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Market surveillance on non-halal additives incorporated in surimi based products using polymerase chain reaction (PCR)–southern hybridization analysis

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Abstract

In Malaysia, halal certification status for some surimi-based product is still suspicious due to the incorporation of non-halal plasma protein additives as part of the food ingredient. This study was conducted to determine the presence of plasma protein additives that have been incorporated into surimi-based product using Polymerase Chain Reaction (PCR)-Southern Hybridization method which able to differentiate 7 type (beef, chicken, duck, goat, buffalo, lamb and pork) of species on a single chip. A random of 17 (n = 17*3) different brands of surimi-based product was purchased from Selangor local market in January 2013. Of 17 brands, 3 (n = 3*3) brands were positive for chicken DNA and 1 (n = 1*3) brand was positive for goat DNA, while remainder 13 brands (n = 13*3) has no DNA species detected. In presence study, it is evidence that PCR-Southern Hybridization analysis offered a reliable result due to its highly specific and sensitive properties in detecting plasma protein incorporation in surimi-based product.

Introduction

Islam has been the official religion and the largest practiced religion in Malaysia. In the year 2010, the Department of Statistics Malaysia has stated that, 61.3% of the Malaysia population comprised of Islam religion. Life based on Islam is strongly emphasized to the hygiene concept concerning foods, drinks, feeds, pharmaceuticals and cosmetics. It has become one of the most important issues discussed globally when relating Halal quality and safety in a product. In most cases, the Muslims could determine whether a product is permissible (*halal*) or prohibited (*haram*) based on the explicit injunctions of the Islamic Laws. However, the industrialization of food processing in the late centuries has seen an improvised technology which has replaced the traditional method of production. This at once has caused a great concern among many Muslims in determining products which are permitted or not under the Islamic law.

According to Okada (1992), the term surimi refers to the protein concentrate myofibrils extracted from fish meat by mechanical separation, washed with water and mixed with a cryoprotectant. Today, surimi food has been popularized not only in Japan but also in many other countries due to its unique texture and its high nutritional value (Park and Morrissey, 2000). It has been estimated that about 315.800 million tons of surimi products have been produced in the Southeast Asian region in 2005 (Laong and Siriraksophon, 2007).

Gel strength was most commonly used to assess the qualities of surimi as a crude ingredient for analog. Surimi has several functions including gelling, binding and emulsifying characteristics that may be used as a functional protein ingredient in some products (Alina, 2012). A three dimensional protein gel network formation is essential for surimi production. According to Park (2005), network was formed by cross-linkage between actin and myosin of fish muscle proteins, which solidifies during heat treatment. Surimi gelling is a process that involves decomposition and aggregation of protein. During heat treatment, the protein breaks down exposing the reactive clusters. On exposure, there will be formation of bonds between the neighboring protein molecules. When sufficient bonding force have been obtained, a series of three-dimensional will be created producing gel (Lanier, 2000). Gelatin hydrocolloid product was initially used for its specialty and uniqueness in serving multiple functions with a wide range of applications in various industries including food ingredient as gelling, foaming agent, thickener, plasticizer, emulsifier, foaming agent, moisture retention, improve texture and binding agent (Sahilah *et al*., 2012). Gelatin is considered the best gelling

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protein, but it may cause weakening of myofibril protein gelatins in surimi products (Sun and Holley, 2011). As an alternative, some protein additives have been used in surimi and surimi based products to improve the characteristics of the gel as well to reduce protein degradation caused by endogenous proteinases.

Protein supplements have been widely used to increase surimi gel strength. It functions as a proteinase inhibitor and / or to increase surimi gel (An *et al*., 1996; Alina *et al*., 2012). Protein is normally used as an additive due to its ability to improve surimi gel structure formation and inhibit proteolytic action enzyme when compared with concentrated as well as other types of carbohydrates that are widely used (Piestrasik and Li-Chan, 2002). Whey protein, egg whites, plasma protein and soy protein isolates are several types of proteins that are widely used (Benjakul *et al.*, 2004). Recently, blood from animals and treated products of slaughtered animals which is known as cheap protein supplement is used as emulsifiers, stabilizers, clarifiers, or food components to enhance the properties of the food (Rawdkuen *et al.*, 2004).

Plasma proteins are a complex mixture of over 100 different proteins such as albumin (60%), globulin (35%) and fibrinogen (4%), where the serum is the major protein involved in the formation of heat-induced gels (Elena *et al*., 2012). Without prejudice to the smell, taste and color of the final product, plasma protein has been used in surimi production. In fact, the use of plasma protein produces better gel when compared to low-quality raw materials (Marquez *et al.*, 1997). This is because plasma proteins act either as an inhibitor of proteolytic enzymes and increase the protein-protein interactions to increase the capacity of 3-dimensional network formation (Benjakul *et al*., 2004). Use of this blood protein in food products is considered 'Haram' to Muslims. Not by just concerning the 1.6 billion Muslims population acceptance and suitability, the halal standard is as well said to be practice by the non-Muslim community (Sahilah *et al.*, 2012).

Food grade enhancer such as Bovine Protein Plasma (BPP), Pig Protein Plasma (PPP), Egg White (EW) and potato powder may be used in surimi production (Lee *et al*., 2000; Benjakul *et al.*, 2001). A research conducted by Benjakul and Visessanguan (2000) found that PPP successfully inhibits proteolysis of Pacific proteinase whitening activity and surimi autolytic activities. Besides PPP, Chicken Protein Plasma (CPP) is able to increase the gel strength by acting as surimi gel filler in the matrix as well as proteinase enhancer. Blood plasma can increase or decrease myofibril protein gel strength, and this differs with different meat products (Sun and Holley, 2011). To avoid the misuse of halal certification, some scientific methods have been developed for the detection of additional materials such as plasma proteins in surimi based food products. Several techniques for detection have been done based on deoxyribonucleic acid (DNA) such as electrophoresis (Kim and Shelef, 1986), High Pressure Liquid Chromatography (HPLC) (Ashoor *et al.*, 1998), Polymerase Chain Reaction (PCR) (Calvo *et al.*, 2001) and Species-Specific Polymerase Chain Reaction (Herman, 2001). In the present study, we examined the presence of different DNA species incorporated in surimi based products in addressing halal status for quality assurance and lifestyle choice using PCR-southern hybridization analysis.

Materials and Methods

Samples for analysis

A total of 17 (n = 17*3) surimi based product were purchased from local market in area of Selangor in January 2013 and stored in 4°C prior to use. The true brand was not mentioned here; instead batch number of the brands was assigned (P1 to P17).

DNA extraction

The DNA extraction was done using Qiagen DNeasy Blood and Tissue Kit (Germany) commercial kit as described by the manufacturer in the guidebook supplied. Each labeled brand was masticated individually and triplicate was performed for each brand by random pick. DNA was extracted and stored at -20°C prior to use. The concentration and purity of the extracted DNA was determined using Implen Nanophotometer.

PCR amplification

The working primers used in this research were obtained from Olipro™ Meat ID PCR kit (OLIPRO, MY). A total of eight primers were incorporated in the master mix reaction of the Olipro™ Meat ID PCR kit. The designed primers specifically target the mitochondrial DNA of cytochrome *b* of each species. A valid PCR conducted is determined by the presence of internal control. Beef (Bovine), chicken (*Gallus gallus domesticus*), duck (*Anatidae*), goat (*Capra hircus*), buffalo (*Bison bison*), lamb (*Ovis aries*) and pork (*Sus scrofa*) were among the species that was able to be detected using the kit.

A final volume of 50 µL for the PCR reaction was taken place which includes: 22.7 µL of Olipro™ Meat ID MasterMix, 0.5 µL Taq DNA Polymerase

(Promega, Research Biolabs, MY), 1 µL Internal Control DNA template, 10 µL of DNA template (depending to DNA concentration at 25 ng/µL) and 15.8 µL of Nucleases Free Water (NFW). The Eppendorf thermocycle profile consist of 95°C for 3 min, 45 cycles of 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec, with a final extension at 72°C for 5 min. Negative controls (NFW) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination.

PCR products were visualized on a 2.5% (w/v) agarose gel in 0.5X TBE buffer at 180 V for 30 min and stained by ethidium bromide. A 100 bp DNA ladder (Fermentas, MY) was used as size reference. The gels were visualized using a Kodak Gel Imager (Kodak GL200).

Southern hybridization analysis

The amplicons (amplified PCR products) were denatured to single stranded at 95°C for 10 min before proceeding to hybridization. Denatured product is placed on a cold block immediately to avoid formation of double stranded DNA. Southern-hybridization was performed according to Olipro™ Meat ID Hybridization kit protocol. A total of 400 µL of Reagent A will be pipette out into every chip. 10 µL of denatured amplicons will then be mixed onto the Reagent A liquid according to chips assigned and incubated at 68-70°C in a hybridization oven at maximum vibration for 1 hour. The chip will undergo a series of washing step to form colorimetric development on the membrane and dried at 37°C for 5 min.

Interpretation of data on biochip

Results obtained from the hybridization method were analyzed through Olipro™ Scanner. Figure 1 indicates the morphology of the chip where the formation of the internal control (IC) spot shows that the PCR amplification and hybridization was successful. Reading on the scanner should give a 'grey value' count that exceeds 2.5 to verify the sample was positive.

Results and Discussion

A total of 17 (n = 17*3) surimi brands were successfully extracted using the Qiagen DNeasy Blood and Tissue Kit. Sampling was done in triplicates for each brand in order to control the validity of the results obtain and to rule out experimental bias or some random error. High quality of intact pure DNA is very important in many scientific applications. During analysis, the probability of getting an accurate result is depending on the quality of DNA concentration (Sahilah *et al.*, 2012). The observation showed that there is a relatively large range of concentration distribution of samples ranging from 0.5 to 500 ng/µL. The intensity of DNA in a gel differ where high intensity of DNA will result in thicker band while low intensity produce faint bands (Sahilah *et al.*, 2012). Olipro™ Meat ID Gene Chip was developed with the ability to detect at a specifications and sensitivity of 1 ng/µL.

Table 1. Surimi based product tested for meat DNA.

Samples	Detected Positive for Meat DNA	Detected Negative for Meat DNA	Total
Surimi Based Products	4	13	17
Total (%)	23.53	76.47	100

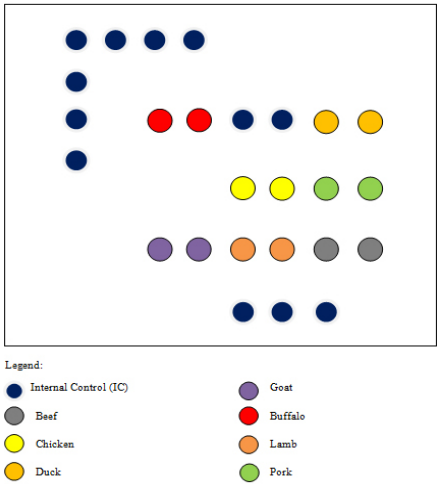

Figure 1. Olipro™ Meat ID Gene Chip morphology.

The Olipro Bitotechnology Meat ID kits that has been used, have the ability to detect a total of 7 known species of the gene sequence encoding the mitochondrial cytochrome *b* for *Bos taurus* (202 bp), *Gallus gallus domesticus* (206 bp), *Anatidae* (311 bp), *Capra hircus* (175 bp), *Bison bison* (565 bp), *Ovis aries* (390 bp), *Sus scrofa* (265 bp) and Internal control (439 bp). The internal control that was used in the kit was derived from genes of leguminous plants. The plant gene was used as internal control as to avoid cross-reactivity between animal species studied. Positive results for each PCR carried out can be determined by reference to an agarose gel band with a sequence size of 439 bp. As tabulated in Table 1, out of the 17 (n = 17*3) brands that were examined, 4 (n = 4*3) brands showed positive for the presence of meat DNA and the remaining 13 brands (n = 13*3) showed negative for the presence of meat DNA. 3 brands labeled P6, P8 and P12 indicates the presence of cytochrome *b* that codes for chicken (*Gallus gallus domesticus*) and 1 brand labeled P9, showed the presence of cytochrome *b* for goat (*Capra hircus*). This may be identified with reference to the band size 206 bp and 175 bp on Figure 2 and Figure

Table 2. The 'grey value' reading of tested sample.

Brands	Internal Control	Beef	Chicken	Duck	Goat	Buffalo	Lamb	Pork
P1 (i)	32	-	-	-	-	-	-	-
(ii)	26	-	-	-	-	-	-	-
(iii)	21	-	-	-	-	-	-	-
P2 (i)	30	-	-	-	-	-	-	-
(ii)	34	-	-	-	-	-	-	-
(iii)	35	-	-	-	-	-	-	-
P3 (i)	41	-	-	-	-	-	-	-
(ii)	34	-	-	-	-	-	-	-
(iii)	30	-	-	-	-	-	-	-
P4 (i)	28	-	-	-	-	-	-	-
(ii)	42	-	-	-	-	-	-	-
(iii)	38	-	-	-	-	-	-	-
P5 (i)	33	-	-	-	-	-	-	-
(ii)	36	-	-	-	-	-	-	-
(iii)	38	-	-	-	-	-	-	-
P6 (i)	38	-	15	-	-	-	-	-
(ii)	45	-	12	-	-	-	-	-
(iii)	38	-	11	-	-	-	-	-
P7 (i)	40	-	-	-	-	-	-	-
(ii)	52	-	-	-	-	-	-	-
(iii)	46	-	-	-	-	-	-	-
P8 (i)	47	-	18	-	-	-	-	-
(ii)	35	-	19	-	-	-	-	-
(iii)	45	-	18	-	-	-	-	-
P9 (i)	43	-	-	-	19	-	-	-
(ii)	40	-	-	-	17	-	-	-
(iii)	38	-	-	-	19	-	-	-
P10 (i)	40	-	-	-	-	-	-	-
(ii)	32	-	-	-	-	-	-	-
(iii)	35	-	-	-	-	-	-	-
P11 (i)	44	-	-	-	-	-	-	-
(ii)	44	-	-	-	-	-	-	-
(iii)	37	-	-	-	-	-	-	-
P12 (i)	42	-	20	-	-	-	-	-
(ii)	38	-	18	-	-	-	-	-
(iii)	35	-	20	-	-	-	-	-
P13 (i)	40	-	-	-	-	-	-	-
(ii)	47	-	-	-	-	-	-	-
(iii)	30	-	-	-	-	-	-	-
P14 (i)	50	-	-	-	-	-	-	-
(ii)	49	-	-	-	-	-	-	-
(iii)	33	-	-	-	-	-	-	-
P15 (i)	47	-	-	-	-	-	-	-
(ii)	50	-	-	-	-	-	-	-
(iii)	75	-	-	-	-	-	-	-
P16 (i)	52	-	-	-	-	-	-	-
(ii)	63	-	-	-	-	-	-	-
(iii)	49	-	-	-	-	-	-	-
P17 (i)	55	-	-	-	-	-	-	-
(ii)	53	-	-	-	-	-	-	-
(iii)	54	-	-	-	-	-	-	-

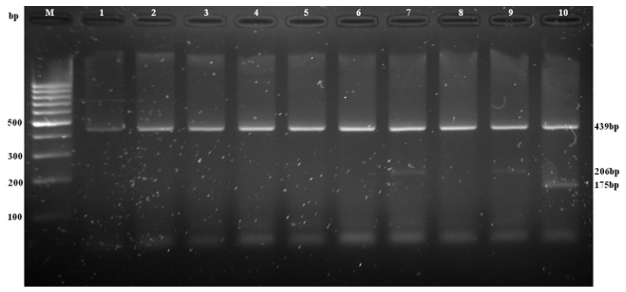

Figure 2. Gel electrophoresis of PCR analysis using Olipro™ Meat ID PCR kit. Lane M: Ladder Marker (100 bp), Lane 1: Negative Control (NC), Lane 2 – 10: Brand P1 to P9.

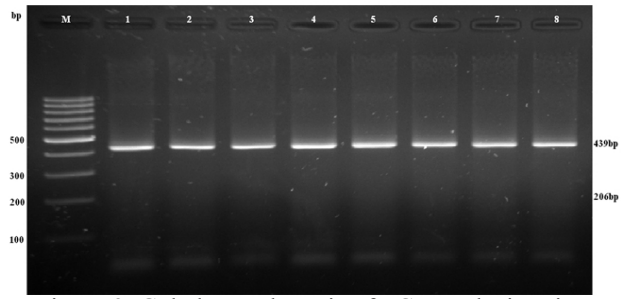

Figure 3. Gel electrophoresis of PCR analysis using Olipro™ Meat ID PCR kit. Lane M: Ladder Marker (100 bp), Lane 1 – 8: Brand P10 to P17.

3 of the agarose gel when compared with the 100 bp marker that were used.

The formation of spot on the membrane at the chicken and goat probes location has provided a conformation relating to the agarose gel reading obtain. The reading of 'grey value' for the detected sample is as tabulated in Table 2. The consistency of 'grey value' of the triplicate reading for each positively detected brands from Table 2 indicates that there are high chances that protein plasma was added in surimi based food product in a very low concentration to ensure gel formation. In general, too much of protein plasma may cause a decrease in gel strength (Eakpetch *et al*., 2008). The formation of gel was able to increase the gel strength by acting as filler in surimi gel matrix as well a proteinase inhibitor during surimi production. Characteristics of protein plasma in improving the texture of surimi that has undergone high pressure treatment and frozen condition have make it as the main ingredient used by many manufacturers for quality improvement.

Conclusion

Many studies have been conducted in detecting the presence of additives based on beef and pork. However, the current study has proven that the use of alternatives source of other protein source such as chicken and goat. This shows the awareness of surimi manufacturers in current issues associated with surimi and finds an alternative to shroud from certain parties. As such, it is recommended that the authorities monitoring the surimi based food product marketed in Malaysia take more stern action to combat fraud in usage of non-halal additives and giving away the Halal certification. In conclusion, the PCR-southern hybridization analysis is useful in identifying non-halal DNA species in surimi based product, thus determining the Halal status of the products and to curb abuses of halal certification.

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