed matrices down to a limit of detection of about 0.1% was reported (Fumière *et al*., 2006). The detection quantification of bovine, porcine, lamb, chicken, turkey, and ostrich DNA in complex samples was achieved by using TaqMan real-time polymerase chain reaction (PCR) systems using minor groove binding (MGB) probes. Species-specific amplification was achieved by combining only two fluorogenic probes and 10 oligonucleotide primers targeting mitochondrial sequences. The limits of detection ranged from 0.03 to 0.80 pg of template DNA. Analysis of experimental mixtures containing two to four different species showed the suitability of the assay for detection of more than 1% of pork, chicken, or turkey and of more than 5% of cattle or lamb. The quantification accuracy in samples containing 10-100% of beef or pork DNA was close to 90% (López-Andreo *et al*., 2005; Hunt, 1997; Janseen, *et al*., 1998; Montiel-Sosa, *et al*., 2000; and Partis, *et al*, 2000). In a latest research, 3 food samples (2 chocolates and one chicken nugget) were tested positive for containing the 152bp fragments of the porcine leptin (Farouk, *et al*., 2006).

PCR based methodology, used in this publication where pig-specific primers were used to amplify a 152 bp fragment from the procaine leptin gene that is the homologues of murine obese, is a successful technique of identification. As it is PCR-based, it is the most convenient for critical samples in which DNA is largely degraded such as with processed food (Farouk, *et al*., 2006; Stratil, *et al.*, 1997; Wintero*, et al*., 1990, and Wolf, *et al*., 1999).

The main target of the work was to develop a fast, cheap and reliable molecular technique in foods and to check foods bearing "*Halal*" tags for porcine contamination by a simple species-specific band identification of PCR products in the Saudi market.

Materials and Methods

Meat Samples

Thirty-three samples of frozen meat (beef steaks, beef sausage, minced beef, beef roast, turkey sausage, chicken sausage, chicken nuggets and chicken balls) were purchased from nearby hypermarkets.

DNA isolation

Frozen meat was thawed and grinded. 30 milligrams of the grounded meat were transferred into 1.5 ml Eppendorf tube. 200 µl ASL buffer (Qiagen) was added into the individual tubes containing the sample material. The mixtures were vortexed for 5 minutes and incubated overnight for sufficient lysis to maximize DNA extraction. After incubation the samples were vortexed for 2 minutes and centrifuged at full speed (13,000 *rpm*) for 15 minutes using the Biofuge Pico, Germany to remove any solid particles. 40 µl of the supernatant was transferred into a new Eppendorf tubes and stored at -4^{o}C for subsequent PCR application.

DNA Quantification

The concentration and purity of the extracted DNA were measured by absorbance at 260 nm using the Anthelie series UV - visible spectrophotometer (Secoman, France). 10 µl of DNA sample was diluted in 990 µl of nuclease-free water (Promega) for PCR. For both the DNA extraction with the Qiagen kit and the microwave extraction the DNA extracted was measured to be in the range of 1000-1500 ng.

PCR Mix

DNA (200 ng) was amplified using puRe Taq™ Ready-To-Go™ PCR Beads (Amersham, USA). In a final volume of 25µl, each reaction contained 2.5 units of puRe Taq™ DNA polymerase, 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, 1.5 mM $MgCl_2$, 200mM dATP, dCTP, dGTP and dTTP, and stabilizer, including BSA. Amplification was performed using 20 pmol of each forward and reverse primer on a thermalcycler (Mastercycler Gradient, Eppendorf, Germany). The forward primer was 5'–TGCAGTCTCTCCTCCAAA-3' and the reverse primer was 5'–CGATAATTGGATCACATTCTG-'3 (Meyer *et al*., 1995). The amplification was run using the following program: 94^{o}C for 3 minutes to denature the template DNA completely, followed by 35 cycles at 94^{o}C for 1 minutes, 55^{o}C for 1 minutes, and 72^{o}C for 1 minute, and closed by the extension step at 72^{o}C for 7 minutes. The amplified DNA was determined by gel electrophoresis (Electrophoresis,

Power Supply EPS 301, amersham Pharmacia biotech) in 1.7% agarose gel of 1x TBE buffer and made visible by staining with ethidium bromide at a constant voltage of 70 for 2 hours. The resulting fragments were visualized by UV transillumination (MacroVue UV-20 Hoefer, Germany).

Results

The gel electrophoresis suggests that by just using ASL buffer for lyses to isolate DNA from meat samples was reliable and subsequently DNA primers could be used to amplify the species-specific 152-bp porcine leptin gene fragment. Showing that DNA primers are able to detect leptin gene in pork samples, the next stage of work aimed at finding out the possibility of using the same protocol to detect porcine contaminants in non-pork food products – as established in Figure 1. The amplification of the 152bp porcine leptin gene fragment as shown in lane 2 "processed pork", beef sausage sample in lane 4 and beef steak sample in lane 7, illustrates the successful detection of pork contamination or elements in food samples. Whereas lanes 3, 5 and 6 containing other meat products did not show any existence of the porcine leptin gene fragment (Fig.1).

Discussion and Conclusion

In a previous work (Farouk *et. al,* 2006) the use of the commercial *Qiagen* Stool Kit (Germany) was an easy, rapid and reliable method to obtain sample extracts suitable to be used for the study of DNA by PCR assays. There are PCR inhibitors in the processed food products having high levels of proteins and fats, which is why different protocols were explored and tested for the preparation of DNA that remove all PCR inhibitors or reduce them in the DNA sample. However the current method of using just the ASL buffer for the lyses step and eventual PCR- ready DNA is cheaper than the use of various different reagents in the commercial kit and faster than the use of the conventional methods.

Comparable to the current methodology another reagent

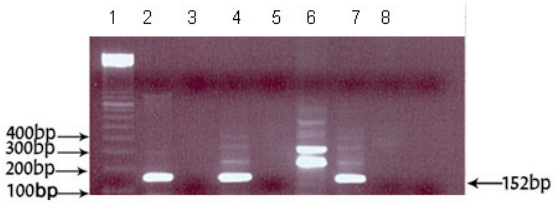

Fig 1. Gel electrophoresis showing the detection of the specific 152bp leptin gene fragments in processed food products. Lane 1: 100bp molecular marker, Lane 2: Pork Sausage, Lane 3: Chicken Nuggets, Lane 4: Beef Sausage (Brand A), Lane 5: Chicken Sausage, Lane 6: Turkey Sausage, Lane 7: Beef Steak (Brand B), Lane 8: negative control.

DNAzol® Direct is a universal reagent for processing biological samples for direct PCR. It is manufactured by the Molecular Research Centre Company (Cincinnati, USA). The DNAzol Direct procedure is simple; where a sample is lysed in DNAzol Direct for 15 minutes, and the resulting lysate used in PCR and amplification of a selected DNA fragment(s) is performed (Mackey, *et. al*., 1997).

According to the manufacturer's protocol the standard DNAzol Direct procedure supports PCR amplification of DNA fragments up to 8-kb long. The patent-pending DNAzol Direct composition and procedure are based on the use of an alkaline solution containing polyethylene glycol and other additives (Mackey, *et. al*., 1996). DNAzol Direct quickly and effectively lyses biological samples, releasing DNA into the lysate. The combined effects of the alkaline pH and chaotropic properties of DNAzol Direct sufficiently inactivate PCR inhibitors including proteases and nucleic acid degradation enzymes. After processing a sample in DNAzol Direct, DNA is denatured into a single-stranded form, RNA is hydrolyzed, and proteins are denatured and partially hydrolyzed. Due to its unique composition, the DNAzol Direct lysate does not require neutralisation before its use in PCR. The resulting pH of a PCR mix containing less than 10% of the lysate is within the effective range for PCR which is between 8-8.4. (Chomczynski, *et. al*.,1997). Thus, from the above references it is of no doubt that single solution reagents can be used for PCR ready DNA.

In conclusion, the detection method, which uses minute DNA quantities and resources, has been established thus, so far out of the food samples tested, 2 samples have been tested positive shown by the amplification 152-bp fragment of the porcine leptin gene. Although this specific methodology using PCR is well known, however it is of utmost importance that this strategy and experiment was successfully done in Saudi Arabia. In addition it is vital to note that this porcine contaminant discovery is just a small quantity from the thousands of different food products in the Kingdom and thus more food has to be tested in future research endeavors and a systematic food screening methodology be established.

This should initiate more systems where various religious, political, educational and scientific bodies of the Kingdom of Saudi Arabia work together to create an awareness program for its citizens with regard to the religious and R&D aspects of the food being consumed. Furthermore specialized initiatives could be implemented to share this technology and knowledge to the higher schools of the Kingdom to develop and spark the talented young minds of the Kingdom to create and foray into the area of food technology to establish the Kingdom as a platform of a

Halal-Hub for the Middle East and the Muslin World.

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استخدام طريقة محسنة لفصل الـ DNA من أجل الكشف عن مشتقات لحم الخنزير في منتجات اللحوم المستوردة للسوق السعودي

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الملخص

في هذا البحث تم اعتماد طريقة للكشف عن مشتقات الخنزير في منتجات اللحوم المستوردة للمملكة العربية السعودية، تعتمد هذه الطريقة على فصل الـ DNA, ثم استخدام طريقة تفاعل سلسلة إنزيم البوليمريز ((PCR للكشف عن قطع الـ DNA الخاصة بالخنزير. مع القدوم المتسارع للعولمة، والنمو السريع والمتغير في مجال صناعة الطعام الحلال في السوق العالمي، أصبحت هناك حاجة ملحة لتأسيس وتطبيق نظام علمي، وعملي في المملكة العربية السعودية لرصد، وكشف محتويات الطعام خصوصاً مشتقات الخنزير. تم تصميم تقنية معدلة، وسهلة لفصل الـ DNA من العينات الغذائية. تتضمن هذه الطريقة حضن العينة فيASL BUFFER من شركةQiagen لسلسة من الفترات المختلفة من اجل إنحلال جدر الخلايا، ثم استخدام الـ DNA المستخلص في تفاعل سلسلة انزيم البوليمريز ((PCR من اجل تضاعف الـ DNA المراد كشفه. هذه الطريقة المثلى تم استخدامها للكشف عن مشتقات لحم الخنزير في منتجات الطعام المستوردة، التي تم شراؤها من الأسواق المحلية الكبيرة. تعتبر هذه الطريقة مضمونة ويمكن الاعتماد عليها للكشف عن مشتقات لحم الخنزير.تم فحص 33 عينة تم شراءها من الأسواق الكبرى في المملكة العربية السعودية من المفترض ان تكون خالية من مشتقات الخنزير. وجد ان عينتين هي: (1) ستيك عجل, و(1) نقانق عجل تحتوي على مشتقات لحم الخنزير.من خلال هذه النتائج نستطيع أن نخلص إلى أن الـ DNA المستخلص في هذه الطريقة يكفي لاستخدامه في الـ PCR. بالرغم من انه يوصى بحضن العينات خلال الليل لتفكيك جدران الخلايا من أجل استخلاص الـ DNA, إلا أن رخص تكلفة التجربة، والبساطة النسبية، والموثوقية، وكذلك إمكانية إجراء عدد كبير جدا من الاختبارات في وقت قصير، مما يسمح بالاستفادة منها تجارياً في المملكة العربية السعودية. إن من الأهمية بمكان اتخاذ كافة الإجراءات الوقائية العلمية للتأكد من ان تكون جميع المنتجات الواردة للمملكة العربية السعودية حلال، لان أكل لحم الخنزير محرم في الشريعة الإسلامية، كما قال تعالى: «حُرِّمَتْ عَلَيْكُمُ الْمَيْتَةُ وَالدَّمُ وَلَحْمُ الْخَنْزِيرِ.....» (سورة المائدة, آية 3).