Journal Halal Science, Industry, and Business, Vol. 3 No. 1 (2025)https://doi.org/10.31098/jhasib.v3i1.3178

Research Paper

Minimum Limit of Detection of Pork Fat DNA in Halal Food Mixtures Using Real-Time Polymerase chain reaction (PCR) technique

Duaa Mughal[1*], Syeda Areeba[2], Shakil Ahmed[3], Ishtiaq Ahmed[4], Syed Ghulam Musharraf[5],

[1,2,3,4,5]University of Karachi, Pakistan

Abstract

A practical scientific approach was developed and implemented to detect raw pork meat and fat. This study aimed to determine the minimal limit of detection (LOD) of pork fat DNA in a halal food mixture using Real-time PCR. The minimum LOD was determined for 0.01% and 0.001% pork fat DNA in a mixture of halal meat. A comparative analysis was conducted on DNA isolated from raw pork meat and pork fat. DNA extraction was performed using the Qiagen DNeasy Mericon Food Kit. Meat samples, including chicken, camel, and bovine, were collected from the local market, whereas pork fat samples were obtained from customs. Pork fat was mixed at 0.1%, 0.01%, and 0.001% (w/w) concentrations in the halal meat mixture. DNA band size identification of pork meat and pork fat was conducted using gel electrophoresis, and results were validated through test repeatability. The findings confirmed that Real-time PCR could detect pork fat at a minimum concentration of 0.001%, establishing it as the LOD. This study first identifies the minimum LOD of pork fat (0.001%) in halal food mixtures. Time constraints limited the research and manual DNA extraction yielded higher DNA quantities but was more time consuming than kit-based methods.

Keywords *Pork fat; Raw Pork meat; DNA Isolation; Porcine DNA quantification; limit of detection (LOD)*

INTRODUCTION

Consumer protection has become a significant issue for national and international bodies in the food industry (Poms et al., 2001). Consumers are concerned about various problems, such as food adulteration and authenticity. This has raised awareness about the content of food goods. In many countries, food makers use lard instead of oil because it is inexpensive and more readily accessible. Pork meat and pork fat have been considered important problems by certain religions, including Islam and Judaism. Daily ingestion may cause biological problems and health concerns. Food derived from pigs is considered Haram (unlawful or banned) in Islam (Nakyinsige et al., 2012). As a result, in the modern era of open trade and mass market globalization, some food companies use every chance to boost profits while ignoring necessary food safety measures based on religious or cultural convictions. The analytical procedures used to identify the adulteration of fats and oils, including lard, are based on variations in the nature and content of the adulterant's minor and major components against those of pure fats or oils. These techniques frequently depend on their physiochemical constants or Biological and Chemical data (Kowalski, 1989).

Species mislabeling is a standard type of economically motivated adulteration (EMA) in which a product is purposefully mislabeled for financial advantage (FDA). Meat species mislabeling has economic incentives because different meat species have different prices. There is a financial incentive to use pork instead of beef in processed products because, for instance, US$13.31/kg was the average retail value of beef in 2015. In contrast, in the same year, the average retail value of pork was around US$8.49. From slaughterhouses to the moment of sale, substitutions can occur at any stage of the supply chain. According to earlier research, cross-contamination can also result in mislabeled meat products being mislabeled when processing different kinds of meat on the same machinery (Premanandh, 2013). For example, several meat fraud incidents have been highlighted

Corresponding author's email: duaamughal62@gmail.com

in the literature, providing incentives for meat detection efforts. The recent Johor Baharu meat cartel issue, in which mixing of non-halal with halal food was sold as halal food using a fake Halal label, outraged Malaysians, especially Muslims (Riza et al., 2022). In Europe, some traces of horse meat are found in precooked foods marked as 100% buffalo (Premanandh, 2013). In Malaysia, another example of adulteration of beef meatballs with buffalo, and in Bali, Indonesia, sliced marinated chicken meat was replaced with dog meat (Hossain et al., 2017). In China, rat meat is sold as mutton. Therefore, to prevent food fraud, sustain food companies and protect consumer confidence, proper labelling of meat and meat-related product ingredients and field monitoring is important.

Adulteration of Pork origins in food items is strictly prohibited in Islam. According to Islam, this religious restriction applies to any part of the pig, including skin, meat, and derivatives (lard, enzyme, and others). As a result, lard-containing goods must be prominently identified on food labels. Several strategies have been developed to help customers select food goods. Examples of these methods are FTIR spectroscopy, HPLC, and PCR. Muslim scholars should be motivated to investigate detecting food adulteration strategies to protect Muslim customers while selecting food goods for consumption (Fadzlillah et al., 2011).

Pork detection in halal food mixtures can be easily performed by genomic DNA analysis or using proteins as the targeted molecules (Ali et al., 2014). Protein-based assays are particularly sensitive for raw meat analysis but less effective for analyzing pork fat. This is because protein molecules may differ between different tissues or body parts, resulting in inaccurate decision-making regarding the absence or presence of the pork contaminant (Ibarguren & Villamarín, 2017). In this paper, a minimum limit of detection using the qPCR technique was developed. 0.1%, 0.01%, and 0.001% (w/w) pork in a mixture of beef, chicken, and camel meat was detected along with the comparative analysis of the Genomic DNA of pork meat and pork fat.

Real-time PCR assays, among various PCR methods, based on targeted DNA have recently been applied to food samples to identify species of animal origin. Two main techniques are based on introducing fluorescent dyes, such as the TaqMan fluorogenic probe and SYBR Green. The TaqMan® probe method utilizes an additional probe and primer, which bind exactly to the targeted DNA sequence at the time of the annealing step. Probe-based chemistries typically depend on TaqMan fluorescence probe technology. On one end, TaqMan probes have a fluorescent reporter dye, and on the other side, a quencher dye that prevents fluorescence. During the extension stage, the probe is split into two parts for detecting the targeted species using the TaqMan-based real-time qPCR method (Rodríguez et al., 2005).

Compared with other previous studies, Karabasanavar et al. (2014) detection limit was 10 pg, which is 0.1%, indicating that pork meat was detected through species-specific PCR. Real-time qPCR is one of the most sensitive methods for identifying DNA molecules. Also, Martín et al. (2009) reported a study of mixtures that demonstrated the method's suitability for identifying pork DNA in mixtures containing as low as 0.1%.

The study's main objective was to develop a cheap, reliable, and fast molecular technique for foods containing "Halal" tags for pork impurity by specific gene targets. In this study, Genomic DNA from pork fat was chosen as the target of investigations along with the minimum detection limit in a halal food mixture. This research aimed to identify a method for identifying pork samples in halal food mixtures. Taq-man-based real-time PCR analysis was used to quantify Pork fat DNA, i.e. 0.1%, 0.01%, and 0.001% (w/w) in the mixture of halal food samples. The real-time PCR procedure is commonly based on the TaqMan fluorescence probe principle. The probe is labelled with a quencher and reporter dyes, targeting DNA attached to the primers. During the reaction, fluorescence emission is identified and is directly related to the amount of specific amplified PCR products (Holland et al., 1991).

Establishing a real-time PCR test to precisely measure the amount of pig fat DNA in commercial beef, chicken, and camels was the goal of this study. For this purpose, a kit-based technique was developed to amplify a portion of the pig's genomic DNA, which was subsequently applied to real-time PCR.

LITERATURE REVIEW

Food adulteration and misrepresentation are increasing globally, impacting trade and food safety standards. Authorities have enforced strict regulations following incidents like the horse meat scandal. Adulteration refers to making food impure by adding inferior or improper ingredients. In the meat industry, high-value species are often replaced with lower-value ones for profit. Accurate labelling is essential for informed consumer choices. Research by Mohamad et al. (2024) demonstrated the assay's effectiveness in detecting lard in processed foods, with a detection limit of about 1%.

As a result, food authenticity is now a standard monitoring criterion for confirming food labelling and legal concerns (Bonne & Verbeke, 2008). One crucial factor in determining the safety and quality of food is authenticity. For Muslim and Jewish consumers who are forbidden from consuming pork and its derivatives in any form, foods certified as Halal or Kosher are readily accepted. To detect meat from other animal species as contaminants or to identify pig adulteration in mixtures of meat samples, a variety of analytical techniques have been employed (Ali et al., 2015)

These include ELISA (enzyme-linked immunosorbent assays), high-performance liquid chromatography, Fourier transform infrared (FTIR) spectroscopy, and electrophoretic techniques (Cai et al., 2012). According to Al-Kahtani et al. (2017), real-time PCR (qPCR) and conventional PCR techniques were used to find pork DNA in meat combinations. We used 75 commercial food items and 30 meat mixes with different proportions of pig (0%, 1%, 5%, 10%, and 20%), including beef, chicken, camel, rabbit, goat, and sheep.

Pork DNA assay combined with polymerase chain reaction (PCR) offers a quick, sensitive, and particular substitute for protein-based techniques. Real-time PCR is a helpful tool for determining minute amounts of different species, especially in complex foods, and conventional PCR procedures typically produce qualitative results of the detected species (Soares et al., 2010). Sheep, chicken, beef, and pork are among these goods' most frequently unreported species. From a religious perspective, these results are troubling because several religions forbid eating beef or pork (Perestam et al., 2017). In addition, previous research in the 2013 European horsegate scandal revealed that horsemeat was an unreported ingredient in many beef products (Issa et al., 2024).

According to Chiş and Vodnar (2019), real-time PCR analysis was effectively refined and verified for processed meat products, enabling the detection and measurement of meat levels as low as 0.1% for beef and pig and 0.02% for chicken. The technique's correctness, precision, and repeatability were confirmed through successful validation using low-meat products (beef, swine, and chicken sausages). With an accuracy of detecting 0.1% DNA in the examined products, real-time PCR is a quick and easy way to identify different types of meat. We hypothesized that pork fat DNA can be detected with high sensitivity and specificity in mixed food matrices using Real-Time PCR, with a limit of 0.01% concentration.

RESEARCH METHOD

Table 1 summarizes the step-by-step process for preparing samples to detect pork fat DNA in halal meat mixtures. The procedure includes sample collection, weighing, mixing, and homogenization to ensure uniform DNA distribution. Proper storage at -20°C preserves sample integrity before DNA extraction using the Qiagen DNeasy Mericon Food Kit, which was selected for its efficiency and reliability. The DNA concentration was quantified using a Qubit Fluorometer 3.0

to ensure quality before Real-time PCR analysis, which was optimized for pork DNA detection. Each step was conducted under sterile conditions using specialized equipment to maintain accuracy and prevent contamination.

Table 1. Process of Research Methodology

Step	Description	Equipment Used
Sample Collection	Chicken, camel, and bovine meat were obtained from the local market; pork fat was obtained from a custom sample.	Sterile gloves and collection bags
Sample preparation	Pork fat was mixed with halal meat at 0.1%, 0.01%, and 0.001% (w/w) concentrations.	Analytical balance
Homogenization	Samples were ground into a fine paste to ensure an even distribution of the pork DNA.	Tissue lazer and microcentrifuge tubes
DNA Isolation	DNA was isolated using a Qiagen DNeasy Mericon Food Kit.	Qiagen DNeasy Mericon Food Kit, centrifuge
DNA Quantification	DNA concentration was measured before PCR.	Qubit Fluorometer 3.0
PCR	DNA amplification was performed using Real-time PCR.	Qiagen rotor gene

Sample Preparation

Samples of beef, chicken, and camel meat were obtained from the local market. The pork fat sample was obtained from the customs. After that, all meat was cut, and the remaining portions were rejected. All samples were finely minced in a separate tray, and reference mixtures containing 0.1%, 0.01%, and 0.001% (w/w) pork fat in beef, chicken, and camel meat mixture as shown in Table 2 were prepared to a final sample of 200mg as shown in the table—one by gradual additions of minced meat samples.

Table 2. Sample Preparation of Halal Food Mixture Containing 0.1%, 0.01%, and 0.001% (w/w) Pork

	Sample 1 (0.1%)	Sample 2 (0.01%)	Sample 3 (0.001%)
Pork fat	0.2 mg	0.02 mg	0.002 mg
Halal food mixture (Chicken, Beef, Camel) Meat	199.8 mg	199.98 mg	199.998 mg

DNA Isolation

DNA was isolated from a 200mg sample of each homogenized meat mixture according to the user manual of the Qiagen DNeasy® Mericon Food Kit (QIAGEN, Germantown, MD, 93 US). Using a mortar and pestle, 2.0 g of each admixed sample was homogenized in liquid nitrogen, and the manufacturer's protocol extracted the gDNA. The Qiagen DNeasy Mericon Food Kit was chosen for DNA extraction because of its efficiency, reliability, and ability to extract high-quality DNA from complex food matrices. Compared with manual methods, this kit reduces contamination risks and ensures efficacy. The Qubit High-Sensitivity dsDNA test kit (Thermo FischerScientific, MA, US) was used to measure the amount of the isolated gDNA. 1% agarose ge electrophoresis was used to

evaluate the quality of the extracted gDNA. The purity of the gDNA was evaluated using a Qubit Fluorometer 3.0 (Invitrogen Life Technologies, US). The isolated DNA wasz stored at −20 °C until further study.

DNA Quantification using Florescent Technique

The extracted samples were quantified using Qubit Fluorometer 3.0 (Invitrogen Life Technologies, US). Qubit standards S1 and S2 were prepared by adding 189 µL of stabilizing buffer along with 1ul of fluorescent dye and 10 µL of standard, which were provided along with the Kit. Sample preparation was performed by adding 198ul of buffer into a tube (Qubit Assay Tubes, Thermo Fisher SCIENTIFIC) containing 1 µL dye. 1 µL DNA sample was added, incubated in the tubes at room temperature (25 ± 5 ºC) for 1 minute and inserted in a Qubit fluorometer to take readings (Demeke & Jenkins, 2010). Hence, the quality of the extracted genomic DNA was determined via 1.5% agarose gel electrophoresis.

Real-Time (qPCR) Assay

The use of qPCR for pork DNA detection has the benefit of being a practical approach for detecting, identifying, and quantifying pork in various types of meat-derived products. Real-time PCR combines target-specific primers with TaqMan probes for targeted amplification (Muflihah et al., 2023), as shown in Figure 1. Real-time PCR amplification with TaqMan® probes was performed using a Real-time PCR (QIAGEN Rotor-Gene Q, Germany). The total volume is 25 µL PCR reaction mixtures that contain 12.5 µL Basic Mix and 7.5µL of Oligo Mix with 5 L of DNA template (approx. 10 ng.) Real-time PCR was set on conditions where the initial denaturation step was 95°C for 10 min and 45 cycles at 95 °C for 15, whereas amplification was done at 60 °C for 90 sec. The results were interpretation was done using QIAGEN rotor gene Q series software (version 2.3.1). For the negative control, a no-template control (NTC) was used for this method. Real-time PCR assay was done using a based amplification method. The Eurofins|GeneScan DNAnimal Identification Halal RT PCR kit, which contained the pork target gene, was used.

Limit of Detection

For the identification of the quantitative method for meat products, the limit of detection was determined using the threshold value (Ct) obtained from the pork fat DNA. Amplification curves were constructed using DNA samples containing known 0.1%, 0.01%, and 0.001% (w/w) concentrations of pork fat in beef, chicken, and camel meat mixtures. Potential sources of error in DNA extraction and PCR quantification are:

1. DNA Extraction Errors: Incomplete lysis, low DNA yield, or contamination from non-target DNA.
2. PCR Quantification Errors: Pipetting inconsistencies, primer inefficiency, PCR inhibitors in food samples, or cross-contamination leading to false positives/negatives.

FINDINGS AND DISCUSSION

DNA Quantification using Florescent Technique

The extracted DNA was quantified using Qubit Fluorometer 3.0. It is a suitable and specific technique for identifying the DNA concentration at the nanogram level. The extracted DNA samples were quantified using a wavelength range of 430–495 nm (blue) and 510–580 nm (green) to detect the purity and yield of all extracted DNA. Pork meat contains a higher DNA yield than pork fat. The average concentrations and standard deviations of pork fat DNA in different mixture percentages were calculated using three independent replicates (n = 3). The concentration of DNA from Pork fat and pork meat, along with different concentrations, are shown in Table 3.

Table 3. Concentrations (ng/µL) of Extracted DNA

Sample	Mean DNA Concentration (ng/µL) ± SD
0.1% Pork fat in Halal mixture	75.6 ± 2.3 (n = 3)
0.01% Pork fat in Halal mixture	6.4 ± 0.4 (n = 3)
0.001% Pork fat in Halal mixture	1.5 ± 0.2 (n = 3)

Identification of band size by Gel Electrophoresis

DNA was extracted from pork meat and pork fat and visualized using the agarose gel electrophoresis. DNA is separated based on molecular mass and polar charges. Both samples (Pork meat and Pork fat DNA) were loaded onto agarose gel and allowed to run for 60 min at 120 Volts. Short DNA fragments move faster and faster than large molecular weight fragments. The DNA band of pork meat DNA appeared slightly above the standard ladder, as shown in Figure 2

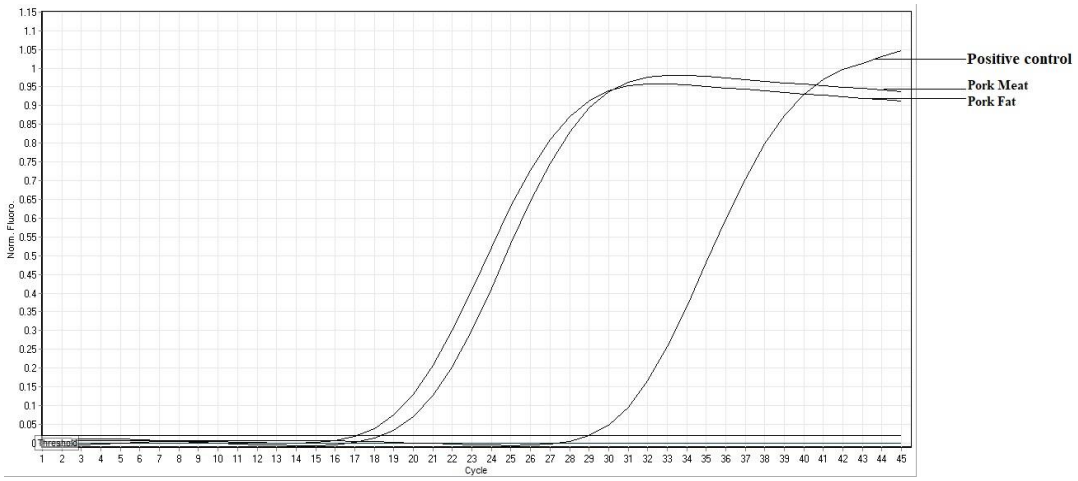

Figure 3. Amplification Curves of Raw Pork Meat and Pork Fat

The authenticity of Halal labelling in processed goods depends more and more on verifying hog fat in meat products. Here, we report a highly accurate and repeatable Real-Time qPCR assay for detecting the DNA of pork fat in meat mixtures. Although the qPCR method provided high specificity and sensitivity, several limitations were observed, such as the presence of proteins and fats in complex food matrices, which may hinder DNA extraction efficiency and lead to reduced PCR sensitivity. Future studies should explore additional pre-treatment methods to enhance DNA recovery. Specific components of processed food products, such as polyphenols and salts, may act as PCR inhibitors, affecting DNA amplification. Using an internal control gene in future studies may help detect inhibition.

Limit of Detection

Research by Mohamad et al. (2024) demonstrated the assay's effectiveness in detecting lard in processed foods, with a detection limit of about 1%. In this research work, 0.001% pork fat DNA was detected in halal food mixtures by qPCR, which was considered the minimum limit for detection. 0.1%, 0.01%, and 0.001% (w/w) concentrations of pork fat in beef, chicken, and camel meat mixtures were analyzed to determine the minimum detection limit using qPCR. Figures 4, 5, and 6 show the amplification curve where 0.1% shows a high Ct value compared to others because

of the high concentration of pork contamination. Among all three concentrations, 0.001% pork fat DNA was detected in the halal food mixture via qPCR, which was considered the minimum limit for detection. The mean Ct value for 0.1% pork fat was 28.53 ± 0.12 (n = 3). The threshold (Ct) value for 0.01% pork fat concentration was 26.5 ± 0.23, and The mean Ct value for 0.001% pork fat was 32.2 ± 0.35 (n = 3)

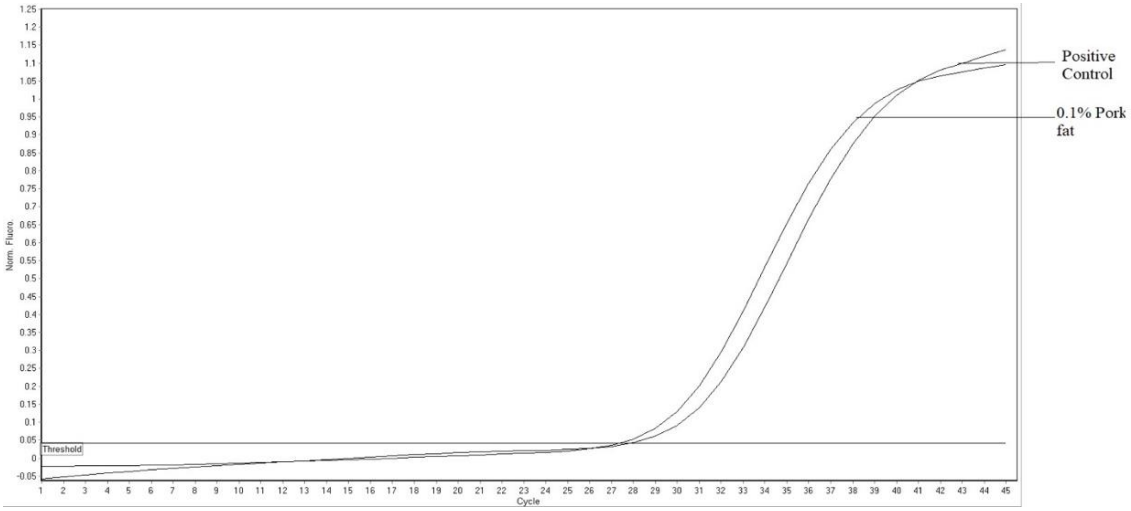

Figure 4. Amplification Curve Showing 0.1% Pork Contamination.

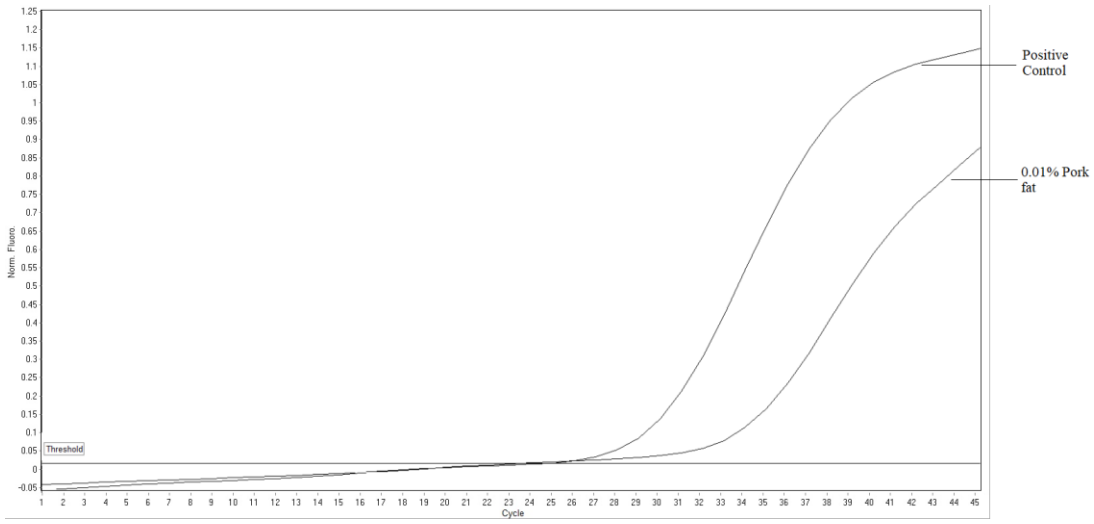

Figure 5. Amplification Curve Showing 0.01% Pork Contamination.

Discussion

Mixtures of halal meat spikes with 0.1%, 0.01%, and 0.001% pork fat were used for halal identification at the minimum level. In this study, a method for determining the minimum limit for detecting pork fat was developed. Halal authentication using detecting 0.001% pork fat in halal food mixture was performed using a real-time PCR technique. DNA isolation from pork fat is critical because fat content can cause DNA hindrance, so the genomic DNA kit-based approach was used to isolate it. Detection of the genomic DNA band size of pork meat and Pork fat agarose gel was performed. The gel electrophoresis technique identified the genomic DNA of Pork Meat and Pork fat, showing the large band size of pork meat compared with pork fat. The real-time PCR approach is a sensitive and specific technique for identifying the minimum limit of pork fat in a complex mixture of halal foods. Due to the low DNA concentration in the 0.001% sample mixture, the Ct value is low compared to others.

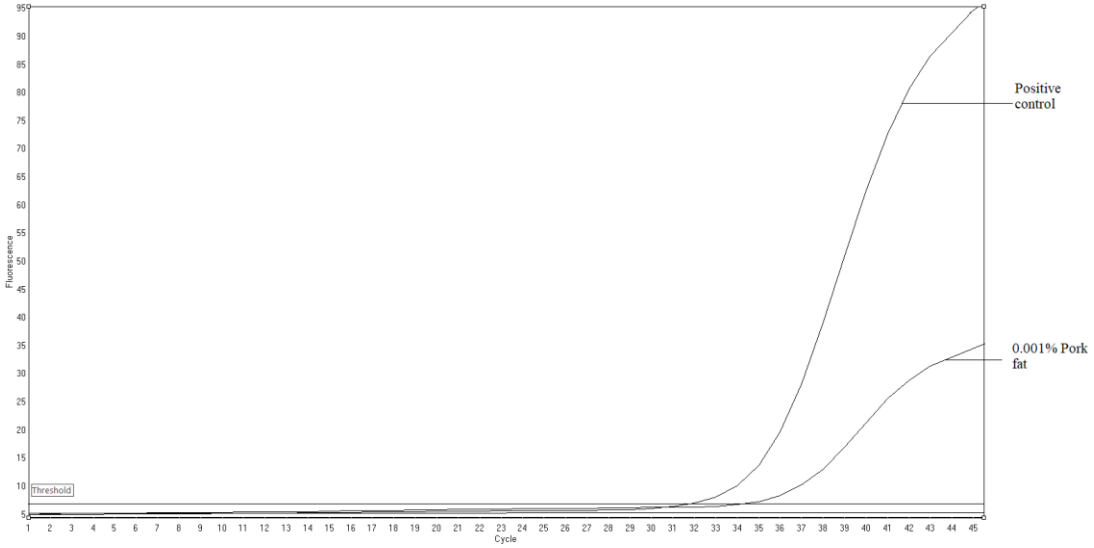

Figure 6. Amplification Curve Showing 0.001% Pork Contamination.

In order to give customers correct information about the items they purchase, it is becoming more important to identify the species in food while evaluating its composition (Rodríguez et al., 2005). Meat products must be appropriately labelled concerning their species by EU labelling laws. The adulteration of meat species in-ground and commented products has recently become a significant issue in retail marketplaces (Asensio et al., 2008). Typically manufactured from beef, chicken, lamb, and turkey, meat extract is a highly concentrated meat stock used to make drinking broth and to add flavor to food. Iran's use of commercial meat extracts has increased significantly over the past ten years, and the majority of goods bearing the Halal label are imported from non-Muslim nations. Muslims who observe their religion want protection from meat products with misleading labels, and the detection of hog DNA in meat extracts is crucial for Halal authenticity. In this sense, accurate techniques for detecting pig byproducts in food items are essential for food ingredient analysis (Farrokhi & Joozani, 2011). Compared with previous study by (Karabasanavar et al., 2014), sensitivity for the detection of pork meat in other species meat using unique pork targeted PCR was developed to be at 0.1% limit of detection (LOD) of pork DNA was 10 pg (picograms). A TaqMan real-time PCR was also done to identify pork contamination in chicken nuggets. Research conducted using chicken nuggets mixed with pork showed that the method could quantify 100–0.01% of pork adulteration (Ali et al., 2012).

Compared with reverse transcription polymerase chain reaction (RT-PCR) based on single and nested gels, the real-time PCR approach often achieves better sensitivity, is less time-

consuming, labor-intensive, and less susceptible to cross-contamination. Since an annealed probe is needed in addition to the annealing of both primers, probe-based techniques like TaqMan may have higher specificity than the SYBR Green method (Gyarmati et al., 2007). The main objective of this research was to create a quick, affordable, and accurate molecular assay for food testing and to use the simple species-specific band identification of PCR products to screen items with "Halal" tags for pig contamination (Alaraidh, 2008).

CONCLUSIONS

The adulteration of halal food with pork is a fraudulent practice that affects the halal status and quality of halal food products. Multiple methods have been identified for the detection of food fraud. Among all the methods, real-time PCR is currently known as the most sensitive method to detect (DNA) nucleic acid molecules because most spectroscopic methods produce vast quantities of data, have a long computation time, and are unsuitable for real-time applications. However, real-time PCR-based techniques are particular and sensitive. The DNeasy Mericon Food Kit (Qiagen) developed a technique appropriate for regularly isolating gDNA from meat and fat samples. Genomic DNA was deemed appropriate for use as PCR templates in this kit. A dependable and valuable technique for identifying technically undesirable contamination or intentional mixtures in highly processed food, such as meat products, is the TaqMan probe-based assay, which can be suggested for detecting animal tissue by laboratories or food agencies. In this work, real-time PCR was used to assess the detection sensitivity of DNA in pork fat. DNA from hog fat was added to a complicated mixture of beef, chicken, and camel meat at concentrations of 0.1%, 0.01%, and 0.001%. At a minimum concentration of 0.001%, or as little as one copy of pork DNA in the mixture, the technique detected the DNA.

The ability to detect pork DNA at concentrations as low as 0.001% has significant implications for halal food certification and consumer trust. This method enhances the reliability of halal labelling by ensuring that trace contamination can be identified. Regulatory authorities, including halal certifying agencies, such as JAKIM (Malaysia), SANHA (South Africa), and the Pakistan Halal Authority, can adopt this sensitive real-time PCR technique to routinely screen imported and processed meat products. The findings help prevent fraudulent practices, ensure compliance with religious dietary laws, and support international trade where halal integrity is critical.

LIMITATION & FURTHER RESEARCH

This study successfully extracted and detected pork DNA from raw pork meat and fat, with a limit of detection (LOD) as low as 0.001% using real-time PCR. However, certain limitations highlight areas for refinement and further investigation. One primary limitation encountered during the study was time constraints. The choice of DNA extraction method played a critical role in balancing the time efficiency and DNA yield. This study used a kit-based DNA extraction method due to its simplicity and rapid processing capabilities. Although effective, manual DNA extraction methods generally yield more DNA. Manual extraction requires meticulous handling and optimized protocols for increased nucleic acid recovery. However, this approach is considerably more time-consuming than kit-based methods, which makes it less feasible when working within strict timelines. Future studies could address this limitation by allocating sufficient time to comprehensively explore manual extraction techniques to compare yield and quality against kit-based methods.

Another key limitation of the present study is the sensitivity and sample size for detecting pork DNA. Although the study demonstrated the ability to detect pork DNA at a LOD of 0.001%, this low detection threshold may pose challenges in precise quantification, particularly in complex

sample mixtures. When mixtures of various components, such as beef, chicken, and camel meat, achieving consistent and accurate detection of trace amounts of pork DNA becomes increasingly challenging. Variability in the sensitivity of the detection method can lead to potential discrepancies in the results, especially when working with highly diluted or heterogeneously mixed samples. In addition, the minimum sample size used in this study limits the findings. Although the results are promising, the method must be validated across a broader range of conditions and sample types to ensure reliability and reproducibility. For example, detecting pork DNA in processed foods, heat-treated products, or samples containing inhibitors may yield different results, necessitating further testing.

Future research should aim to refine and optimize DNA extraction and detection processes to overcome these limitations. Improving the sensitivity of the detection method, such as through enhanced PCR protocols or the use of advanced molecular tools, can help achieve consistent LODs across various sample types and conditions. Furthermore, studies should consider using larger and more diverse sample sizes to validate the robustness of the method across different matrices and product types. Another avenue for future investigation is exploring the detection method's applicability to various industrial and regulatory contexts. This could include testing its effectiveness in detecting pork DNA in processed or adulterated products, ensuring compliance with labelling standards, or addressing consumer concerns about food authenticity. Additionally, integrating novel technologies, such as next-generation sequencing or digital PCR, could enhance detection precision and address the limitations of traditional real-time PCR.

In conclusion, while this study provides valuable insights into detecting pork DNA with a low LOD, addressing its limitations will be crucial for advancing the methodology. Future research should optimise DNA extraction protocols, improve sensitivity, expand sample diversity, and validate the method under broader conditions to achieve more accurate and reliable outcomes.

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