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Mini Review

Identification of pig DNA in food products using polymerase chain reaction (PCR) for halal authentication-a review

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Abstract

Muslim societies prohibit the inclusion of pig-derived materials in any food. As such, analytical methods that accurately and sensitively detect the presence of porcine materials are critical to ensure the halal authenticity of foods. Among molecular biology techniques, polymerase chain reaction (PCR) is used with preference to identify the presence of pig-derived DNA in foods and gelatins. Using specific primers, PCR can be successfully used to test for the presence of pork DNA in food products. In addition, 90% of gelatin used in food and pharmaceutical products has a porcine origin, so PCR is frequently used to test gelatins. This article describes the use of conventional or real-time PCR to test for the presence of pork DNA and porcine gelatin DNA.

Introduction

Muslim communities in Muslim countries such as Indonesia, Malaysia, and Pakistan have become increasingly aware about the food they consume. As a consequence, the global market for halal products and halal services has significantly expanded. The halal food market currently represents 16% of the entire global food industry, and is expected to grow to 20% of the world food trade in the future (van der Spiegel *et al*., 2012). Accurate labelling of food products allows consumers to make informed choices and assures that food products adhere to halal standards (Nakyinsige *et al.*, 2012). Globally, Muslim consumers are concerned about a number of issues concerning meat and meat products, especially the inclusion of pork-derived materials in food and the authenticity of halal claims.

Technological advances enhanced the economic benefit of food adulteration and fraud. Non-authentic foods can take different forms: (1) complete or partial absence of valuable constituents; (2) whole or partial substitution of food components with undeclared alternative materials (which are usually cheaper); (3) concealed damage to food or use of inferior foodstuffs; and (4) adulteration (addition of undeclared substances or material to increase product bulk or weight to increase the perceived value of a product) (Hargin, 1996; Nakyinsige *et al*., 2012). Pig-derived materials are commonly cheaper than those that come from halal animals (e.g., buffalo and cow) (Rohman and Che Man, 2012). Pig derivatives refer to any component that is derived from pigs, including lard extracted from pork adipose tissue, pork (pig meat), and porcine gelatin. Muslims are not allowed to consume pork products. Therefore, to assist regulatory bodies that test for the presence of pig derivatives in food products, standardized analytical methods have been proposed, developed, and tested (Mursyidi, 2013).

The analytical techniques used in food authentication depend on the type of pig derivative. Gas chromatography as well as FTIR spectroscopy and other spectroscopic methods, are widely used to analyze lard contents in some products (Rohman and Che Man, 2008). Meanwhile, liquid chromatography can indicate the presence of porcine gelatin by detecting specific markers (Cheng *et al.*, 2012). An electronic "nose" coupled with mass spectrometry can rapidly detect volatile components released

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from products containing lard and pork (Nurjuliana *et al*., 2011). Due to its specificity, PCR-based DNA analysis is the most frequently used approach to test for the presence of pig DNA in any product, and in particular to check whether pork or porcine gelatin is present in food and pharmaceutical products (Rahmawati *et al.*, 2016).

Some described techniques above are proposed and developed by halal scientist to analysis pig derivatives and one huge practical due to simple and easy application is the strip trap test based on immunosorbent assay. Some of methods also already developed in laboratory scale including spectroscopic methods using infrared radiation and nuclear magnetic resonance spectroscopy, chromatographic based methods, electronic nose, and differential scanning calorimetric have also been developed. Among analytical methods, the polymerase chain reaction based on the molecular genetic analysis was believed as the high sensitive, valid and judgeable as well as reliable for the analytical instrument, therefore this review describe and detail discussing PCR and real time PCR to guide some researchers and scientist to refer and develop new methods for the pig derivatives detection of various complex of the food matrices and products.

Polymerase chain reaction

Given that DNA has greater thermos-stability than most proteins, nucleic acid methods are less likely to be affected by food processing methods. The ubiquitous presence of DNA enables the identification of any appropriate sample containing DNA, regardless of the tissue of origin. Furthermore, DNA sequence data can potentially provide more information than protein tests. Among the DNA-based techniques, polymerase chain reaction (PCR) is the most common approach to identify the presence of species-specific materials in food products, as species-specific PCR primers can be used to detect minute amounts of DNA (Mafra *et al.*, 2007).

Due to their high specificity and sensitivity, several PCR-based methods have been proposed to identify species-specific materials in foods (Mafra *et al.*, 2007). These approaches include qualitative PCR (conventional PCR), detection of restriction fragment length polymorphisms (RFLP), and real-time PCR or quantitative PCR (Popping, 2002). PCR is a simple technique to understand and use, and yields rapid results. Moreover, PCR is highly sensitive, and has the potential to produce multiple copies of a specific product for use in sequencing, cloning, and analysis (Garibyan and Avashia, 2013). However, PCR does have some limitations: (1) DNA polymerases used in PCR are prone to errors and can produce mutations in the generated fragments; (2) the specificity of the PCR products may be altered by non-specific primer binding to other similar sequences on the template DNA; and (3) DNA sequence information is needed to design specific primers for use in PCR reactions (Garibyan and Avashia, 2013). Specifically, PCR assays can be limited by insensitivity and lack of quantitation for end-point analyses, as well as the dependence on a low throughput technique (e.g., agarose gel electrophoresis) for product analysis (Zhang *et al*., 2007; Novak *et al.*, 2007).

PCR amplification process

Each PCR assay requires single- or double-stranded template DNA and two oligonucleotide primers to specify the exact DNA product to be amplified. The primers are short DNA fragments with a defined sequence that is complementary to the DNA targeted for detection or amplification. The primers serve as an extension point for the heat stable DNA polymerase to extend the DNA strand by adding adenine, thymine, cytosine, and guanine (A, T, C, G) nucleotide bases. The presence of magnesium ions in the buffer improves the quality of PCR reactions (Weier and Gray, 1998).

There are three major steps for PCR reaction: (1) denaturation, (2) annealing, and (3) amplification (Kubista *et al*., 2006). During denaturation, a high temperature incubation separates double-stranded DNA into single strands and loosens secondary structure in single-stranded DNA. The highest temperature that the DNA polymerase can withstand is usually about 95°C. If the template DNA GC content is high, the denaturation time should be increased. During annealing, complementary sequences hybridize, so the temperature used is based on the calculated melting temperature (T_m) of the primers (5°C below primer T_m). During annealing, the temperature is lowered (usually to 45–60°C) to allow primers to anneal to the template strand. Inappropriate annealing temperature can inhibit primer-template interactions, so the temperature must be optimized. During elongation or amplification, the reaction temperature is 70-72°C to maximize DNA polymerase activity that allows primer extension to proceed at a rate of ~100 bases per second. When the target sequence for amplification by real-time PCR is short, the annealing and elongation steps are often combined and the reaction temperature is 60°C (Life Technologies, 2015).

Analysis of pig derivatives using conventional PCR

PCR amplification products, or amplicons, can be

analyzed in several ways: (1) species-specific PCR primers, (2) restriction digestion of PCR products, (3) single strand conformational polymorphism analysis, and (4) random amplified polymorphic DNA (RAPD) analysis (Lockley and Bardsley, 2000). Table 1 and Table 2 list some PCR techniques for analysis of pig derivatives in food and pharmaceutical products. Meyer *et al.* (1994) tested for the presence of pork DNA using species-specific PCR primers targeted to the porcine growth hormone gene, which enabled the detection of pork in fresh or cooked mixtures of beef that contained pork at levels below 2% (w/w). The specific oligonucleotides yielded a 108 bp (base pair) fragment amplified from the porcine growth hormone gene.

Meyer *et al.* (1995) detected pork material in marinated, processed, and fermented products by amplifying the mitochondrial cytochrome *b* gene and analyzing the resulting restriction fragments (PCR-RFLP). Using this method, the detection limit of pork in meat mixtures of pork and beef was below 1% (w/w). Erwanto *et al*. performed PCR using restriction fragment length polymorphism (PCR-RFLP) of the porcine cytochrome *b* gene to identify pork in meatball products obtained from different markets in Surabaya and Yogyakarta, Indonesia (Erwanto *et al.*, 2014). DNA isolated from these samples was amplified, and the purity of the PCR products was confirmed by ultraviolet spectroscopy before digestion with the restriction enzyme *Bse*DI that can cleave the porcine cytochrome *b* gene PCR product into two fragments (131 bp and 228 bp).

PCR–RFLP using *Bse*DI was also used to identify pork DNA in beef sausage and chicken nuggets, including before and after frying (Erwanto *et al*., 2011). Increasing amounts of pork samples (1%, 3%, 5%, 10%, and 25%) were added to mixtures of beef and chicken meats used for processing sausage and nuggets. The oligonucleotide primers to analyze pork content were: CYTb1 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') and CYTb2 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3') (Kocher *et al*., 1989), which together amplify 359 bp fragments from pig mitochondrial DNA. These amplicons were subjected to *Bse*DI restriction enzyme digestion to cleave the amplified DNA fragments into 131 and 228 bp fragments (Figure 1). This method could detect as little as 0.1% pork in meat products.

Aida *et al.* (2005) developed a method for species identification from pork and lard samples using PCR-RFLP analysis of a conserved region in the mitochondrial (mt) cyt *b* gene. The quality of genomic DNA from lard was good and produced clear PCR ~360 bp products upon amplification of the mt cyt *b*

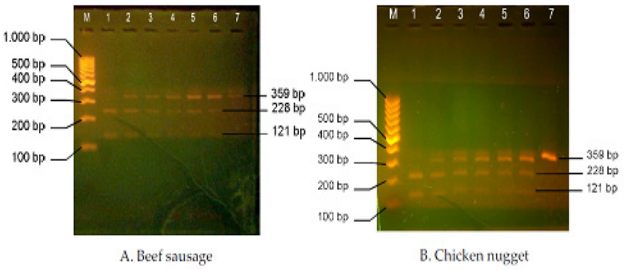

Figure 1. *Bse*DI restriction profile of cytochrome *b* PCR product amplified from samples. (A) M= 100 bp ladder size standard; 1= pork (100%); 2 = (beef 75%; pork 25%); 3 = (beef 90%; pork 10%); 4 = (beef 95%; pork 5%); 5 = (beef 97%; pork 3%); 6 = (beef 99%; pork 1%); 7 = beef 100%. (B) M = 100 bp ladder, 1 = pork (100%); 2 = (chicken 75%; pork 25%); 3 = (chicken 90%; pork 10%); 4 = (chicken 95%; pork 5%); 5 = (chicken 97%; pork 3%); 6 = (chicken 99%; pork 1%); 7 = chicken (100%) (Erwanto *et al*., 2011). Taken from Bogor Agricultural Technology.

gene. To distinguish between species, the amplified PCR products were cut with the restriction enzyme BsaJI to generate porcine-specific RFLPs. This cyt *b* PCR-RFLP species identification assay showed excellent detection of pig-derived materials, which is useful for halal authentication. PCR identification of pork derivatives in different food products (e.g., sausages and sausage casings, breads, and biscuits) wherein pork mitochondrial genes (12S rRNA and cytochrome *b*) were amplified was also a reliable and suitable technique in routine food analysis for halal certification (Che Man *et al*., 2007).

Montiel-Sosa *et al*. (2000) designed highly species-specific primers that target pork D-loop mt-DNA. Using these primers and restrictive PCR amplification conditions was a reliable and rapid method for detecting a PCR-amplified 531 bp band from pork. This method is useful for detecting both pork meat and fat in meat mixtures.

PCR and evaluation of a semi-quantitative PCR technique to detect pork in ground beef and pâté by densitometry using a specific and sensitive repetitive DNA element has also been developed. The results showed that 0.005% (w/w) of pork in beef (raw and heated) could be specifically detected and up to 1% pork contamination could be semi-quantitatively detected. The application of the referred technique to commercial pâtés provided evidence for the fraudulent additions of pork meat to the product (Calvo *et al*., 2002). The quantitative assessment of pork species in meat mixtures was achieved by competitive polymerase chain reaction (QC-PCR) using a new porcine-specific PCR system that targets Sus scrofa growth hormone (Wolf and Luthy, 2001).

Species-specific polymerase PCR using the Cyt *b* gene was also used to evaluate gelatin sources

Table 1. Polymerase chain reaction (PCR) techniques used for authentication of meat in different food products.

Analytical purpose	PCR technique	Primers (gene target)	DNA isolation	Detection limit	References
Analysis of pork in meatball	Real time-Evagreen	D-Loop P22 Forward: 5'- TCG TAT GCA AAC CAA AAC GCC -3' D-LoopP22 Reverse: 5'- ATG CAT GGG GAC TAG CAG TTA -3'	Phenol-chloroform-isoamyl alcohol	5 ng DNA	Fatimah (2013)
Identification of pork DNA in abon	Real time-Evagreen	D-Loop P22 Forward: 5'- TCG TAT GCA AAC CAA AAC GCC -3' D-LoopP22 Reverse: 5'- ATG CAT GGG GAC TAG CAG TTA -3'	Phenol-chloroform-isoamyl alcohol	10 ng DNA corresponding to 0.5% meat	Rahmawati *et al.* (2016)
Analysis of pork in sausage, bread and biscuits	Specific-specific PCR	12SRNA; FW (5'—CCA CCT AGA GGA GCC TGT TCT ATA AT—3') 12SRNA: R (5'—GTT ACG ACT TGT CTC TTC GTG CA—3')	DNeasy® Tissue Kit and DNeasy® Plant Mini Kit (Qiagen, Hilden, Germany)	Not reported	Che Man *et al.* (2007)
Analysis of pork in a mixture with chicken, lamb and beef	Specific-specific PCR	Designed wtih ClustalW software Sus-loop FWD: 5'- CACACCCTATAACGCCTTGC-3', Sus-loopRVS: 5'- GATTGGCGTAAAAATCTAGGG- 3')	DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). DNA concentration is determined spectroscopically	0,001 ng/µL Pork DNA	Che Man *et al.*, (2012)
Authentication of halal meat with pork	Specific-specific PCR	Designed with Primer-Select (Lasergene software; DNAStar, Inc.). Forward VPH-PF: 5'-AAT TTT TGG GGA TGC TTA GAC T-3' Reverse VPH-PR: 5'-TAT TTT GGG AGG TTA TTG TGT TGT A-3'	Wizard_ Genome DNA purification kit (Promega, Madison, USA).	1 pg	Karabasanavar *et al.*, (2014)
Analysis of pork and lard DNAs	PCR-RFLP	CYTb1 (5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3') dan CYTb2 (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3')	Qiagen DNeasy_ Tissue Kits	Not reported	Aida *et al.*, (2005)
Analysis of pork in meatballs	PCR-RFLP	Cyt b Fw: 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3' Cyt b Rev: 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'	Phenol-chloroform-isoamyl alcohol	0.1% contamination with pork could be detected.	Erwanto *et al.* (2014)
Analysis of pork DNA in sausage	PCR-RFLP	CYTb1: (5'-CCA TCC AAC ATC TCA GCA TGA TGAAA-3'). CYTb2: (5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3').	High Pure PCR Template Kit (Roche, Germany)	1% contamination with pork could be detected.	Erwanto *et al.* (2011)
Analysis of pork DNA in meatballs	PCR-RFLP	CYT b FW 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3'. CYTb REV 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA-3'.	High Pure PCR Template Kit (Roche, Germany)	1% contamination with pork could be detected.	Erwanto *et al.* (2012)
Analysis of pork and wild boar meat	Real-time PCR	CYT b1: 5'-CCA TCC AAC ATC TCA GCA TGA TGA AA-3' CYT b2: 5'-GCC CCT CAG AAT GAT ATT TGT CCT CA Pork FW: 5'-AAC CCT ATG TAC GTC GTG CAT-3' Pork R: 5'-ACC ATT GAC TGA ATA GCA CCT-3' 12SL FW: 5'-AAA CTG GGA TTA GAT ACC CCA CTA-3' 12SH: 5'-GAG GGT GAC GGG CGG TGT GT-3'	Extraction kits (SureFood® Animal ID, Congen Biotechnology GmbH, German)	Not reported	Mutalib *et al.* (2012)
Analysis of pork in some products (sausage, hamburger)	Real-time PCR using SYBR Green	Cyt b F: 5' AAACATTGGAGTAG TCCT ACTATTTACC-3' Cyt b R: 5'- CTACGAGGTCTGTT CCGATATAAGG-3' 18S rRNA F: 5'- CTGCCCTATCAAC TTTCGATGG-3' 18S rRNA R: 5'-TAATTTGCGCGCCTGCTG-3'	1×iQ™SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA)	As low as 0.1% pork can be detected	Soares *et al.* (2013)
Analysis of fresh pork and cooked pork	Real-time PCR using fluorophore FAM (6-carboxy-fluorescein) and *VIC (4,7,2'-trichloro-7'-phenyl-6-carboxy-fluorescein)*	Primers included in SureFood® Animal ID Pork SENS Plus V kit or SureFood® Animal ID Beef or SureFood® Animal ID Chicken or SureFood® Animal ID Turkey)	For fresh meat using SureFood® Prep Animal kit, and for cooked meat: SureFood® Prep Animal X kit (CONGEN, R-Biopharm, Germany)	0.1% pork	Ulca *et al.* (2013)
Analysis of pork and other meat	Real-time PCR multiplex-Evagreen	FW: 5' GCCTAAATCTCCCC TCAATGGTA 3' RV: 5' ATGAAAGAGGCAA ATAGATTTTCG 3'	Nucleospin Tissue Kit (Macherey-Nagel).	0.003% of pork	Safdar and Abasıyanık (2013)
Analysis of pork in sausage and in the mixture with horse meat	PCR-duplex	SIM 5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3', PIG 5'-GCT GAT AGT AGA TTT GTG ATG ACC GTA-3' and HOR 5'-CTC AGA TTC ACT CGA CGA GGG TAG TA-3'	Tissue mini kit (QIAGEN, Hilden, Germany).	Not reported	Di Pinto *et al.* (2005)
Analysis of pork in a mixture with other meat	Real-time PCR tetraplex	SUS 1 FW: 5'- CGA GAG GCT GCC GTA AAG G-3' SUS 1 RV: 5'- TGC AAG GAA CAC GGC TAA GTG-3' SUS 1 TMP: 5'- TCT GAC GTG ACT CCC CGA CCT GG-3'	Kits Wizard Plus Miniprep DNA purification system (Promega, Madison, USA).	Limit of quantification: 0.3% of pork; limit of detection: 0.1% pork	Köppel *et al.* (2008)
Analysis of adulteration of halal meat with pork	PCR specific-duplex	Pork-F: 5'-ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C-3' Pork-R: 5'- CTA CGA GGT CTG TTC CGA TAT AAG G-3' (target pada sitokrom b)	Wizard method using buffer TNE (10 mM Tris, 150 mM NaCl, 2 mM EDTA, 1% SDS), 100 µL 5 M guanidine hydrochloride and	0.25 ng DNA	Soares *et al.* (2010)

			40 µL proteinase K (20 mg/mL).		
Analysis of pork and beef	Real-time PCR-Triplex	Sus1-F_pork: 5'-CGAGAGGCTGCCGTAAAGG-3' Sus1-R_pork: 5'-TGCAAGGAACACGGCTAAGTG-3' Sus1_TMP (HEX): 5'-TCTGACGTGACTCCCCGACCTGG-3' (sebagai *probe*)	DNA extraction kit (Surefood® Animal X Kit, Congen Biotechnology, Germany)	20 genome equivalent	Iwobi *et al.* (2015)
Analysis of pork and other non-allowed meat	Real-time PCR-multiplex	[Forward: (50-CCATCCCAATTA TAATATCCAACTC-30) and reverse (50-TGATTATTTCTTGGCCTGTGT GT-30)]	Yeastern Genomic DNA Mini Kit (Yeastern Biotech Co., Ltd., Taipei, Taiwan)	0.02 ng pork DNA	Ali *et al.* . (2015)
Analysis of pork and other meat	PCR-TaqMan	12STAQMANFWM: 5'-AAAGGACTTGGCGGTGCTT-3'; 12STAQMANS: 5'-GTTACGACTTG TCTCTTCGTG CA-3'. 12SPROBE: 5'-TAGAGGAGCCTGTTCTATAATC GATAAACCCCG-3') (as probe)	Wizard_ DNA Cleanup system (Promega, Madison, WI, USA),	0.01 ng DNA corresponding to 0.1% meat	Rodnguez *et al.* (2005)
Analysis of pork in meatballs	PCR-TaqMan	Designed with primer3Plus software (www.bioinformatics.nl/cgibin/primer3plus/primer3plus.cgi). Swcytb F=TCC TGC CCT GAG GAC AAA TA dan SwcytbR=AAG CCC CCT CAG ATT CAT TC) TaqMan probe (SwcytbTqM=6-FAM/AGC TAC GGT/ZEN/CAT CAC AAA TCT ACT ATC AGC T/3IABkFQ) (as *probe*)	MasterPure™ DNA Purification Kit (Epicenter Biotechnologies, Madison, USA). Annealing at 61 °C	0.01% (wt/wt) pork	Ali *et al.* (2012)

(Shabani *et al.,* 2015). After isolation of DNA from gelatin powders with a known origin, conventional PCR using species-specific primers was carried out on the extracted DNA. PCR products of 212 and 271 bp were amplified for porcine and bovine gelatin, respectively, and a minimum amount of 0.1% w/w for both gelatin types was detected. Moreover, eight food products labeled as containing bovine gelatin and eight capsule shells were subjected to PCR examination. All samples contained bovine gelatin, and the absence of porcine gelatin was verified (Shabani *et al*., 2015).

Analysis of the presence of pig derivatives using real-time PCR

The limitation of conventional PCR was resolved in 1992 by the development of real-time PCR (Higuchi *et al.,* 1992). In real-time PCR, the amount of DNA product formed can be monitored in real time, i.e., during the course of the reaction, with accuracy and high sensitivity over a dynamic range. This monitoring can be assisted by the use of fluorescent dyes or probes introduced into the reaction. The fluorescence intensity of these probes will be proportional to the amount of DNA product formed. Moreover, in real-time PCR the number of amplification cycles required to obtain a particular amount of DNA molecules is recorded. Assuming a certain amplification efficiency, which typically is close to a doubling of the number of molecules per amplification cycle, the number of DNA molecules carrying the target sequence that were initially present in the sample can be calculated. A number of highly efficient detection chemistries, sensitive instrumentation, and optimized assays are now available that allow quantification of the number of DNA molecules of a particular sequence in a complex sample with unprecedented accuracy and sensitivity that can detect a single molecule (Kubista *et al*., 2006).

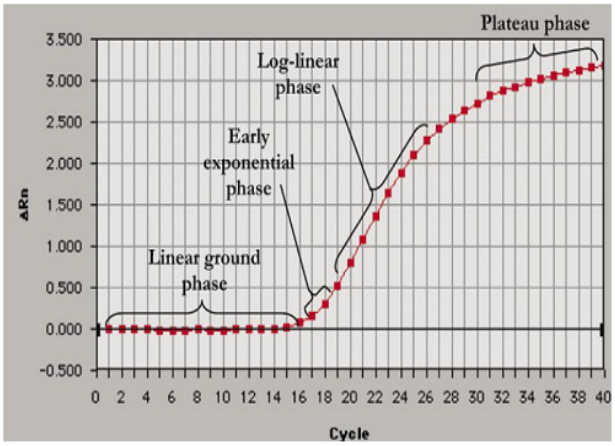

Figure 2. Real-time PCR response curve. The PCR amplification curve charts the accumulation of fluorescence intensities at each reaction cycle (taken from Applied Biosystems, 2015.

The response curve of real-time PCR can be divided into 4 separate phases: (1) linear ground phase, (2) early exponential phase, (3) log-linear (also known as exponential) phase, and (4) plateau phase (Figure 2, Tichopad *et al*., 2003). During the linear ground phase (usually the first 10–15 cycles), the fluorescence intensity for each cycle has not yet risen above background and baseline fluorescence can be calculated during this period. For the early exponential phase, the fluorescence intensity is significantly higher (usually 10 times the standard deviation of the baseline) than background levels. During the log-linear phase, PCR amplification is maximal and the PCR products double after each cycle. Finally, when the plateau stage is reached, reaction components are limited and fluorescence intensity is no longer reliable for DNA quantitation (Wong and Medrano, 2005).

Table 2. PCR techniques to identify gelatin sources in different matrix samples.

Analytical purpose	PCR technique	Primers (gene target)	DNA isolation	Detection limit	References
Analysis of porcine gelatin	PCR-southern hybridization chip and conventional PCR	Target (cytb) SimP-F: 5'-GAC CTC CCA GCT CCA TCA AAC ATC TCA TCT TGA TGA AA-3' SimP-R: 5'-GCT GAT AGT AGA TTT GTG ATG ACC GTA-3'' Target Cytochrome oxidase II: Pork 1: 5'-GCC TAA ATC TCC CCT CAA TGG TA-3' Pork 2: 5'-ATG AAA GAG GCA AAT AGA TTT TCG-3' Target ATP6: PPA6 F: 5'-CTA CCT ATT GTC ACC TTA GTT-3' PPA6 R: 5'-GAG ATT GTG CGG TTA TTA ATG-3'	QIAGEN DNeasy® Blood and Tissue Kit (Qiagen, USA)	0.25 ng (cyt b), 0.1 ng (cytochrome oxsidase II), and 0.001 ng (Olipro™ Chip)	Mutalib *et al.* (2015)
Analysis of porcine gelatin in food products	Real-time PCR with FAM	using Sure Food® Prep Animal X kit (CONGEN, R-Biopharm, Germany)	Primer is included in kits	1% porcine gelatin	Demirhan *et al.* (2012)
Analysis of gelatin sources in capsule shells	Real-time PCR, primer specific	FW: 5'-ATT TCC ATC CCA CAG CCC-3' RF: 5'-AAC AGA TGC TGA CTC ACA GAC-3' Probe: 5'-CCC AAC CCC CAA ACT GTC TCC T-3'	MasterPureTM DNA purification kit (EpiCentre, Madison, WI).	1 pg/mL	Cai *et al.* (2012)
Analysis of porcine gelatin in soft candy	Real time PCR-Evagreen	D-loop 318 Forward: TGCATTAACTGCTAGTCCCCA Reverse: GCTCGTGATCTAGTGGTGGT	DNA isolation KitK280-50 (BioVision Inc.)	10 pg	Sepminarti *et al.* (2016)
Analysis of porcine gelatin in capsule shells	Real time PCR-Evagreen	D-Loop 108 forward: 5'-CGT ATG CAA AAA ACC ACG CCA-3'; reverse: 5'-CTT ACTATA GGG AGC TGC ATG-3'	DNA isolation Kit K280-50 (BioVision Inc.)	5 pg	Sudjadi *et al.* (2016)

Real-time PCR results can be used either for qualitative analysis (presence or absence of a sequence) or quantitative analysis (number of copies of DNA). Real-time PCR for quantitative analysis is also known as qPCR, as opposed to conventional PCR, which is at best semi-quantitative. Because real-time PCR data can be analyzed without gel electrophoresis, the experimental time is reduced. In real-time PCR, reactions are run in a closed-tube system, which minimizes the potential for contamination and post-amplification manipulation is eliminated (Bio-Rad, 2015).

Two types of chemistries are typically used to detect RT-PCR amplification products, fluorescent dyes (e.g., SYBR green) and sequence-specific DNA probes (e.g., Taqman probe) (Cai *et al.*, 2012). Fluorescent dyes intercalate into double-stranded DNA to yield fluorescent complexes that can be monitored in real-time. Meanwhile, the Taqman probe is an oligonucleotide linked to a fluorescent dye and quencher. Taqman degradation frees the fluorescent dye from the quencher to produce fluorescence emission that is proportional to the amount of template. The fluorescence signal after each PCR cycle can be measured and used for DNA quantitation (Lovatt, 2002).

There are two types of quantitative PCR, relative quantification and absolute quantification (Wong and Medrano, 2005). Absolute quantitation uses serially diluted DNA standards with known concen¬trations to generate a standard curve. The standard curve produces a linear relationship between Ct (cycle threshold) and initial amounts of DNA, to allow the concentration of unknowns to be determined based on their Ct values. Relative quantification eliminates the need for standard curves and mathematical equations are instead used to calculate the relative expression levels of a DNA target relative to DNA standards or a calibrator (Arya *et al*., 2005).

Real time-PCR applications to detect the presence of pig derivatives

Real-time PCR is currently the most commonly used approach to identify and quantify pork DNA in food production, as well as to determine the presence of porcine gelatin in food and pharmaceutical products.

Application of real-time PCR to analyze pork DNA content in meat-based foods

Species-specific real-time PCR assays were successfully used to detect beef, pork, lamb, chicken, and turkey, with levels of detection below 0.1% (w/w). These assays target small (amplicons <150 base pairs) regions of the mitochondrial cytochrome *b* (cyt*b*) gene (Dooley *et al*., 2004). Two TaqMan real-time PCR systems have been developed and can distinguish between beef and pork in a total of eighteen animal species with sensitivities below 0.1% (w/w) for both species (Laube *et al*., 2003).

Analysis of pork DNA and porcine DNA in gelatin using real-time PCR was initially achieved using design and optimization of species-specific primers for real-time PCR reactions. Good primer design is a critical parameter in real-time PCR analysis, and primer sequences should be selected according to standard PCR guidelines (Quellhorst and Rulli, 2012). The designed primers must be specific for the

target DNA sequence. In order to provide practical annealing temperatures, primers should be between 18 and 24 nucleotides long. Primer sequences carrying extended homopolymer sequences (e.g., poly (dG)) or repeating motifs should be avoided as they can inappropriately hybridize (Life Technologies, 2015). Several software packages such as OligoPerfect™ and Primer Express® are available to assist with primer design. To confirm the specificity of the designed primers, Basic Local Alignment Search Tool (BLAST) *(http://blast.ncbi.nlm.nih.gov/Blast.cgi)* searches of public databases should be performed to ensure that primers recognize only the DNA target of interest. Tables 1 and 2 list some primers designed for analysis of porcine DNA.

RT-PCR using primers specific for the mitochondrial Displacement Loop (D-Loop) 686 cytochrome *b* (cytb) gene was exploited to identify the presence of pork DNA. The primers used are: Forward: 5'- GTTACGGGACATAACGTGCG-3'; Reverse: 5'-GGCAAGGCGTTATAGGGTGT -3' (D-Loop686, Soares *et al.,* 2013) and Fw: 5'- ATGAAAC ATTGGAGTAGTCCTACTATTTACC-3'; Rev: 5'-CTACGAGGTCTGTTCCGATATAAG G-3' (cytb, Kesmen *et al.*, 2014). The primers were tested for their specificity for DNA sequences in four species types: beef, chicken, goat, and horse. RT-PCR using D-Loop686 and cytb gene primers specifically distinguished between pork DNA and the other species. The lowest concentration of pork DNA (0.5%) in a mixture of processed pork and beef products was detected by both primers and produced of 114 and 134 bp amplification products for the D-Loop686 and 149 bp for cytb gene (Maryam *et al.,* 2016). Rahmawati *et al.* (2016) used primers designed by Fatimah (2013) to analyze for pork DNA contamination in beef meatballs. The primers amplified the target DNA at 22nd-197th basal orders and produced a 176 base pair amplicon. A specificity test revealed that the primers specifically amplified DNA from pork against other tested species (cow, chicken, goat, and horse). The detection limit of pig DNA in shredded (abon) pork was 10 pg, whereas the limit for a mixture of shredded pork and beef was 0.5%. Primers specific for mitochondrial rather than nuclear DNA are preferred for analysis of meat products because the copy number of the mitochondrial genome is several-fold lower than that of the nuclear genome. Moreover, mitochondrial DNA tends to be maternally inherited and has a high mutation rate compared to nuclear genes. As such, mtDNA tends to accumulate a sufficient number of point mutations to allow the differentiation of closely related species (Kocher *et al*., 1989; Lockley and Bardsley, 2000).

Application of real-time PCR to analyze DNA in products containing porcine gelatin

Commercial gelatin is typically obtained from porcine and bovine sources, and 90% of gelatin has a porcine source (Widyaninggar *et al*., 2012). Cai *et al.* (2012) identified gelatin sources (bovine or porcine) in capsules used in pharmaceutical manufacturing. For their studies, they developed species-specific RT-PCR assays based upon repetitive elements. The detection limit of porcine and bovine DNA was as low as 1 pg/mL. They designed their primer and probe sets using PrimerQuestTM software (Integrated DNA Technologies, IDT, Coralville, IA, http://www.idtdna.com/). The primers used for porcine DNA were: forward: 5'-ATT TCC ATC CCA CAG CCC-3' and reverse 5'-AAC AGA TGC TGA CTC ACA GAC-3'. The probe used was 5'-CCC AAC CCC CAA ACT GTC TCC T-3'.

Demirhan *et al.* (2012) used real-time PCR based on a multi-copy target cytochrome *b* (cyt b) and porcine-specific primers for halal authentication of gelatin. They extracted and purified DNA from the gelatin samples using the SureFood® PREP Animal system, and carried out real-time PCR using the SureFood® Animal ID Pork Sens kit. The minimum level of adulteration they detected was 1.0% w/w in marshmallows and gum drops. A survey of processed food products and capsule shells conducted in Turkey showed that some products did in fact contain porcine gelatin.

RT-PCR detection of porcine gelatin in soft candy was performed by Sepminarti *et al*. using DNA isolated with the mitochondrial DNA Isolation Kit K280-50 (Bio-Vision) (Sepminarti *et al*., 2016) and the D-Loop 318 primer. They examined fresh tissue samples from pig, cow, chicken, goat and rat, as well as different gelatin sources (beef, pigs, and catfish). The D-loop318 primer can amplify porcine DNA at an optimum temperature of 61.4°C. The repeatability test performed on soft candy had a relative standard deviation value of 1.06% and showed that the commercial soft candy samples they tested did not contain porcine DNA.

Sudjadi *et al.* (2016) designed some primers for analysis of gelatins used in commercial capsule shells. Only the primer D-Loop 108 (forward: 5'-CGT ATG CAA AAA ACC ACG CCA-3'; reverse: 5'-CTT ACT ATA GGG AGC TGC ATG-3') can identify the presence of porcine DNA in fresh tissue and gelatin sources at an optimum annealing temperature of 58.4°C. The sensitivity of this method expressed as limit of detection of DNA in gelatin and capsule

shells is 5 pg. None of the 23 commercial capsule shells they evaluated contained porcine DNA.

Constraints of conventional and real time PCR in porcine derivative detection

Technical constraints of PCR applications include the melting temperature (T_m), which is specific for each amplicon, and represents the temperature at which 50% of the DNA amplicon is double-stranded. The Tm depends on various factors, including amplicon length and nucleotide sequence. A common problem with real-time PCR that uses intercalating dyes is the production of unpredicted amplicons. The most frequent unpredicted amplicons are derived from primer dimers, which are most evident in samples that have low levels of the specific target template (Sakalar and Kaynak, 2016).

Development of methods to detect DNA of porcine derivatives should also take into account the broad inter- and intra-species variations among the wide varieties of domestic and wild pigs. Chen *et al.* (2007) reported that there are currently 58 pig breeds that can be considered as "transboundary" (i.e., present in more than one country), and these include 25 regional transboundary breeds and 33 international transboundary breeds.

The data for pig breeds suggest that researchers should develop molecular markers for genetic diversity and phylogenetic analysis in pigs. Molecular markers are needed for the Halal council to consider regulations and fatwas concerning halal, and they could serve as a standard for halal detection methods.

Conclusion

Because PCR is sensitive and specific, yet also rapid, simple, and economical, this approach is well suited to confirm food authenticity and to detect the presence of pork DNA in food products. PCR is also the method of choice for identification of gelatin sources in food and pharmaceutical products. Together, these qualities make PCR an ideal approach for use in halal authentication.

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