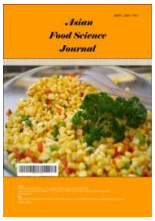

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Halal Food Safety: PCR Based Detection of Porcine DNA in Imported Chocolate

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Authors' contributions

This work was carried out in collaboration among all authors. Author GMSU designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors KA and MK managed the analyses of the study. Author SM managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Halal food and food products consumption is a major part of living in Muslim community. Pork meat or meat items are not considered halal in Muslim countries and consumers. Ensuring pork-free food items is a challenge for the food industry and exporters to Muslim nations. This study aims to detect porcine DNA in imported chocolate products in Bangladesh for halal safety assurance. The imported chocolate samples were collected from various multi shops in Dhaka. Polymerase chain reaction (PCR) method is used in our research to detect the porcine DNA. Two primer sets are used for the detection of porcine mitochondrial cyt-b (cytochrome-b) gene fragments in chocolate samples. To visualize the amplified DNA, agarose gel (1%) was used. After electrophoresis, DNA band in agarose gel indicated that the gene fragments are amplified properly. In our research, out

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of 42 chocolate samples, only 2 samples were found positive. The chocolate samples were branded as Wild Berry Flavor Chocolate and Cadbury Milk Tray Chocolate. In comparison with the positive pork sample, these two samples also containing the 165bp and 359bp fragment of the porcine cytochrome b gene. We reported that chocolate products contain the pork contamination were not labeled as halal. While other samples that did not have any halal logo originated from outside Bangladesh and imported also showed negative result. The present study established the DNA-based porcine detection system based on mitochondrial cyt-b that is viable in highly processed products. It can be used in the halal certification process to determine the pork items presence in food and halal safety. Our research also reported that imported chocolates should halal certify before release into the market.

Keywords: Porcine DNA; Polymerase Chain Reaction (PCR); cytochrome b gene; chocolate; halal.

1. INTRODUCTION

Studies have found that Muslims are the most conservative and often obeyed with the Sharia law [1,2]. According to the Muslim beliefs, pork and pork items are considered contraband to eat. So, the halal trademark on consumer goods is used as a sign of Shariah-compliant goods and is trusted by 1.8 billion Muslim consumers of the world [3,4]. The projected yearly business of Halal food sector is over US $661 billion and will be booming in the upcoming days [4]. To cope up with market competition and to realize unwarranted return, fraudulent tagging of Halal brand is frequently occurring also [5,6,7,8,9,10,11]. Moreover, in the globalization era, a single processed food can contain ingredients sourced from a dozen nations and its origin is not questioned due to lack of technology. So, food producers tried to use elements that are cheap and prohibited, such as pork, contraband in Muslim consumers. Pork is a potential contaminant in halal and satisfactory foods because of the convenience at low-cost than those of Halal (allowable) meats [11,12,13]. To halt this trend most countries in the world have regulatory bodies to ensure transparency in food labeling [12,14,15,16]. According to the European Commission (EC) legislation (178/ 2002) on food safety [17], manufacturers are obliged to clearly label all raw materials used in the preparation of food products.

Among many consumed foods, chocolate is the most popular sweet delicacy in the world and in Bangladesh. Majority of the market share of chocolate is occupied by imported foreign branded chocolates. So, there might have porcine mixture in the chocolate. Unfortunately, there is no established molecular identification system of pork adulteration in processed chocolates yet in Bangladesh. So, we selected chocolate as our research sample for identification of pork contamination. There are a number of methods that have been published on the detection of pork or its derivatives in Halal meat products [6,7,8,9,10,12,18,19,20,21]. Few methods have also been used for the detection and quantification of adulterated meat products [6,22,23] such as, the detection of species-specific proteins [24] and ELISA [25], HPLC [6,26]. However, the performance of these methods are not tested in commercial samples. In this case, molecular techniques are the best option to identify the prohibited materials commercially. For example, the contamination of pork can be detected by PCR or RT-PCR method. Several studies already reported the detection of porcine DNA by PCR method [12,27-32]. A fragment of the mt-DNA 12S rRNA gene, a simple band of PCR products, has been used to identify the pork derivatives in sausages, casings, bread, biscuits [33]. A streamlined DNA extraction method and a quantitative SYBR Green quantitative polymerase chain reaction (SyG-qPCR) assay were combined to form a ready-to-use kit for rapid detection of porcine admixtures processed meat products [34]. Besides, species-specific primers to selectively amplify a short fragment (109 bp) of porcine cytochrome b gene were used to identify processed ternary mixture of pork, beef, and wheat flour [35]. Cytochrome b gene is a genetic marker and species specific and not easily degraded in processed food sample. Therefore, we use the gene specific primers to amplify 165bp and 359bp mitochondrial cytochrome b (Cyt-b) gene. It is a suitable identification technique for critical samples where DNA is largely degraded such as processed food. For the first time in Bangladesh, by the research we have detected porcine DNA in imported chocolate samples using PCR method. It is noted that, for halal certification, this PCR methods may use for the identification of porcine DNA in processed chocolate.

2. MATERIALS AND METHODS

2.1 Collection of Food Sample

The chocolate samples were collected from various multi store shop in Dhaka region and categorized by randomly numbering from S_1 to S_{42} (sample -1 to sample -42). The Brand names, Product type, Country of origin, Email/Web addresses are mentioned in the Table 1. In addition, laboratory-prepared porcine and bovine gelatins were used as control.

2.2 Extraction of DNA

DNA from the samples was extracted using Wizard® magnetic DNA purification kit for food (Promega, USA) following referred protocol (Instructions for products use FF3750 and FF3751). The extracted DNA were stored at 4°C before further use. For each sample DNA was extracted for three times and the highest concentrations of isolate were used for PCR assay.

2.3 Quantification of DNA

The DNA was quantified using fluorimeter kit (QuantiFluor® dsDNA System, Promega Corporation, USA) by fluorimeter (Fluorimeter, E6150 Promega Inc, USA). For quantification a standard DNA sample (2 µl std DNA, 98 µl 1X TAE buffer & 100 µl dye), test samples (2 µl sample DNA, 98 µl 1X TAE buffer & 100 µl dye) and blank (100µl 1X TAE buffer & 100µl dye) were prepared. Quantities of isolated DNA are given in Table 2. Each sample was measured for three times.

2.4 Primer Collection

The primers were taken from two published paper which were targeted for PCR of porcine Cytochrome-b gene; (Set-A): F3 primer (5-TCGAGACGTAAATTACGGATGAG-3), R3 primer (5-GGATCCGTAGTATAGACCTCGG-3) [19], (Set-B): CYTb1 Forward primer (5'-CCATCCAACATCTCAGCATGATGAAA-3') and CYTb2 Reverse primer (5'-GCCCCTCAGAATGATATTTGTCCTCA - 3') [36].

2.5 PCR Amplification Process

The PCR assay was conducted by PCR machine (Astec Co. Ltd, GeneAtlas G2, Japan). Then 20 µl reaction mixture consisted of 1µl of forward and 1µl reverse primers, 10µl of Master Mix (Promega, Madison, WI USA), 8 µl of template DNA was carried out for PCR assay. The PCR program was initiated at a pre-denaturation step at 95°C for 5min, followed by 35 cycles of denaturation at 95°C for 30s and annealing temperature was set by calculating the Tm temperature of forward and reverse primer to lower 5°C was 47°C (for set-A) 60°C (for set-B) for 30s adjusted with a slow heating of 72°C for 10s (for set-A primer) and 24s (for set-B primer). Finally, the elongation part was done in 72°C for 5 minutes. For each sample by using two set of primer the reaction was carried out three times.

2.6 Gel Electrophoresis of PCR Products

The PCR product was used for gel electrophoresis assay. For this work 1% agarose gel was prepared by using agarose powder (Carl Roth GmbH. Co. KG). 0.5 gm agarose weighted to add 50 ml 1X TAE buffer (Carl Roth GmbH. Co. KG) and melt it by hotplate magnetic stirrer. Ethidium bromide 6µl was added to mix thoroughly. Then it was poured into casting tray with comb and allowed to solidify. The ladder was added, positive sample & tested samples into the well of gel. Gel was run at constant voltage (100 V) until band separation occurs. DNA band viewed by UV light box and results showed.

3. RESULTS

The identification of porcine DNA was done using mitochondrial Cytocrome-b gene primer by conventional PCR method in the chocolate samples. The specificity of this PCR detection system was evaluated by isolating DNA from fresh pork meat and used as a positive sample to compare with the 43 chocolate samples. A range of temperatures for amplification using the PCR method was from 50 to 60°C, and the optimal temperature for primers attachment was 59°C. Porcine primer combinations were evaluated in the PCR detection system to assess the presence of pork DNA in the chocolate samples. Results demonstrated on agarose gel showed that the amplification of PCR was done successfully, when the band of desired amplicon size was appeared in the gel image. Pork DNA was observed in the two chocolate samples out of 42 samples, which were mentioned in the Figs. 1and 2 of gel electrophoresis.

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Table-1. The product collected from different shops, brand name, product type, country of origin and website available on the product cover

SN	Brand name	Product type	Country of origin	Website/ Email address
S1	Choc Coin	Chocolate	Thailand	Not available
S2	Amul Milk Chocolate	Chocolate	India	customercare@amul.coop
S3	Ritter Sport	Coconut & Milk	Germany	www.ritter-sport.com
S4	Tayas	cream filling	Turkey	www.tayas.com.tr
S5	Belgian	Dark chocolate	Belgium	www.thebelgian.com
S6	Nestle Kitkat	Chocolate	UAE	www.nestlecocaplan.com
S7	Cadbury Bournville	Dark chocolate	UK	www.cadbury.co.uk
S8	Toblerone	dark chocolate	Switzerland	www.toblerone.com
S9	Lindt Assorted Napolitains	premium chocolate	Switzerland	www.lindt.com
S10	LindtLindor	Dark chocolate	Switzerland	www.lindt.com
S11	Hershey's	Milk chocolate	Malaysia	www.askhershey.com
S12	Cadbury Wispa	cadbury chocolate bar	UK	www.cadbury.co.uk
S13	Cadbury fuse	chocolaty feast	Mumbai, India	suggetions@mdlzindia.com
S14	Cadbury Sandwich Snack	Milk chocolate	UK	www.cadbury.co.uk
S15	m&m chocolate	Chocolate	France	contactchoc-za@mars.com
S16	Cadburry dairy milk	Chocolate	Mumbai, India	suggetions@mdlzindia.com
S17	Nestle black magic	Dark chocolate	UK	www.qr.nestle.co.uk
S18	Nestle Aero Bubbles	Incredi bubble chocolate	UK	www.aerochocolate.co.uk
S19	Snickers	Chocolate	Russia	www.planetmarsme.com
S20	Nestle Kitkat Chunky	Peanut butter	UK	www.nestlecocoaplan.com
S21	Nestle Dairy Box	Milk chocolate	Spain	www.qr.nestle.co.uk
S22	Ritter Sport Hazelnuts	Chopped hazelnuts	Germany	www.ritter-sport.com
S23	GizoChokoku	Chocolate	Indonesia	www.gizitas.com
S24	Cadbury Twirl Bites	Milk chocolate	UK	www.cadbury.co.uk
S25	Elit Love Chocolate	Milk chocolate	Turkey	info@elitcikolate.com.tr
S26	Ritter Sport Fine	Dark chocolate	Germany	www.ritter-sport.com
S27	Cadburry dairy milk	Chocolate	Mumbai, India	suggetions@mdlzindia.com
S28	LindtHellow	Milk chocolate	Switzerland	www.lindt.com
S29	Ferrero Rocher	milk chocolate	Italy	Customercare.india@ferrero.com
S30	Elit 1924	Chocolate fruit & nuts	Turkey	info@elitcikolate.com.tr
S31	Cadbury Gems	Gems chocolate	Mumbai, India	suggestions@mdlzindia.com
S32	Milka	Milk chocolate	Portugal	www.milka.com
S33	Storck Merci	Chocolate	Germany	www.merci.com

SN	Brand name	Product type	Country of origin	Website/ Email address
S34	Lindt	Dark chocolate	Switzerland	www.lindt.com
S35	Belgian	Dark chocolate	Belgium	www.thebelgiun.com
S36	Cadbury Bournville	Chocolate	Mumbai, India	suggestions@mdlzindia.com
S37	Cadbury Milk Tray	Chocolate	UK	www.cadbury.co.uk
S38	Skittles	Wild berry flavor chocolate	UK	www.mars.co.uk
S39	Bounty	Chocolate bar	South Africa	www.mars.com
S40	Coted'or	Chocolate	Nederland	www.mondelezinternational.nl
S41	Cream & Fudge Hazelnut	Hazelnut Ice-cream	UAE	Not Available
S42	Bellissimo	Strawberry ice-cream	Italian	www.bellissimo.bd.com

Table 2. Quantity of isolated DNA from chocolate samples

Sample Number	Food type	Quantity of DNA (ng/2µl)	Sample No	Food type	Quantity of DNA (ng/2µl)
SP	Positive Pork DNA	0.377 ng	S23	GizoChokoku	0.0597 ng
S1	Choc Coin	0.00 42 ng	S24	Cadbury Twirl Bites	0.0127 ng
S2	Amul Milk Chocolate	0.0044 ng	S25	Elit Love Chocolate	0.0253 ng
S3	Ritter Sport	0.0011 ng	S26	Ritter Sport Fine (Extra Dark Chocolate)	0.0362 ng
S4	Tayas (Magic)	0.0019 ng	S27	Cadburry dairy milk Chocolate (large size chocolate)	0.0148 ng
S5	Belgian	0.0021 ng	S28	LindtHellow	0.0177 ng
S6	Nestle Kitkat	0.0052 ng	S29	Ferrero Rocher	0.0238 ng
S7	Cadbury Bournville	0.0102 ng	S30	Elit 1924 (Gourmet Dragee)	0.0274 ng
S8	Toblerone	0.0047 ng	S31	Cadbury Gems	0.0151 ng
S9	LindtAssorted Napolitains	0.0006 ng	S32	Milka	0.0062 ng
S10	LindtLindor	0.0061ng	S33	Storck Merci	0.0067 ng
S11	Hershey's	0.0061 ng	S34	Lindt	0.0072 ng
S12	Cadbury Wispa	0.0023 ng	S35	Belgian	0.0135 ng
S13	Cadbury fuse	0.0045 ng	S36	Cadbury Bournville	0.0066 ng
S14	Cadbury Sandwich Snack	0.0076 ng	S37	Cadbury Milk Tray	0.005 ng
S15	m&m chocolate	0.0029 ng	S38	Skittles	0.0108 ng
S16	Cadburry dairy milk	0.0048 ng	S39	Bounty	0.0085 ng
S17	Nestle black magic	0.0017 ng	S40	Coted'or	0.0097 ng
S18	Nestle Aero Bubbles	0.0065 ng	S41	Cream & Fudge Hazelnut	0.0033 ng
S19	Snickers	0.0025 ng	S42	Bellissimo	0.0002 ng
S20	Nestle Kitkat Chunky	0.0555 ng			
S21	Nestle Dairy Box	0.0029 ng			
S22	Ritter Sport Hazelnuts	0.0128 ng			

4. DISCUSSION

Processed food has been imported in Bangladesh and sale by multi super shop in our country. In Dhaka city, such kinds of imported processed foods are available, for example fish, meat, chocolate, ice-cream etc. Among the imported food products, chocolate is one of the most popular among consumers, especially among children. There is currently no established lab in our country for detection of food adulteration and food related forensic analysis. But, as a Muslim country, pork DNA identification in processed food is necessary as the buyer has the right to be informed about products being bought and consumed [12]. PCR assay has been widely used to detect pork in raw and heat-treated meat mixtures. It is also the relevant method for detection of DNA in food and processed food sample. High-performance liquid chromatographic procedure can also be used. In this method primers were designed to hybridize species specific DNA sequence to determine targeted DNA in samples [37]. Therefore, we used PCR assay for the detection of porcine contamination in some imported chocolate products. For the detection of pork contamination we used two set of primer to target cytochrome b gene. The cytochrome b gene (Cyt b) from mitochondrial genome contains species specific information and used in polygene as well as in forensic investigation. Thus, it has been confirmed that the use of Cyt-b gene for the detection of food adulteration is reliable [38]. Gel electrophoresis result suggests that the isolated DNA from various samples was appropriate and the specific porcine cytochrome gene fragment by two different primers were amplified. DNA band of the food samples in the gel indicate the replication of target DNA was amplified which confirmed the occurrence of pork DNA and/or pork samples contaminations in chocolate products. From the gel electrophoresis results the bands of positive DNA (marked as S37, S38 in Fig. 1 and Fig. 2) indicate that the species specific DNA was amplified in PCR reaction. Besides, two samples marked as S35, S36 in the Fig. 1 and Fig. 2. do not show the bands for porcine DNA which representing the absence of pork derivatives in the chocolate products. For other samples, DNA band in the gel not appeared (figure not presented) indicates that the pork contamination is not present.

In 2011, Malaysia's Islamic Development Department tested Cadbury chocolate and found pork DNA though companies disputed the finding. Indonesia also tested it but did not find any positive result and said Cadbury chocolate in their country complied with Islamic standards. But the question is, why will chocolate be contaminated with pork? One possibility is that industrial food packaging sometimes uses lubricants and stabilizers (known as stearates) are made from animal derivatives, including pigs in some cases. Several reports reveal that food matrices such as polyphenol and even cocoa powder which is key ingredient in chocolate production may contain insignificant amount of PCR inhibitor that can obstruct DNA extraction from lard or pork-adulterated chocolate. This might be the reason why only 2 out of 42 samples were found to be positive [39]. Lubricants made from animals may be the

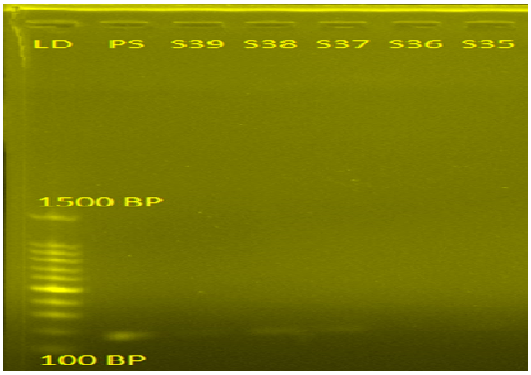

Fig. 1. Gel electrophoresis analysis in 1% agarose gels using primer F3/R3 showing the detection of specific 165 bp cytochrome gene fragment in the imported chocolate in Bangladesh. [Here, Lane LD: Ladder (100bp molecular marker), lane PS: Positive sample (pork meat), lane S38: Skittles (wild berry flavor chocolate), lane S37: Cadbury milk tray (Chocolate), lane S36: Cadbury bourneville, lane S35: Belgium]

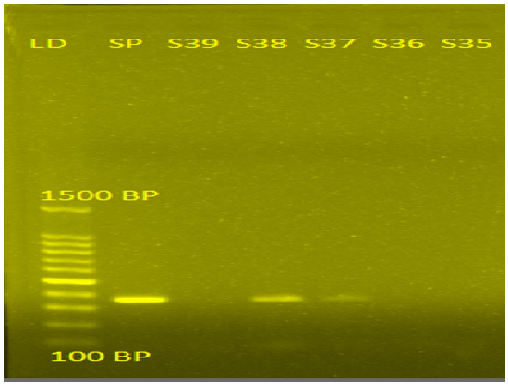

Fig. 2. Gel electrophoresis analysis in 1% agarose gels using cytb1 and cytb2 primer for pig showing the detection of specific 359bp cytochrome gene fragment in the imported chocolate in Bangladesh. [Here, Lane LD: Ladder (100bp molecular marker), Lane PS: Positive sample (pork meat), lane S38: Skittles (wild berry flavor chocolate), lane S37: Cadbury milk tray (Chocolate), lane S36: Cadbury bourneville, lane S35: Belgium]

culprits in the adulteration of the chocolate. In Bangladesh, southeast university tested chocolate for the first time to ensure it adherence to the Islamic dietary laws and found pork DNA in Wild berry flavor chocolate (UK) and Cadbury Milk Tray, Chocolate (UK). It is also interesting that both chocolates are manufactured from UK by MARS Company Ltd and Cadbury Company Ltd. In our findings, there was no particular difference between two sets of primer. For both primer DNA band were generate in their specific length of target sequences. So, the result was accurate and verified by revised tests. We also investigate the labeling indications on the packaging materials of the chocolates and found there is no appropriately labeled. Some of the producer have no website even in some case website indicates on the packaging materials but not valid yet. In 42 chocolate indication of halal certification also not present. Therefore, during import buyer or consumers should investigate the matter or take a halal certification from exporters.

5. CONCLUSION

Chocolate samples collected from commercial market and retail store were not pork or pork elements noticeable in the ingredients list or even not labeled as “pork –face”. It could be an ethical issue for not only in Muslim country’s but also in Muslim consumers. So, consumers are getting cheated regularly. Identification of accurate and extremely sensitive animal species and the detection of substitutes are the two major challenges in the food industry. In this study we used foreign processed sample for identification of pork contamination. Conventional PCR methods which required minute quantities of DNA as well as resources are used for the detection of pork derivatives and out of 42 samples 2 samples were positive. We have reported that magnetic based DNA isolation and PCR technique represents a valuable new method for the detection of food adulteration with pork. More research can be done in this area in order to improve the purity of the products and find out whether the method is reliable of many other products as well. This can also be used for halal authentication and quality control approaches in the future.

DATA AVAILABILITY OF STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

DISCLAMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

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