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Halal market surveillance of soft and hard gel capsules in pharmaceutical products using PCR and southern-hybridization on the biochip analysis

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Abstract: The study was conducted to detect the porcine DNA in pharmaceutical products in local market using polymerase chain reaction (PCR) and southern-hybridization on the biochip. A total of 113 (n=113) of hard (82 samples) and soft gel (31 samples) capsules from pharmaceutical products were purchased and tested for the presence of porcine DNA for Halal authentication. All capsules were gelatin-based purchased from local over the counter (OTC) markets. Of all samples tested, 37.2% (42/113) contained porcine DNA. While, none porcine DNA band was detected for 62.8% (71/113) of capsules tested. All samples which were positive toward porcine DNA were imported pharmaceutical products with none Halal logo. Results in the presence study demonstrated that the PCR techniques and southern-hybridization on the biochip is suitable tool for monitoring the Haram component in highly processed product of soft and hard capsule.

Keywords: Halal market surveillance, soft and hard gel capsules, pharmaceutical products, PCR and southern-hybridization on the biochip analysis

Introduction

Scientifically and empirically based Halal certification process would give Malaysia a unique edge globally to conform the highest qualities of processed foods and non-food products (pharmaceutical, cosmetic, leather goods and others) viewed from religious aspect of fulfilling Muslim's obligations and business perspectives. To increase Malaysian participation and dominance in the global Halal industry, innovating Halal products and their standards will best position Malaysian Halal certification (Malaysian Halal Certificate) as 'Halal Malaysia for the World' to pave worldwide acceptance of Halal products. The Halal standard is not only suitable and acceptable for 1.6 billion Muslims but also for the non-Muslim community.

Gelatin is one of the widely used raw materials in foods, pharmaceutical (gelatin capsules) and cosmetic products (creams, facemasks, lotions), which could be extracted from bones, fat, meat waste, used cooking fats and oils of animals. There are a few types of gelatins, and the most preferred ones are from porcine and bovine source (Morrison *et al*., 1999; Karim and Rajeev, 2009; Sahilah and Aminah, 2010). Gelatin is a hydrocolloid product which are special and unique, 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. Due to these characters, gelatin is widely use in dairy and bakery products especially in ice creams, yogurt, cheese and cakes (Karim and Rajeev, 2009). Besides that gelatin is also applied in other food industries of jelly desserts, gummy jelly, chocolate, ice-cream, marshmallow, soft candy, toffees, chewing gum, butter , meat products and pet food. In fitness product, gelatin has been used due to its easily digestible, low in calories and contains no cholesterol. In pharmaceutical industry, gelatin is used as hard and soft capsules, sugar-coated pills, tablets, serum substitute and vitamin encapsulation. The use of gelatin in pharmaceutical is invitable because it helps to protect the medicines against harmful influences, such as light and oxygen. The soft capsules for instance are mainly used for liquid fillings, while hard capsules are used for powders. Thus, the use of gelatin is an all rounder. Chances of Muslims exposed to haram gelatin is becoming greater (Sahilah and Aminah 2010).

Although the food products are strictly monitored during Halal certification applications through Jabatan Kemajuan Islam Malaysia (JAKIM) that complied with Halal standards and integrity, unfortunately there are no such requirements in non-food products such as pharmaceuticals. No study has been reported on the Halal market surveillance in pharmaceutical products. Identification of porcine DNA contents in food or pharmaceutical products is possible using

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polymerase chain reaction (PCR) amplification of a segment of the mitochondrial cytochrome b (Partis *et al*. (2000), Lenstra *et al*. (2001), Aida *et al*. (2005); Kesmen *et al*. (2007); Chandrika *et al*. (2009); and Sahilah *et al*. (2011)). In this study, we extracted the DNA from capsules (hard and soft gels) and conducted the PCR analysis in attempt to detect the porcine DNA in the capsule samples for Halal authentication. Further confirm was done using southern-hybridization on the biochip.

Materials and Methods

Samples for analysis

A total of 113 (n=113) of hard (82 samples) and soft (31 samples) capsules from pharmaceutical products were purchased from local over the counter (OTC) market in area of Selangor in June 2009 to July 2010. The true brand was not mentioned here, instead of that we put batch number of the samples for hard and soft capsule namely, B1 to B113. The products were from local and oversea companies.

DNA extraction

The DNA of soft gel capsules and cosmatics were extracted using DNA using QIAGEN Purification Kit as provided by the manufacturer. A total of 50-100 mg soft gel was grinded (for the hard capsule) and minced (soft capsule) in a 1.5 ml sterile microfuge tubes. Quality and quantity of purified DNA was determined using spectrophotometry analysis. The DNA was stored at -20°C until used as PCR templates.

PCR amplification

The primers used in this work were obtained from PCR OLIPRO porcine kit (OLIPRO, MY). The primers were designed for targeting the mitocondrial DNA of cty b and positive results were indicated by single band of 276 bp. Amplification of was performed in a final volume of 50 µl as provided by the manufacturer. Each reaction mixture contained 50 µl volume containing 25.0 µl of Dream *Taq* Master Mix (Fermentas), 1.0 µl of 5 µM each primer (Forward and reverse for porcine DNA), 14.0 µl of nucleas free water (NFW) and 10 µl of 50 ng DNA template. A negative-DNA control was performed by adding 3 µl of NFW and a positive control was performed by adding 3 µl of the known porcine DNA sample. PCR was carried out in Eppendorf thermal-cycler (Eppendorf, Germany) with a temperature program consisting of the initial denaturation at 95°C for 5 min to complete denature the DNA template, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds at 55°C, polymerization at 72°C for 30 seconds and final elongation at 72°C for 5 min. Negative controls (water) were included in each PCR amplification, in order to verify the PCR efficiency and to detect contamination. The amplification products were analyzed by electrophoresis using 2.5% agarose gel in 1X TAE buffer (40 mM Tris-OH, 20mM acetic acid and 1mM of EDTA; pH 7.6) at 60 V for 60 minutes and stained by ethidium bromide. A 100 bp DNA ladder (Vivantis, MY) was used as size reference. The gels were visualized using UV transilluminator (AlphaImager™ Gel Documentation). As comparison the, all PCR amplification products were also tested using Biofood Mixed Kit (Biotools, Spain) as provided by the manufacturer. The kit is PCR based detection by amplifying of a cytochrome *b* region.

Multiplex PCR amplification for southern-hybridization analysis

Three primers were used in this work were supplied with OLIPRO Porcine Gene Biochip (OLIPRO, MY). The primers were designed for targeting the mitocondrial DNA of *cty* b and actin gene. Positive results were indicated by band of 276 bp and 205 bp, respectively. The internal control DNA of biochip was also supplied which produced a band of 195 bp. Amplification was performed in a final volume of 50 µl except the multiplex primers were added, 1.0 µl of 5 µM each primer (Forward and reverse for porcine DNA, actin and internal control DNA supplied by OLIPRO, MY.) and the volume of nuclease free water (NFW) was adjusted according to the final volume.

Southern-hybridization analysis

The amplification PCR products (amplicons) were denatured at 95°C for 10 min and placed into ice immediately. The hybridization was carried out using OLIPRO Porcine Gene Biochip (OLIPRO, MY) at the specific temperature as provided and mentioned in the protocols. The amplicons were mixed with reagent A on the chips and incubated at 70°C in hybridization oven at maximum vibration for 1 hour. The chips were then washed to form colorimetric development on the chip according to the manufacturer instruction. The chips were rinsed with reagent G and dried into oven at 37°C for 5 minutes. The chip is readied for scanning and recorded immediately after completed.

Interpretation of data on biochip

The OLIPRO Porcine Gene Biochip contained specific target probe for *cty* b and actin. Positive result for actin indicated sample contains animal

DNA. Results showed positive for *cyt* b and actin or positive to only *cyt* b indicates sample contains porcine DNA. While, positive for internal control indicates PCR and hybridization reaction were successful.

Results and Discussion

Concept of Halal (permissible) is a religious believed and global issue developments related largely to those under Muslim society which concern to Halal quality and safety of foods, food products, feeds, pharmaceuticals and cosmetic. The Muslim communities would like to know whether or not the ingredients or the finished food products contain any Haram (impermissible) substance. Thus, the market surveillance for Haram component was conducted on pharmaceutical products in the local markets, to view the quality assurance of Halal for customers.

A total of 113 hard and soft gelatin-based pharmaceutical products and cosmetics were analyzed using PCR technique. As tabulated in Table 1, of 113 samples 37.2% (42/113) gave positive results for porcine DNA which indicated positive towards *cyt* b. While, 62.8% (71/113) gave negative results for porcine DNA as tabulated in table 2. Figure 1 is an example of positive porcine DNA samples which produced a band of 276 bp in size (using PCR OLIPRO porcine kit).

Table 1. Hard and soft gel gelatin-based capsule tested for porcine DNA in the samples.

Samples	Positive for porcine DNA	Negative for porcine DNA	TOTAL
Hard and soft capsules	42	71	113
Total (%)	41 (37.2)	71 (62.8)	113 (100)

The intensity of DNA extracted from capsule samples were different where the high intensity of DNA showed thicken band while faint band indicated low intensity of DNA (Data not shown). Due to this reason, the southern–hybridization analysis using OLIPRO Porcine Gene Biochip was conducted and found that this technique was sensitive where the faint band appeared in the agarose gel were visible on the chip (Figure 2 and Table 2). As shown in Figure 2, the PCR for southern–hybridization on the biochip analysis indicated capsul which was positive toward porcine DNA produced a band of 276 bp in size with internal control (IC). The IC produced a band of 195 bp in size. In all experiments conducted no actin band was observed. This could be probably due to the copy number of nuclease DNA (nDNA) was low or none could be detected in the capsule sample comparing to mitochondria *cyt* b DNA (MtDNA) which was reported 100 to 1,000 per cell or more (Monteil-Sosa *et al*., 2000). Since the actin was gene detected on nuclease DNA this may explain why the PCR amplification product of actin gene was not appeared on the agarose gel in each experiments.Table 2 showed the two methods (PCR OLIPRO porcine kit and southern–hybridization on the biochip). yielded similar results which showed positive DNA porcine.

Table 2. The detection of porcine DNA in hard and soft gel gelatin-based tested

Batch no.	Brief description of the capsule content	Type of capsule	Positive to *cyt* b gene		Southern-hybridization analysis using Porcine Gene Biochip
			PCR OLIPRO porcine kit	Biofood Mixed Kit	
B1	Calsium, Magnesium and Zinc	Soft	+	-	+
B2	Omega-3	Soft	+	-	+
B3	Vitamin E-200	Soft	+	-	+
B4	Drug	Hard	+	-	+
B5	Steroids	Soft	+	-	+
B6	Fibric Acid derivatives	Hard	+	-	+
B9	Fish oil	Soft	+	-	+
B18	Herb	Hard	+	-	+
B20	Anti-inflammatory	Hard	+	-	+
B26	Drug	Hard	+	-	+
B28	Sulpiride	Hard	+	-	+
B30	Doxymycin	Hard	+	-	+
B34	Drug	Hard	+	-	+
B42	Drug	Hard	+	-	+
B45	Ginseng	Hard	+	-	+
B46	Drug	Hard	+	-	+
B48	Plant derived	Hard	+	-	+
B58	Vitamin E	Soft	+	-	+
B62	Drug		+	-	+
B63	Metanamic acid	Hard	+	-	+
B64	Bittergourd seed	Hard	+	-	+
B71	Drug	Hard	+	+	+
B72	Drug	Hard	+	+	+
B73	Drug	Hard	+	+	+
B76	Ginseng	Hard	+	-	+
B78	Herb	Hard	+	-	+
B81	Drug	Hard	+	-	+
B82	Drug	Hard	+	-	+
B84	Garlic	Soft	+	-	+
B85	Slimming purposes	Hard	+	+	+
B86	Slimming purposes	Hard	+	-	+
B88	Drug	Hard	+	-	+
B89	Vitamin D3	Hard	+	-	+
B90	Drug	Hard	+	+	+
B91	Drug	Hard	+	-	+
B92	Drug	Hard	+	-	+
B93	Drug	Hard	+	-	+
B97	Herb	Hard	+	-	+
B98	Drug	Hard	+	-	+
B104	Drug	Hard	+	-	+
B107	Ursodeoxycholic acid	Hard	+	-	+
B112	Nutrients and antioxidants	Hard	+	-	+

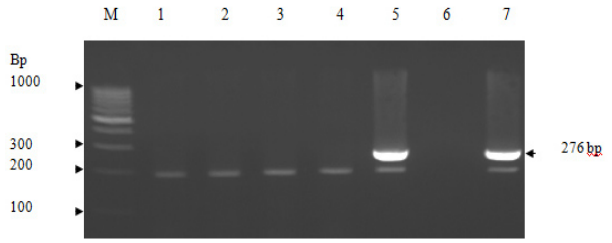

Figure 1. Gel electrophoresis of PCR analysis for porcine DNA detection using PCR OLIPRO porcine kit. Lane M; Marker (100 bp ladder); Lane 1: Negative control; Lane 2-4: Sample negative with porcine DNA; Lane 5: Sample positive with porcine DNA; Lane 6: No sample was loaded and Lane 7: Positive control.

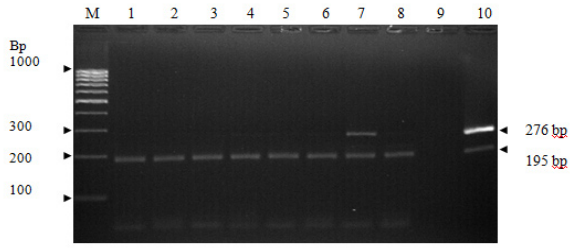

Figure 2. Gel electrophoresis of PCR analysis for porcine DNA detection using PCR OLIPRO southern–hybridization on the biochip. Lane M; Marker (100 bp ladder); Lane 1: Negative control; Lane 2-6 and 8: Sample negative with porcine DNA and positive internal control (IC) indicated by band 195 bp in size; Lane 7: Sample positive with porcine DNA and positive IC; Lane 9: No sample was loaded and Lane 10: Positive and IC control.

However, the PCR and biochip sensitivity levels of both techniques were different where 1 ng and 0.1 ng of genomic DNA per sample, respectively. The biochip has shown more sensitive toward porcine DNA amplified gene due to its specific probes targeted *cyt* b on the biochip. Thus, the biochip showed the greatest level of sensitivity.

Capsules which were positive (42 capsules) for porcine DNA using OLIPRO Porcine Gene Biochip was also tested using Biofood mixed Kit (Biotools, Spain) as a comparison. Biofood mixed kit is PCR based kit adapted for homogeneous samples. Comparing with PCR OLIPRO assay, similar (1 ng DNA) sensitivity level of genomic DNA per sample was detected with Biotools mixed kit. Of 42 samples analyzed using Biofood mixed kit only 9.5 % (4/42) samples were detected (Table 2) positive for porcine DNA which showed the PCR amplification product of 398 bp in size. The results showed here although both methods is based on PCR techniques, the detection level of those kit was different which Biotool mixed kit showed low sample numbers when compared to PCR OLIPRO kit (Table 2). It is difficult for us to draw any conclusion why the sensitivity of both method different. Reliable and sensitive methods to detect porcine DNA in food and non-food products are necessary in determining Halal authentication of highly processed food such as hard and soft capsule gelatin-based. Though PCR possess has been shown rapid and simple method, confirmation through southern-hybridization on the biochip is adviceable for Halal standard and integrity since, the OLIPRO Porcine Gene Biochip demonstrated higher sensitivity and specificity for porcine DNA detection. Both methods (PCR and southern-hybridization on the biochip) were very useful if they could be applied in combination as results showed in this finding.

It was noticed that all (42 capsules) of the capsule which were positive to porcine DNA were supplied by oversea manufacturers and none of them were certified with Halal logo from JAKIM. There has been increasing level of awareness concerning for Halal pharmaceuticals, cosmetics or personal care products, which is being driven by increased consumer knowledge of the ingredients used, ways of productions and alternative consumers' choice of selection. Due to this finding, the pharmaceutical products which are imported from oversea is suggested to be monitored by JAKIM or other authority agencies resolve in the similar issue in Malaysia.

Conclusion

In conclusion, hard and soft gelatin-based capsules associated with DNA porcine were easily detected using PCR approaches though its sensitivity level was not shown greater compared to southern-hybridization on biochip. Both methods are suitable tool and could be used in combination to obtained precise results for monitoring the Halal integrity to customers.

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