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RAPID DNA-BASED ANALYTICAL TECHNIQUES TO VERIFY THE AUTHENTICITY OF HALAL FOOD

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A SEARCA Regional Professorial Chair Lecture

by

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Universiti Putra Malaysia

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Foreword

"Rapid DNA-based Analytical Techniques to Verify the Authenticity of Halal Food" is a publication under the SEARCA Professorial Chair Lecture Monograph series. SEARCA began its Professorial Chair program in 1974, initially, for highly deserving faculty members from the University of the Philippines Los Baños—SEARCA's host institution. In 2012, the Center expanded it to the Regional SEARCA Professorial Chair program to highlight the significant academic contributions of outstanding experts in Southeast Asia. To date, SEARCA has awarded 47 regional professorial chair grants in the fields of agriculture and related sciences.

This issue of the monograph series showcases DNA-based authentication for halal food, which is reported to be accurate, sensitive, and reliable compared to protein- and lipid-based techniques. In this paper, several rapid DNA techniques such as multiplex PCR, convection PCR, PCR-RFLP, PCR-strip, real-time PCR, LAMP, nanotechnology, and commercial rapid test kits for the detection of porcine DNA and DNA from other animals for halal verification have been identified and discussed.

This monograph is a timely publication of the Regional SEARCA Professorial Chair program as the Center navigates its Eleventh Five Year Plan on Accelerating Transformation through Agricultural Innovation or ATTAIN. The impetus of this topic has come at an opportune time when adulteration and illegal halal labeling of food products have reached a global scale. These advanced methods of DNA-based authentication for halal food are needed to ensure that the authorities can provide a halal certificate in a rapid, sensitive, and accurate manner.

For its part, SEARCA will continue to recognize the contribution of institutions and support outstanding individuals contributing to ARD in Southeast Asia through instruction, research, innovation, and extension work. This Professorial Chair Lecture Monograph series is a testament to the contributions that would hopefully inspire others to emulate and act on.

Glenn B. Gregorio
Director, SEARCA

About the Authors

Dr. Shuhaimi Mustafa has been the dean of the Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM) since 2020. He was born in Hutan Melintang, Perak in 1972. He obtained his BSc (Hons) degree in Food Science and Technology from the Faculty of Food Science and Biotechnology, Universiti Pertanian Malaysia in 1996. He completed his MSc in 1999 and was awarded a scholarship to pursue a doctoral degree in Food Biotechnology at UPM, Selangor. Upon graduation, he was appointed in 2003 as a lecturer at UPM's Department of Microbiology. He taught courses related to his area of expertise such as Applied Food Microbiology and Mycology as well as several post graduate courses. He was promoted to associate professor in 2006 and full professor in 2012. Apart from teaching, research, and networking, he is also active in non-governmental organizations and sports. He was honorary secretary and president of the Malaysian Society for Microbiology. He also represents UPM in cycling and golf events.

Muhamad Firdaus Syahmi Sam-on was born in Kuala Pilah, Negeri Sembilan, Malaysia in 1997. He obtained a BSc (Hons) degree in Microbiology in 2020 from the Faculty of Biotechnology and Biomolecular Sciences of UPM. He accomplished his final year project under the supervision of Prof. Dr. Shuhaimi Mustafa. He is currently pursuing his PhD studies in Microbiology under the same supervisor since 2020. He recently published a journal article and attended a conference under the Malaysian Society for Molecular Biology and Biotechnology. Previously, he had an internship as a biotechnologist at the Halways Sdn Bhd under the supervision of Dr. Akhmal Hakim Roslan. He also participated at the International University Carnival on E-Learning (or IUCEL) in 2017 and 2018, the 1st Microbiology Undergraduate Symposium of UPM, and the NASA Space App Challenge 2017.

List of Tables and Figures

Acronyms and Abbreviations

AMSR-E	Advanced Microwave Scanning Radiometer-Earth Observing System
bp	base pairs
Ct	cycle threshold
cyt	cytochrome
DNA	deoxyribonucleic acid
DSC	differential scanning calorimetry
dsDNA	split double stranded DNA
ELISA	enzyme-linked immunosorbent assay
EPC	external positive control
FAM	fluorescein amidites
FTIR	fourier transform infrared
GNPs	gold nanoparticles
IPC	internal positive control
LAMP	loop-mediated isothermal amplification
µg	microgram
ng	nanogram
nm	nanometer
PCR	polymerase chain reaction
pg	picogram

RFLP	restriction fragment length polymorphism
rRNA	ribosomal RNA
rt-PCR	real-time PCR
SEM	scanning electron microscopy

Rapid DNA-Based Analytical Techniques to Verify the Authenticity of Halal Food

Shuhaimi Mustafa
Muhamad Firdaus Syahmi Sam-on

Abstract

The halal food industry across the world is experiencing a period of extraordinary growth and development. However, violation of the principle of halal for profit is a major concern as Muslims are only allowed to consume halal food. Recent episodes of adulteration and illegal halal labeling of food products indicated that food fraud is reaching global scales. Therefore, ensuring the authenticity of halal food is a crucial step that all must seriously implement. Compared to protein- and lipid-based techniques, DNA-based authentication is reported to be accurate, sensitive, and reliable. Moreover, DNA has a good stability at elevated temperature, exists in the entire tissue, and has better variation among organisms. In this paper, several rapid DNA techniques such as multiplex polymerase chain reaction (PCR), convection PCR, PCR-restriction fragment length polymorphism (RFLP), PCR-strip, real-time PCR, loop-mediated isothermal amplification (LAMP), nanotechnology, and commercial rapid test kits for the detection of porcine DNA and DNA from other animals for halal verification have been identified and discussed. These advanced methods are needed to ensure that the authorities can provide a halal certificate in a rapid, sensitive, and accurate manner.

1 Introduction

It is mandatory for Muslims to eat and consume halal products. The general principle concerning food for Muslims is that everything should be halal except those that are with impurities (or mixed with impurity), harmful, and intoxicant. These exceptions are clearly prohibited by shariah laws, including pork, *khamr*, blood, meat from cadavers, and meat of animals that have not been slaughtered according to Islamic rules (Riaz and Chaudry 2004). As mentioned clearly in Al-Quran, "Mankind! Eat what is good and lawful on the earth…" (2:168). Allah says in another surah in Al-Quran, "He has only forbidden you what dies of itself, and blood, and flesh of swine, and that over which any other (name) than (that of) Allah has been invoked; but whoever is driven to necessity, not desiring, nor exceeding the limit, no sin shall be upon him; surely Allah is Forgiving, Merciful" (2:173). Prophet Muhammad says, as narrated by An-Numan bin Bashir, "What is halal is clear, and what is haram is also clear, and in between those two is doubtful in which many people do not know about. So, whoever distanced himself from it, he has acquitted himself (from blame). And those who fall into it, he has fallen into state of haram…"

The global halal trade is experiencing tremendous growth and development. For halal food alone, the Imarc Group (2021) reported that the global demand was valued at more than USD 1.9 trillion in 2020. New Zealand and Australia are leading worldwide in exporting halal meat. On the other hand, Brazil and Argentina are the largest poultry producers in the world. Manufacturers

from European countries and China are among the largest producers of halal processed food in the globe. Consequently, the authenticity of halal products sold in bazaars, wet markets, supermarkets, hotels, and fast-food restaurants needs to be verified through credible halal certification and laboratory analysis.

2 The Need for Laboratory Analysis

The concern of food authenticity and adulteration has resulted in increased awareness regarding the composition of food products. Several cases involving adulteration of meat and meat-based products have been reported. For instance, pork tainted with cow blood was marketed as beef in the wet market in Thailand (Denyingyhot et al. 2022). Sultana et al. (2018), on the other hand, detected trace amounts of porcine deoxyribonucleic acid (DNA) in halal-labelled gelatin while Naaum et al. (2018) identified undeclared pork species in beef sausage produced in Canada. Hence, verification that the meat components are authentic and from sources acceptable to Muslim consumers is essential.

Sensitive and reliable methods for detection of halal products adulteration are of paramount importance for the implementation of halal food labelling, regulations, and product quality control. Various techniques have been specifically proposed for the analyses of pork, lard, *khamr*, and gelatin, including DNA-based methods, gas chromatography, liquid chromatography, differential scanning calorimetry (DSC), and fourier transform infrared (FTIR) spectroscopy.

Table 1 shows the comparison of the available detection methods for halal verification based on diverse targeted sites such as leather, lipid, protein, and DNA. According to Mirghani et al. (2009) and Jaswir et al. (2016), microscopic examination can be used to observe the halal authentication

procedure for leather products. Light microscopes and scanning electron microscopes can be utilized to examine the sample product, but it comes with some advantages and disadvantages. Lipid can also be used to verify the halal status in the sample product by using several methods, including chromatography-based techniques (Rohman and Che Man 2012; Reid, O'Donnel, and Downey 2006); electronic nose (Nurjuliana et al. 2011; Gliszczyńska-Świgło and Chmielewski 2017); differential scanning calorimetry (Nurrulhidayah et al. 2015; Rohman and Che Man 2012); and FTIR (Rahayu et al. 2018; Rohman and Che Man 2012). In addition, western blot (De Cicco et al. 2018; El-Sheikha et al. 2017) and species-specific proteins by enzyme-linked immunosorbent assay (ELISA) (Lubis et al. 2016; Ofori and Hsieh 2017; El-Sheikha et al. 2017) are techniques that can be used to detect protein, while nanotechnology (Ali et al. 2012a; Ali et al. 2012b; Krishna et al. 2018), DNA barcoding (Luo et al. 2011; Ali et al. 2012a), and polymerase chain reaction (PCR) (Shabani et al. 2015; Septiani 2019) can be used to detect the DNA in the sample/product for halal verification. Specifically, this paper focuses on the applications of rapid DNA-based approaches for halal food authentication.

Table 1. Comparison of the published detection methods for halal verification

Target/Methods	Advantages	Disadvantages	References
Leather products			
Microscopic examination	• Easy and fast • Accessible in most laboratories (light microscope) • Accurate at high magnificent scanning electron microscopy (SEM)	• Not accurate (light microscope) • Further clarification/test is needed (light microscope) • Hardly accessible SEM	Mirghani et al. (2009) Jaswir et al. (2016)
Lipid			
Chromatography-based techniques (GC-MS, HPLC, LC-MS)[1]	• Powerful and sensitive • Provides both qualitative and quantitative analyses	• Require more steps for food sample • Expensive and time-consuming • Expert needed in handling	Rohman and Che Man (2012) Reid et al. (2006)

Continued on next page

1 GC-MS - gas chromatography mass Spectrometr; HPLC - high-performance liquid chromatography; LC-MS - liquid chromatography-mass spectrometry

Table 1 continued

Electronic nose	• Non-destructive, relatively low cost, and reliable method • Easy to operate	• Relatively new in halal authentication	Nurjuliana et al. (2011) Gliszczyńska-Świgło and Chmielewski (2017)
DSC	• Provides a direct estimate of the overall enthalpy change of lard transitions • Simple and fast	• Requires an expensive instrument • Tedious technique and involves careful instrument calibration	Nurrulhidayah et al. (2015) Rohman and Che Man (2012)
FTIR	• Accurate and reliable • Improves the speed of spectral acquisition and has excellent wave number reproducibility	• The calibration model developed is only usable with similar functional groups to derive the calibration model. • Can only be used for formulations of samples that are consistent with those tested	Rahayu et al. (2018) Rohman and Che Man (2012)
Protein			
Western blotting	• Low cost • Accessible in many laboratories • Proteins and epitopes are effectively recognised	• Nonquantitative and well-trained supervise a necessity • Require available primary antibodies against the protein of interest • Antibodies often exhibit off-target binding	De Cicco et al. (2018) El-Sheikha et al. (2017)
Species-specific proteins by ELISA	• Easy to perform • Efficient in identifying porcine plasma-derived ingredients in foods and dietary supplements	• A multiplexed analysis of different species in a single run is complicated and, if applicable, quite costly • Less sensitivity than real-time PCR (rt-PCR) and not resistant to high temperature	Lubis et al. (2016) Ofori and Hsieh (2017) El-Sheikha et al. (2017)
DNA			
Nanotechnology	• Sensitive, fast, low cost, and repeatable • No need for DNA amplification	• Relatively new in halal authentication	Ali et al. (2012a) Ali et al. (2012b) Krishna et al. (2018)

Continued on next page

Table 1 continued

DNA barcoding	• Accurate and repeatable • Simple and powerful	• Can identify long fragments that are difficult to avoid degradation of DNA • Costly • Cannot provide quantitative information and cannot detect multiple species at the same time	Luo et al. (2011) Ali et al. (2012a)
Target/Methods	**Advantages**	**Disadvantages**	**References**
PCR	• Fast and accurate • Accessible in most laboratories (conventional PCR)	• Some need to specialize in handling (for example, rt- PCR) • Costly reagents (rt-PCR)	Shabani et al. (2015) Septiani (2019)
Loop-mediated isothermal amplification (LAMP)	• Fast and accurate • No isothermal needed; result can be observed by the naked-eye • Less-expensive method than PCR for the identification of pork meat	• Multiplexing approaches for LAMP are less developed than for PCR. • The larger number of primers per target in LAMP increases the likelihood of primer–primer interactions for multiplexed target sets.	El-Sheikha et al. (2017) Dhama et al. (2014) Abdullahi et al. (2017)

3 DNA-Based Methods in Halal Verification

Advances in molecular biology techniques have led to rapid development of DNA analysis for halal authentication. DNA offers advantages over proteins, including stability at high temperature, presence in all tissue types, and greater variation with genetic code (Mackie 1996). Some of the recent works related to porcine DNA analysis that focused on using PCR as biomolecular technique to amplify the specific fragments of genes of interest are listed in Table 2. Both genomic and mitochondrial genes have been targeted for the detection of porcine DNA in various products.

Table 2 shows the report of targeted gene for non-halal animal species detection using several techniques including conventional PCR, multiplex PCR, Taqman rt-PCR, Molecular beacon rt-PCR, commercial rt-PCR. Dalmasso et al. (2004) stated that conventional PCR detected 12S ribosomal RNA (rRNA) in porcine, Ali et al. (2015) detected ND5, ATPase 6, and ATPase in monkey, dog, and rat, respectively. Furthermore, according to Cai et al. (2012), Yusop et al. (2012) and Demirhan, Ulca, and Senyuva (2012) indicated that Taqman-, molecular beacon-, and commercial rt-PCR were used to identify and detect different sequences in porcine sample.

Species-specific PCR approach for the detection of pork and lard targeting the mitochondrial cytochrome b gene combined with restriction fragment length polymorphism (RFLP) was applied (Aida et al. 2005). The restriction fragment length polymorphism (PCR-RFLP) technique produced

reproducible and reliable result for the detection of porcine DNA from meat and fat samples.

A more sensitive and rapid biomolecular technique, which is the rt-PCR based on molecular beacon chemistry was developed by Yusop et al. (2012). Besides its reliability, this method was also proven to be very sensitive, which can detect the presence of pork for as low as 0.1 percent (w/w) in pork-beef admixtures.

Quantitative rt-PCR targeting repetitive elements of the genomic DNA for detection and quantitation of porcine and bovine DNA in gelatin mixtures and gelatin capsules was developed by Cai et al. (2012). It is surprising to note from their finding that the species-specific gelatins tested were not 100 percent free of DNA of other animal species. While only trace amounts of bovine DNA (0.01 picogram [pg]/mg) were detected in porcine gelatin, a significant amount of porcine DNA (17.83 pg/mg) was found in bovine gelatin. Their method was able to detect a contamination level of as low as 1 percent of the other species in the gelatin blends.

Gelatin production involves non-standardized acidic or basic extraction, and both the proteins and nucleic acids are highly degraded in the final gelatin products. The amount of DNA in gelatin is very low and varies from material to material (Boran and Regenstein 2010). In Europe, approximately 80 percent of edible gelatin is produced from pigskin, but gelatin from fish bones and non-porcine sources is also available (Boran and Regenstein 2010). Once gelatin has been manufactured, it is difficult to ensure that it is pure and that it is free from cross-contamination with porcine gelatin. Equally, the ability to test processed food products for the presence of porcine gelatin is an essential requirement for food control in Muslim communities (Riaz and Chaudry 2004). Therefore, development of a reliable method to detect gelatin adulteration or fraudulent substitution is apt and timely to prevent Muslims from consuming prohibited elements.

Table 2. Reported target genes for non-halal animal species detection

Technique	Target Animal	Target Gene/ Sequence	Reference
Conventional PCR	porcine	12S rRNA	Dalmasso et al. (2004)
Multiplex PCR	monkey	ND5	Ali et al. (2015)
	dog	ATPase 6	Ali et al. (2015)
	rat	ATPase	Ali et al. (2015)

Continued on next page

Table 2 continued

Technique	Target Animal	Target Gene/ Sequence	Reference
Multiplex PCR	donkey	ND2	Galal-Khallaf (2021)
Taqman rt-PCR	porcine	Genomic	Cai et al. (2012)
Molecular beacon rt-PCR	porcine	Mitochondrial cytochrome *b*	Mohd Yusop et al. (2012)
Commercial rt-PCR kit	porcine	Unknown	Demirhan et al. (2012)

Rapid DNA-Based Techniques

There are many cases involving the impurity of food products, mostly imported products in Malaysia and other Muslim countries. Meat authenticity-related issues, especially mislabeling and malpractice in the supply chain, have attracted public scrutiny due to increased awareness among consumers in getting the correct information and in promoting for transparency. Hence, meat authenticity and traceability from production to consumption should receive the government's highest priority in cooperation with national and international regulatory agencies (Chuah et al. 2016). DNA-based species recognition methods have obtained broader approval because of their reliability, superior stability, and DNA universality in all tissues and cells (Ali, Razzak, and Hamid 2014) while immunochromatographic antigen assay showed high false-negative rate (Kenyeres et al. 2021). Thus, rapid DNA-based techniques are studied and commercialized for halal authentication.

Reliable rapid methods are essential for facilitating the authorities' issuance of halal certificates to industries. Hence, each method is mandatory to be sensitive and to generate an immediate result (Ran et al. 2016). The rapid methods can reduce the analysis time and gain the support and trust of industry players and Muslim communities toward the authorities. Hence, this review presents the available fast methods for detecting non-halal meat DNA such as PCR methods, loop-mediated isothermal amplification (LAMP), nanotechnology, and commercial DNA detection kits.

Conventional PCR Methods

Conventional PCR techniques are the globally used system for DNA amplification in identifying pathogenic strains that cause transmissible illnesses. These also aid in the discovery of hereditary syndromes and

are used in numerous studies. Furthermore, these techniques amplify the targeted DNA fragment by applying a set of different temperatures to split double stranded DNA (dsDNA) using a thermocycling machine (Gill and Ghaemi 2008).

PCR operates by involving the chemical reaction in the nucleotide base for the nucleotide matching in the dsDNA helix. The heating process of the DNA molecule disrupts the holding hydrogen bonds together with the double helix. The effect produces dsDNA molecule separation and denatures the dsDNA into ssDNA. As the cooling process takes place, the DNA molecule is reformed into a complementary base pair with a double helix strand. During the PCR run, the exact nucleotide sequence of the gene of interest needs to be known before any DNA amplification process starts (Abdul Karim and Muhamad 2018).

In halal food authentication, the use of conventional PCR can identify non-halal meat such as pork by targeting the DNA site at either cyt b, cyt oxidase II, D-loop, 12S rRNA, 16s DNA, ATP8, or ATP6 gene (Sahilah et al. 2015). However, conventional PCR has weaknesses in terms of total run time as it needs to be performed after DNA extraction and data analysis by agarose gel after PCR reaction is completed. Nevertheless, many researchers in biotechnology laboratories are still using this PCR type due to its cost-effectiveness and equipment accessibility (Yusop and Bakar 2020). Due to the limitations of conventional PCR in detecting DNA, utilization of other types of PCR is essential. Throughout this review, several PCR-based methods are discussed, namely: multiplex PCR, convection PCR, PCR-RFLP, PCR-strip, and rt-PCR. Consequently, each PCR type shows promising outcomes superior to traditional PCR in various aspects such as specificity, sensitivity, and run time.

Multiplex PCR

Multiplex PCR has gained increased interest among scientists and researchers due to its unique multi-target detection system in a single tube (Ali et al. 2014). The establishment of multiplex PCR assays shows vigorous, low-cost, sensitive, and accurate meat species detection. Moreover, the utilization of multiple primer sets in one PCR tube allows simultaneous targeting of different DNA sizes.

In contrast, numerous genes targeted using a singleplex primer set will require many reagents and a significant amount of time to complete the individual PCR runs (Şakalar and Abasiyanik 2011). Hence, several multiplex

PCR approaches were developed in recent years to recognize animal species in food products for rapid halal authentication. The efficiency of multiplex PCR relies on how the primer pairs are designed in terms of specificity to the templates. To optimize the multiplex PCR assay, the primer concentrations and annealing temperatures need to be tested (Sohn et al. 2021).

Ali et al. (2015) reported the first multiplex PCR through species-specific primer design with the sensitivity of 0.01–0.02 ng to detect non-halal animals (i.e., pork, dog, monkey, and cat species). The primer sets designed were amplifying the DNA fragment of 172 base pair (cat), 163 base pair (dog), 141 base pair (pig), 129 base pair (monkey), and 108 base pair (rat) by targeting the site of ND5 region of mitochondrial, ATPase 6, and cytochrome b genes.

Additionally, Indriati, and Yuniarsih (2019) reported that multiplex PCR could differentiate beef and pork DNA by targeting the cytochrome b genes. Their study detected pork DNA and beef DNA admixtures by targeting specific DNA fragments of 398 base pair and 274 base pair, respectively. Verification was done by analyzing the presence of pork DNA and beef DNA in halal sausage and meatball samples. It was detected that one of the sausage samples contained porcine DNA. Therefore, this shows that multiplex PCR could be considered a rapid DNA detection of multiple animal species in halal food products verification. However, as the number of primer pairs increases in multiplex PCR, a few technical problems limiting the detection capability need to be solved, such as primer dimers, primer mismatches, different amplification efficiency of primers, and difficulty in distinguishing product sizes (Tao et al. 2020).

Convection PCR

In principle, the conventional PCR requires precise and long repetitive thermal cycling to perform the denaturation, annealing, extension, and agarose gel electrophoresis stages, which are not suitable for point-of-care testing. Convection PCR technology offers a simplified heating format that can complete the amplification of a target DNA sequence in less than 30 minutes (Krishnan, Ugas, and Burns 2002). In convection PCR, heating of the reaction reagent is performed at the bottom of the capillary tube at a temperature of about 95°C. A steady temperature gradient formed along the capillary tube will automatically induce thermal convection and circulate the reaction reagent up and down between the low and high temperature regions for stable melting, annealing, and extension of target DNA region (Miao et al. 2020).

In the detection of porcine DNA, convection PCR is more time-efficient than conventional PCR (Song, Hwang, and Kim 2017). The convection PCR runs in rapid time within 24 minutes and can detect porcine DNA in a total of 1 hour, including gel electrophoresis. Moreover, the detection of both singleplex and multiplex target DNA sequences of mitochondrial cytochrome b gene presented positive results using this method. Thus, as low as 1 percent of pork DNA in meat samples (beef and lamb) could be detected using this approach (Song, Hwang, and Kim 2017). The convection PCR used by Song, Hwang, and Kim (2017) was portable and battery operated. Therefore, the direct identification of meat species could be done outside the laboratory and could even be made possible during power outages. Thus, Song, Hwang, and Kim's study (2017) demonstrated the possibility of convection PCR applications for rapid on-site halal food verification. However, despite having the edge of being rapid, and having high efficiency and specificity, only a few convection PCR techniques have the capacity of nucleic acid extraction for further analysis such as cloning and DNA sequencing (Song, Hwang, and Kim 2017).

PCR-RFLP

The PCR-RFLP is a detection method that is generally aimed at profiling sequences on the mitochondrial DNA (mtDNA). Mitochondrial DNA is chosen because it is present in high quantities in the cells and is generally more stable compared to chromosomal DNA. Among the target sites for PCR-RFLP method are 12S rRNA gene and cytochrome b (cyt b) gene of mtDNA (Walker et al. 2003; Chen et al. 2010). Chen et al. (2010) reported the reliable and rapid assay for authenticating cattle, yak, buffalo, goat, and pig meat products using PCR-RFLP approach. They discovered that the five animals showed species-specific restriction patterns. The practical application of the method was also tested on commercial beef jerky. They found that the PCR-RFLP profile matched with that of cattle and mismatched with pig.

Ali et al. (2018) investigated the application of multiplex PCR-RFLP assay to determine rabbit, rat, and squirrel meats in frankfurter products. Rat and squirrel were chosen in their assay as they were close relatives of rabbit. The digestion of PCR products with *Bts*I*Mut*I and *Bts*CI enzymes revealed distinctive fingerprints of 115 base pairs (bp) and 8 bp for rabbit, 64 bp and 44 bp for rat, and 176 bp and 67 bp for squirrel, respectively. The multiplex PCR-RFLP assay results on commercial beef and chicken frankfurters did not match with the profiles of rabbit, rat, and squirrel, reflecting that the two

commercial beef and chicken frankfurters did not have any rabbit, rat, and squirrel adulteration.

Another study by Al Amin et al. (2020) showed that PCR-RFLP analysis was able to generate feline specific DNA fragments (69 bp, 43 bp, and 26 bp) when digested with *Alu*1 restriction enzyme. Their PCR-RFLP assay performance was tested against three commercial dummy chicken and beef products such as frankfurter, nuggets, and meatballs containing feline meat and produced a 0.01 percent (w/w) sensitivity level. According to Al Amin et al. (2020), feline meat was a potential adulterant in the food chain. Thus, PCR-RFLP assay was developed to screen for the presence of feline meat in commercial meat products sold in supermarket chains across Malaysia.

PCR-RFLP is considered as a fast and straightforward meat DNA detection compared to the sequencing technique, and the sensitivity is higher, which is 0.001 ng (Guan et al. 2018). Therefore, PCR-RFLP assay could be considered as one of the rapid identification methods for non-halal DNA sample recognition. Table 3 compiled the restriction enzymes and the targeted amplicon applied in PCR-RFLP method for differentiation of some animal species. According to Bravi et al. (2004), cyt b has been used as target amplicon for HaeIII, Alu1, and Hinf1 enzymes to identify horse, donkey, pig, dog, and human. Moreover, Alu1 and Tru9I enzymes targets mitochondrial 12S rRNA gene (Wang et al. 2010), HpaII enzyme targets cytochrome oxidase subunit 1 (COI) gene (Haider et al. 2012), and AluI and Hinf1 enzymes targets cyt b gene, 12S rRNA gene, and 16S rRNA gene, respectively (Mata et al. 2020). Thus, BfaI targets ND5 amplicon in duck and goat (Uddin et al. 2021), FatI targets ND5 amplicon in sheep (Uddin et al. 2021), and Alu1 targets COI amplicon in striped snakehead to differentiate the animal species (Alam et al. 2021).

Table 3. Some restriction enzymes and targeted amplicons applied for differentiation of animal species using PCR-RFLP method

Enzyme	Target Amplicon	Animal Species	Reference
HaeIII, Alu1, Hinf1	cyt b gene	horse, donkey, pig, dog, human	Bravi et al. (2004)
Alu1, Tru91	Mitochondrial 12S rRNA gene	dog, chicken, horse, pig, goat, sheep	Wang et al. (2010)
HpaI1	COI gene	camel	Haider et al. (2012)

Continued on next page

Table 3 continued

Alu1, Hinf1	cyt b gene, 12S rRNA gene, 16S rRNA gene	sea snakes	Suntrarachun et al. (2018)
Taq1, HaeII1	COI gene	bigeye tuna, yellowfin tuna	Mata et al. (2020)
Bfa1	ND5	duck, goat	Uddin et al. (2021)
Fat1	ND5	sheep	Uddin et al. (2021)
Alu1	COI	striped snakehead *(Channa striata)*	Alam et al. (2021)

PCR-strip

PCR-strip is an easy and rapid method for DNA detection using the combination of conventional PCR amplification with specific forward and reverse primers targeting unique DNA sequence and strip format. The strip recognition system only depends on a pipette without any other advanced equipment. Thus, outcomes can also be observed on the strip by the naked eye within 5 minutes (Yin et al. 2015). The immune colloidal gold in the strip is used for DNA recognition. The strip consists of colloidal gold labelling, antigen-antibody response, and lateral flow tests for DNA sample detection.

In contrast, the use of conventional PCR requires gel electrophoresis for endpoint detection; further the use of rt-PCR is costly and skilled technicians are required. The PCR-strip protocol retains the amplified PCR product's reliability while simplifying the gel electrophoresis test and cutting the expense for the real-time detection techniques (Sun et al. 2017).

According to Yin et al. (2020), a Pig-PCR-strip technique combining pig-specific PCR, nucleic acid hybridization, and lateral flow strip detection showed easy, fast, and reliable pork DNA recognition in meat and meat products. The total detection time for the strip test after the PCR run and nucleic acid hybridization was only three minutes. Moreover, it could detect as low as 0.01 percent pork in adulterated meat and the detection limit was 10 femtogram (fg) target DNA. The method was accurate as it was specific to pork DNA and it did not react with other species.

This assay platform is ideal for medium-equipped laboratories with minimally skilled technicians. However, since this method is more sensitive than the conventional PCR, the contamination of samples with aerosol is high, leading to false-positive results. Therefore, the PCR reaction should

be performed in an oil-sealed tube and the strip test should be conducted in a separate room to minimize cross-contamination (Yin et al. 2015).

Real-time PCR

The rt-PCR is a reliable method to identify non-halal DNA in food. Many studies have shown that the results produced by rt-PCR are fast and reliable. Rt-PCR consists of four phases: linear, exponential, log, and plateau (Erwanto et al. 2018). Compared to conventional PCR, rt-PCR minimizes cross-contamination and detects small samples as no gel visualization is needed (Septiani 2019). The process discriminates DNA origin without the need for time-consuming and laborious steps like sequencing, enzyme digestion, or confirmation analysis. The procedure is rapid, allowing routine high throughput testing of multiple samples (Nakyinsige, Che Man, and Sazili 2012).

Analyzing the results can be done by introducing the fluorescent dyes or DNA probes into the sample. The fluorescent dye will react with double-stranded DNA, while the DNA probe will hybridize and amplify the target DNA (Ali et al. 2012c). Moreover, the rt-PCR can be performed by using a singleplex or multiplex form of primer set. Table 4 shows the study of singleplex and multiplex primers with rt-PCR for halal authentication. For the time consumed by the primer sets, the multiplex PCR consumes 40 minutes (Kim et al. 2019) and 30 minutes (Ishida et al. 2018), while the singleplex PCR consumes 90 minutes (Lee et al. 2016). Furthermore, multiplex PCR was able to identify more species in single PCR reaction, identifying pork, chamois, roe, deer and beef at the same time by targeting various mitochondrial genes (Koppel et al. 2020). On the other hand, singleplex PCR could only run one sample at a time (Jeffrey et al. 2007).Various research activities conducted using rt-PCR have shown a reasonable prospect for halal purposes of DNA species recognition. However, in many cases, prior to rt-PCR assay, complicated procedures are required for the extraction of clean DNA from complex matrices such as processed food and gelatin. Mohamad et al. (2015) reported that the extraction for gelatin and gelatin capsules often fails. This failure is due to the poor quality and quantity of the DNA extraction; thus, the PCR cannot produce reliable results.

Table 4. Study of singleplex and multiplex primers with rt-PCR for halal authentication

Primer Set/ Species	Minimum Concentration	Target Site	Time Consumed	Reference
Singleplex				
Porcine	10 fg and 0.001% of DNA	Unknown	90 minutes	Lee et al. (2016)
Pork	10 ng of DNA	Mitochondrial cyt b gene	Unknown	Ali et al. (2012c)
Human, dog, and cat	0.4 pg of DNA (human and cat); 4 pg of DNA (dog)	MC1R gene (dog and cat); THO1 gene (human)	Unknown	Kanthaswamy et al. (2012)
Canine	0.5 pg of DNA	MC1R gene	Unknown	Jeffrey et al. (2007)
Multiplex				
Pork, beef, and chicken	0.1 pg of DNA	D-loop gene (for pork)	within 40 minutes	Kim et al. (2019)
Pork, chamois, roe, deer, and beef	0.1 ng of target DNA	Unknown	Unknown	Köppel et al. (2020)
Dog, cat, rabbit, cattle, pig, chicken, goat, sheep, horse, deer, raccoon-dog, monkey, and bear	0.1–0.01 ng of DNA	Various mitochondrial genes	30 minutes excluding DNA extraction	Ishida et al. (2018)
Duck, pork, beef, and chicken	0.01% DNA adulteration	Species-specific mitochondrial gene sequences	Unknown	Xu et al. (2012)
Porcine, cattle, and buffalo	0.003 ng of DNA	ND5 and cyt b genes	Unknown	Hossain et al. (2017)

However, in the case of porcine capsule, adjusting the pH of the extracted DNA to 8.5 prior to purification with isopropanol greatly increased the total DNA amount compared to the non-adjusted sample (Mohamad et al. 2015). The rt-PCR utilized was able to detect both porcine and bovine adulteration in bovine and porcine gelatin and capsule. It also showed adequate specificity and sensitivity for the detection of impurity, even in the gelatin capsules. The molecular beacon-based rt-PCR method was applied to detect and quantify porcine DNA in gelatin targeting cyt b gene and chromosomal DNA (Mohamad et al. 2018). Higher sensitivity

was observed in chromosomal DNA, detecting as low as 1 pg of gelatin DNA as opposed to the mitochondrial DNA that can only identify 10 pg of gelatin DNA. The higher sensitivity using molecular beacon-based rt-PCR to detect adulteration in gelatin results in rapid detection methods for halal verification.

Loop-mediated isothermal amplification (LAMP)

The advancement in nucleic acid amplification has resulted in the development of LAMP assay. As opposed to PCR amplification of DNA, LAMP reaction takes place at a constant temperature (isothermal condition) within a short period of time (30 minutes to 1 hour) (Tomita et al. 2008). DNA synthesis is performed by DNA polymerase possessing high strand displacement activity and a set of two specific inner and two specific outer primers. Initially in the LAMP reaction, all four primers are used but later during the cycling and elongation reactions, only the inner primers are used for strand displacement DNA synthesis. The inner primers are called the forward inner primer (or FIP) and the backward inner primer (BIP), respectively. The forward inner primer and the backward inner primer contain two distinct sequences corresponding to the sense and antisense sequences of the target DNA, respectively (Notomi et al. 2000). Due to the absence of thermocycling requirements, the process for amplifying DNA can be operated using affordable tools such as a regular water bath (Gadkar et al. 2018).

A study by Lee et al. (2016) demonstrated that the four pork specific LAMP primers set used had a high specificity against 22 eukaryotes. The assay sensitivity of the pork-specific lamp was 1 pg/reaction of pork DNA, whereas in binary mixtures of beef-pork, lamb-pork and chicken-pork, pork concentration was detected for as low as 0.1 percent within 30 minutes (Lee et al. 2016). In another experiment by Liu et al. (2019), the LAMP assay could detect as low as 1 percent of pork in mutton-pork binary mixture. LAMP assay was also conducted for the detection of pork DNA in highly processed food (Girish et al. 2020). It was able to detect thermally processed (121°C for 30 minutes) pork-beef mixture up to the level of 0.1 percent and the limit of detection of DNA was at 0.5 nanogram/microliter (ng/µl). Table 5 summarizes the LAMP detection methods on various animal species. LAMP detection methods showed rapid detection with maximum time processing in 60 minutes (Kanchanaphum et al. 2014) and minimum time at 15–20 minutes (Abdulmawjood et al. 2014). This method indicated different targeted genes such as D-loop gene (Kanchanaphum et al. 2014;

Cai et al. 2020), 12S rRNA (Li and Fan 2017), 16S rRNA (Sul et al. 2018), and Cytochrome B gene (Abdulmawjood et al. 2014) to identify the animal species. Moreover, varieties of result displayed by LAMP such as white ring-shaped precipitate (Kanchanaphum et al. 2014), visible band on immune-chromatographic strip (Li and Fan 2017), annealing curve analysis using a Genie III LAMP detector (Sul et al. 2018), and fluorescence intensity by Genie II (Cai et al. 2020; Abdulmawjood et al. 2014).

Table 5. Reported rapid LAMP detection methods for various animal species

Species/ Result Produced	Minimum Concentration	Target Site	Time Consumed	Reference
Pork				
White ring-shaped precipitate	10 fg and 0.001% of DNA	Unknown	90 minutes	Lee et al. (2016)
Pork, sheep, chicken, duck, and bovine				
Visible band on immune-chromatographic strip	0.1%	12S rRNA gene	50 minutes	Li and Fan (2017)
Chicken (from chicken/pork and chicken/beef mixture)				
Annealing curve analysis using a Genie III LAMP detector	0.1% raw meat/1.0% treated meat	16S rRNA gene	30 minutes	Sul et al. (2018)
Porcine (from meat product of pig, cattle, horse, sheep, chicken, rabbit, rat, dog, fox, duck, donkey, and goat)				
Fluorescence intensity by Genie II	1.76 pg of DNA	D-loop gene	30 minutes	Cai et al. (2020)
Ostrich				
Fluorescence intensity by Genie II	0.01%	cyt b gene	15–20 minutes	Abdulmawjood et al. (2014)

Nanotechnology-based biosensor

Nowadays, advances in nanotechnology can facilitate sensitive and rapid detection of animal species for halal authentication. One of the key accomplishments in nanotechnology is the development of novel sensors and biosensors. In general, biosensors are composed of a bio-recognition receptor (antibodies, enzymes, DNA, RNA, or cells) and a transducer.

The transducer will convert the alteration of the analyte bond with the receptor into a signal that is amplified (Campos et al. 2020).

One of the nanotechnologies used in biosensor for DNA species detection of meat products is gold nanoparticles (GNPs) (Ali et al. 2012b). GNPs deliver a superior nanobiosensing platform while demonstrating excellent capability to identify specific meats in meat items. There are a few advantages of GNPs as a biosensor. Firstly, it is compatible with almost all types of chemically (organic and metallic) and biologically active molecules. Moreover, these molecules can keep on being functionally active even after immobilization on GNPs. Secondly, GNPs can facilitate the immobilization of large quantities of organic or biomolecules. The high surface area to volume ratios may increase the chances of interaction with a target analyte. Thirdly, GNPs (e.g., citrate capped) are mostly negatively charged, making them suitable for electrostatic interaction with specific positively charged biomolecules. This makes highly selective interaction exclusively with the target analyte of interest possible. Furthermore, the advantage of biofunctionalized GNPs is multiplexing in the detection of analytes. In multiplexing, GNPs hosting multiple ligands can interact with various receptors selectively and simultaneously, making it possible to detect numerous target analytes at the same time (Upadhyayula 2012). Thus, Ali et al. (2012b) and Upadhyayula (2012) suggested the application of nanotechnology (GNPs) as a rapid tool in halal verification.

The application of GNPs-biosensor (Ali et al. 2012b) showed a rapid outcome (less than 10 minutes), as well as a consistent and low-cost approach, for detecting adulteration of pork in meatballs. Devoid of any advanced instrument and chemistry modification involved, GNPs directly targeted the non-amplified mixed gDNA. The process is simple; it only depends on the color change of the GNPs (20 nm) with the addition of salt. A change in color from pink to purple indicates the concentration of pork DNA in the vial. A spectrophotometer and reusable cuvette are used as optical instruments, which are available in most scientific laboratories.

Moreover, Ali et al. (2012b) reported that the first swine-specific biosensor depends on GNPs established. GNPs are used to detect pork adulteration at the lowest possible concentration (1%) in ready-to-consume beef meatballs. The time needed by this approach is about 1–1.5 hours. GNPs are coated with highly stable citrate-tannate in large diameter (size 3 nm), four-folds molar, and low point ionic intense of the hybridization buffer. The coated GNPs analyzed by fluorescence displayed high sensitivity and specific pork DNA sensors for the cross-testing of varied commercialized

meatballs. The precision of the technique on the 5–100 percent adulteration in meat product revealed 90–95 percent accuracy. Nevertheless, if pork's adulteration mixed in beef meatball was lower than 5 percent, the accuracy significantly dropped to less than 80 percent. Another concern was the presence of single-stranded DNA that might interfere with the test as this would stick to GNPs. Moreover, this method could not provide quantitative information on the target DNA. Thus, the GNPs method is applicable to examine highly processed samples, which may not be possible by PCR because it requires an amplifiable target DNA.

Commercial DNA Detection Kits for Halal Authentication

Nowadays, varieties of commercialized kits are accessible for halal authentication. Most of the available DNA detection kits are primarily for laboratory testing and not for consumers. This limitation is due to the requirement of PCR run, DNA extraction, and other procedures during analysis that can only be done in laboratories. Authorities play an important role to prevent any false claims on any product, which can cause panic among the public (Lubis et al. 2016). Thus, DNA kits' application is beneficial for authorities to clarify specific products before revealing them to the community due to the reliability of DNA-based detection. This review presents the applicability of commercial DNA kits such as Meat ID Halal, LiliF HALAL Real-time PCR Kit, RapidFinder Halal ID Kit, CarnoCheck, EasyFast Pig/Suidae, and HaFYS.

Meat ID Halal

Meat ID Halal is a commercial kit available for halal detection supplied by Minerva Biolabs Incorporation. It is applied to detect both DNA of porcine and donkey using a qPCR-based screening system. Meat ID Halal allows fast analysis of various food matrices, including raw, highly processed, or cooked meat products, where significant DNA degradation may have occurred. A sensitivity threshold level of 0.5 percent can be obtained with the optimum DNA extraction protocol.

The assay is based on the TaqMan principle and relies on the 5′ to 3′ exonuclease activity of Taq polymerase and the probes' dual labelling with fluorescents and quenchers. During PCR, the Taq polymerase cleaves and removes annealed probes releasing the previously quenched fluorescent signal. Target sequences of this assay are the pork and donkey species-

specific mitochondrial genes, cyt b. Even small amounts of DNA presence (down to 1 pg/PCR-reaction) could lead to positive results. Primer sets are specific to the animal species tested. It is recommended for research purposes but also possible for enforcement or regulatory purposes if the method is validated.

To get a good sensitivity and reproducible result, DNA needs to be extracted first from the sample material using ExtractNow Meat ID Kit by Minerva Biolabs. The shelf life and storage of the kits are essential. It should be stored at 2°C–8°C for the unopened components until the expiry date shown on the label. Once rehydrated, the reagents should be kept at ≤ -18°C. The entire test needs roughly 90 minutes to run, excluding DNA extraction. Morover, the detection of animal species is done by PCR (Minerva Biolabs 2019).

LiliF HALAL Real-time PCR Kit

LiliF HALAL Real-time PCR Kit is another commercialized rapid DNA kit for halal authentication supplied by iNtRON Biotechnology. It is aimed to detect pork DNA from food and feed products, especially dairies. Thus, regulators and auditors can use it to screen for possible pork contamination in food products or animal feeds.

The LiliF HALAL Real-time PCR Kit (pork) is a qualitative Duplex rt-PCR test for detecting the porcine specific gene and the exogenous internal positive control (IPC) using specific primers and probes labelled with the fluorescent dyes. The target sequences are detected through the fluorescein amidites (FAM) and hexachloro-fluorescein (HEX) (VIC) channels. The primer and probe mixture provided exploits the so-called 5′ nuclease assay principle.

During PCR amplification, forward and reverse primers hybridize to the target DNA. A probe included in the same reaction mixture consists of an oligonucleotide labelled with a 5′-reporter dye and a downstream 3′-quencher. Furthermore, the probe is cleaved, and the reporter dye and quencher are separated in the process. The increase in fluorescence can be detected on a range of rt-PCR platforms. Internal control is employed to check the extraction method and to identify PCR inhibition. The sensitivity level of as low as 0.001 percent of pork DNA in beef-pork DNA admixture can be achieved using this technology (iNtRON Biotechnology 2018).

RapidFinder Halal ID Kit

Previously, RapidFinder technology developed by Thermo Fisher Scientific Inc. has been used to detect the presence of non-0157 STEC in meat and vegetables (Costa et al. 2019). Non-0157 STEC was detected in both food matrices with 100 percent sensitivity, specificity, and repeatability. RapidFinder Halal ID Kit is a new halal detection kit supplied by Thermo Fisher Scientific Inc. in March 2020. RapidFinder Halal ID Kit enables rt-PCR detection of pork (*Sus scrofa*) DNA present in food and feed samples.

For fresh or minimally processed meat samples, the cut-off value corresponds to 0.0005 percent (limit of detection) pork DNA when the DNA sample concentration is 10 ng/µL. Two reporter dyes for the TaqMan probe, namely FAM dye and VIC dye, are used during rt-PCR for targeting pork and IPC. Positive FAM channel result in the detection of pork DNA could either be VIC channel positive or negative. On the other hand, a result of a VIC channel positive and FAM channel negative indicates that there is no pork DNA detected. Negative results in both the FAM and VIC channels may be due to problems such as contamination or errors in DNA extraction.

The pork DNA from the positive results is then analyzed using the RapidFinder Halal ID Kit together with the RapidFinder Quant Multi-Meat Set. The RapidFinder Quant Multi-Meat set comprises the multi-meat standard, a plasmid DNA quantitation standard containing individual species-specific DNA targets, and a highly conserved animal-specific mitochondrial genomic region (total animal DNA target). The kit also includes a TaqMan assay for the whole animal DNA target. Both species-specific and animal DNA present in each sample can be quantified relative to the multi-meat standard using primer and probe sets from both kits. Thus, complete setup and run for rt-PCR could take approximately 60 minutes (excluding DNA extraction) (Thermo Fisher Scientific 2020).

CarnoCheck

CarnoCheck is the first DNA chip for determining animal constituents in food products supplied by Greiner Bio-One GmBH. CarnoCheck was developed to help consumers worldwide with concerns about health-, ethical-, religious-, or commercial-related issues by detecting the animal constituents in what they eat. With CarnoCheck, eight different animal species (donkey, cow, chicken, sheep, horse, pig, turkey, goat) can be rapidly and unequivocally identified in processed food and complex-composition products. Consequently, it can be used for halal DNA detection as it can detect pig DNA. The low detection limit (0.5%–1%, depending on the

processing) permits identifying even small traces of animal constituents. The 8-animal species' detection is carried out exclusively with molecular biological techniques and based on species-specific differences in the cytochrome b gene sequence. These differences are also used for phylogenetic and forensic investigations.

Six steps are required for detection using the CarnoCheck kit. The first step requires taking and homogenizing the samples (30 minutes). This is followed by DNA extraction using the commercialized rapid kit available (30 minutes). The third step is the amplification of the cyt b gene of all the animal species present in the sample in a single PCR with the aid of fluorescently labelled universal primers (90 minutes). Afterwhich, hybridization of the labelled PCR-products to the CarnoCheck DNA chip will be done (10 minutes). The fifth step is washing the CarnoCheck DNA chip (5 minutes), and the last step is scanning and evaluating the CarnoCheck DNA chip using any standard scanner for microarrays (10 minutes). The total amount of time needed to finish the detection using CarnoCheck is approximately 175 minutes (Greiner Bio-One GmbH 2021). Hence, this kit has the potential to be used in a rapid DNA detection for halal verification.

EasyFast Pig/Suidae detection and Quantification kit

The EasyFast Pig/Suidae detection and Quantification kit has been developed and has been supplied by Progenus. It is a rt-PCR kit that enables the detection of pig DNA (*Sus scrofa*) and other Suidae in meat-based food products with a rapid and reliable detection. The vertebrate probe serves as an IPC. In fact, the vertebrate PCR should always give a positive result, indicating the sample contains meat. The fluorescence dye acts as a detection parameter with FAM and VIC as a reporter dye. FAM focuses on verifying pig DNA while VIC looks into other vertebrates' DNA.

Apart from this, the PCR reagent consists of two components, MIX (green color) and EPC (External Positive Control, blue color). MIX contains the primers and probes for detecting the pig, vertebrate, and the reagents for rt-PCR, while EPC contains synthetic DNA, which is recognized by the pig and vertebrate probes. The results are expressed in Cycle threshold (Ct) values either for pig or vertebrate DNA. The Ct is the number of PCR cycles necessary to reach the detection threshold. The quantity of detected target is inversely related to the Ct value. Ct (vertebrate) in negative control might be between 35 and 38 if a person touches any material as the vertebrate PCR also detects human DNA. Based on its website, Progenous claimed that this kit is simple, safe, and sure, with fewer manipulations and risk

of errors. As a result, it demonstrates a suitable approach for rapid DNA detection method in halal food verification (Progenus 2017).

HaFYS Pork-DNA Detection Assay

Rapid and simple detection of raw meats and processed meat products is possible by using portable rt-PCR and without complex and laborious DNA extraction protocols. Manufactured by GeneSTAT Molecular Diagnostics at Utah, USA and under a license from Universiti Putra Malaysia (UPM), the HaFYS Pork-DNA Detection Assay is intended for the qualitative detection of pork-specific DNA from raw and processed meat samples as well as samples from food preparation surfaces and cutting instruments, personal care and cosmetic products, and animal feeds. The HaFYS assay consists of the HaFYS specimen pre-treatment kit and the HaFYS cartridge kit. The test cartridge can only be analyzed using the rt-PCR-based GeneSTAT analytical system.

Recently, the HaFYS Pork-DNA Detection Assay was performed to detect the presence of pork DNA in raw meat, processed meat products, capsule products, and animal feeds (cat food). The assay was conducted according to the manufacturer's instructions and was performed at the Halal Products Research Institute, UPM. A piece each of the meat samples the size of a grain of rice was added into the HaFYS specimen pre-treatment kit and was shaken by hand for a few minutes. The kit was then attached to the HaFYS cartridge kit and inserted into the GeneSTAT analytical system. The total run time for the assay was about 1 hour.

Figure 1 shows the representative graph for positive results. The purple line indicates that pork DNA was detected by the system with a Ct value of 24. The internal control (blue line) showed that the run was valid. The internal control was included in the system to ensure proper functioning of the analyzer and internal chemistry control to guarantee that the reagent system was also functioning properly.

As shown in Table 6, two pork samples generated positive results indicating that the HaFYS Pork-DNA Detection Assay was successful. Two cat food samples also showed positive results which meant that these products might contain pork as one of the ingredients. The other samples showed negative results. This HaFYS method was proposed to be able to detect the presence of pork in raw and cooked meats, processed food, prepared food, gelatin powder, gelatin capsules, and even kitchen and manufacturing surfaces (Gulf Bio Analytical Group 2021).

Figure 1. Detection of pork DNA from raw pork sample using HaFYS Pork-DNA Detection Assay

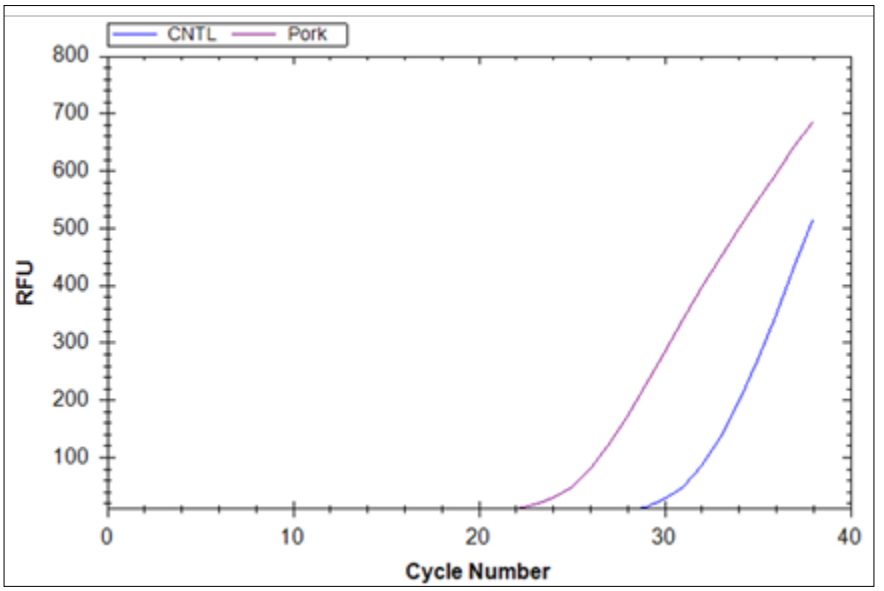

Table 6. HaFYS Pork-DNA Detection Assay results for raw and processed products including gelatin capsule

Product	HaFYS Pork-DNA Detection Assay results (+ve/-ve)
Pork (granular)	+ve
Pork (swab)	+ve
Halal-labelled capsule	-ve
Cat food (sample 1)	+ve
Cat food (sample 2)	+ve
Instant noodle paste	-ve
Halal-labelled chicken stick	-ve
Halal-labelled beef sausage	-ve

4 Conclusion

Various DNA-based technologies have been highlighted in this paper such as conventional PCR, multiplex PCR, convention PCR, PCR-RFLP, PCR-strip, rt-PCR, LAMP, nanotechnology-based biosensor, and commercial PCR kits for halal products authentication. These methods have different mechanisms and advantages depending on the chemistries of the specific DNA amplification and detection used.

However, among these methods, the rt-PCR-based platform seems to be the most promising since the method allows technical staff to see the results almost immediately while the process is still ongoing. The rt-PCR platform also generally offers good reliability, specificity, accuracy, and rapidity. Therefore, this technology could be a worthy complement to the already established document and audit-based halal certification to ensure that a halal certificate is given appropriately with scientific evidence, whenever necessary, to provide clarity and transparency to the consumers, especially when involving animal-based food products.

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