Research Article

Istihalah and Porcine DNA Detection in Chickens Fed with Pig-Derived Feed: Real-Time PCR Assessment of Purification Timeline

Mohd. Nazri bin Abdul Rahman[1,2], Siti Munirah binti Mohd Zaini[2], and Nor Hayati Muhammad[3]

[1]Nutritional Biochemistry Research Group, Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[2]Programme of Food Science and Nutrition, Faculty of Food Science and Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia.
[3]Centre of Foundation Studies, Universiti Teknologi MARA, Cawangan Selangor, Kampus Dengkil, 43800 Dengkil, Selangor, Malaysia.

*mdnazri@ums.edu.my

ABSTRACT

The purification of animals from prohibited dietary substances (al-jallalah) through the process of "istihalah" is critical for ensuring halal compliance. However, the appropriate duration of quarantine required for chickens that have consumed pig-derived feeds to be considered purified remains unclear. This study hypothesised that a short-term quarantine of three days would be insufficient to eliminate porcine DNA from the chickens. To evaluate this, eighty broiler chickens were divided into control and treatment groups. Only two chickens from each group were analysed per day across three consecutive days after the feeding phase. Detection of porcine DNA was conducted using real-time PCR and gel electrophoresis. The results revealed the presence of porcine DNA in several intestinal and faecal samples, with Cq values ranging from 22.6 to 34.7. These findings indicate that the three-day quarantine period was inadequate to fully remove detectable porcine DNA. This study underscores the importance of extending the "istihalah" period and provides scientific support for establishing purification timelines in halal animal husbandry practices.

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1. Introduction

Globally, the Muslim community represents 23.4% or 1.6 billion of the world's population, with the majority of Muslims coming from the Asia Pacific region, which accounts for 61.9%, while the Middle East accounts for 20.1% (Shah & Yusof, 2014). Given that Muslims are very particular about food selection, this has indirectly contributed to the growth of the halal food market, which comprises 16% of the global food industry and is expected to rise to 20% in the future (Azam & Abdullah, 2020). The selection of halal and good food, also known as "halalan thoyyiban," is a mandatory requirement for Muslims. According to Abd Razak & Adaha, (2022), the term "thoyyib" refers to healthy, meaning nutritionally adequate and balanced, suitable for the body's needs, and safe. Therefore, the status of food or ingredients that are doubtful in

their halal status, or "mashbooh" or "syubhah," will cause concern among Muslims due to doubts arising from the use of certain ingredients and processes (Zakaria & Shoid, 2023; Tukiran, Anuar & Jamaludin, 2023; Ahmad Anuar, Tukiran & Jamaludin, 2023). The word "mashbooh" is derived from Arabic, meaning doubtful (Abdallah, Rahem, & Pasqualone, 2021; Al-Teinaz, 2020). In Islamic jurisprudence, "mashbooh" or "syubhah" refers to something whose halal and haram status cannot be determined (Bujang, Abidin, & Nizar, 2023).

Generally, livestock such as cattle, fish, chickens, and similar animals that are regularly or occasionally fed dirty or impure food, or "najasa," are termed "al-jallalah." This is because animals like pigs (*Sus sp*.) and pig-derived sources are considered impure or najis in Islam and are haram to be consumed (Wan Ismail & Mahamad Maifiah, 2023). The prohibition of eating pigs and pig-derived products is clearly stated in the Quran in Surah al-An'am (6):145, which explains that consuming carcasses, flowing blood, or pork is haram. However, "al-jallalah" animals can become halal again if quarantined or "al-istibra'" for a certain period until the smell, taste, and colour are gone (Ghazali & Sabjan, 2024). During the quarantine period, the "al-jallalah" animals must be fed clean food. According to Noordin *et al.*, (2024) only animals that are regularly and consistently fed dirty food in large amounts compared to clean food are considered "al-jallalah," while animals that occasionally consume dirty things are not. Thus, the quarantine period during the "istihalah" process varies for each type of livestock.

Generally, the "istihalah" process is a natural purification process that occurs during the animal's quarantine period. Terminologically, "istihalah" means transformation and change, and in detail, it has three main forms: physical and content change, physical change only, and content change only (Al-Shiha *et al.*, 2024). The structure of "istihalah" involves three main elements: raw material, transformation agent, and final product (Jamaludin, 2012). Scholars have conducted "ijtihad" to resolve issues concerning "mashbooh" in food content, considering the questionable halal status of the food. Malaysia follows the Shafi'i school of thought, and according to it, "istihalah" is viewed more strictly, and its application is accepted only if it involves a natural transformation process without the addition of unnatural substances (Hamdan *et al.*, 2024).

Therefore, due to cases causing doubts about the halal status of food, advanced technology has been intensified for sample analysis to confirm halal status. Techniques used in halal development include Fourier Transform Infrared (FTIR) spectroscopy, chromatography technology, and electronic nose (EiNose) technology (Razak *et al.*, 2020). One of the most frequently used methods is polymerase chain reaction (PCR). PCR is a scientific method in molecular biology used to amplify one or a few DNA templates, producing thousands to millions of copies from the original DNA template (Kadri, 2019). This makes PCR highly effective in identifying species contained in samples. PCR is suitable for food analysis, particularly for halal certification, as it can detect pig DNA in samples (Kua *et al.*, 2022). The "istihalah" period can be determined if pig DNA is no longer found in the animal's digestive tract.

The comparative study of confinement periods for the purification process of chicken meat is crucial to further understand the appropriate duration or method of confinement for "al-jallalah" animals, as scientifically proven. This is because no scientific studies have been conducted on the purification process for chickens fed with impure or "najasa" foods such as pig blood and liver. This scientific research is important to strengthen the Islamic scholars' theories regarding the confinement or quarantine period of "al-jallalah" animals for the "istihalah" process (Abdul Rahman & Mohd Laziz, 2012). The study attempts to mirror free-range chicken farming systems, where chickens are raised freely to forage for their own food, potentially consuming haram sources, such animal excretion, blood, wine, pork and products which can be categorised as impure and haram. Furthermore, this study is also important to reflect the situation where farmers use commercial chicken feed without a halal logo, leading to doubts about the halal status of the chicken feed, as it may have been mixed with by-products from other animals used as additional protein for the livestock.

An example of a similar case occurred around 2007, when Malaysia was shocked by reports of catfish being fed with pig protein sources to promote their growth in the state of Perak (Berita Harian, 2007; Malaya, 2011). According to the 73rd National Fatwa Committee Muzakarah held in April 2006, the status of fish raised in fish farms and similar environments is haram if the fish are intentionally kept in impure water or fed with impurities such as pig fat, carcasses. Different schools of Islamic jurisprudence

(Shafi'i, Hanafi, Maliki, Hanbali) interpret istihalah in varying degrees of strictness, especially concerning natural transformation. In Malaysia, the Shafi'i school governs halal rulings. The National Fatwa Committee (2006) declared that animals intentionally fed with impurities such as pig offal are haram unless purified through proven methods.

On a global issue, according to a report by Morell & Daniel, (2014), it was proven that "hydrolyzed protein" supplements containing pig sources and other animal waste such as cowhide, bones, and others are widely used in European countries, particularly in the Netherlands and Germany. Moreover, due to their low cost, these pig protein supplements are used to shorten the maturation period of chickens from 28 to 30 days compared to the actual period of 35 to 40 days. This situation indirectly challenges Muslims living in these European countries to obtain halal raw chicken meat.

Consequently, according to "Regulation (EC) 999/2002" from the "European Parliament and of the Council," ruminant animals fed with mammalian protein sources are prohibited. This means that non-ruminant livestock such as pigs, horses, and chickens are prohibited from being fed with food formulations from animal proteins such as meat and bone meal, fur, chicken organs, and others. Instead, foods such as blood products, "dicalcium phosphate," and "tricalcium phosphate" from animal sources categorised as restricted protein can be fed to non-ruminant livestock (van Raamsdonk *et al.*, 2019).

This study serves as a basis for determining the duration or period of confinement for the "istihalah" process for broiler chickens fed with food formulated with pig blood and organs (*Sus sp.*). The importance of this study is to support the theory of "fiqh istihalah" from both Islamic and scientific perspectives, as it can help Muslim farmers address the issue of commercial chicken feed that is suspected of being contaminated with pig protein or other impurities.

Istihalah reflects biochemical conversion of substances, aligning with the principle of transformation through metabolism. Halal authentication typically involves confirming the absence of prohibited DNA in animal tissues, although standard regulatory guidelines vary and need clearer scientific support. Literature shows DNA degradation in poultry digestive systems occurs over time, with residual DNA sometimes detectable post digestion (He *et al.,* 2020; Manna & Lanza, 2021). Few studies explore DNA degradation kinetics and clearance timelines in poultry; comparative analysis is needed with existing models (e.g., rodents or fish). Earlier work on catfish (Wan Norhana *et al.,* 2012) and Patin fish (Abidin & Ahmad, 2016) offer comparable models to inform this poultry study. Wan Norhana *et al*. reported porcine DNA clearance in catfish fed with 5% porcine offal after a quarantine period of 14 days. DNA was extracted from muscle, gut, and skin tissues using PCR techniques similar to this study.

Additionally, no scientific writing has been done to explain how to determine the quarantine period for the "istihalah" process for chickens, apart from the reference study by Wan Norhana *et al.* (2012) and Abidin & Ahmad (2016), which studied the quarantine period for the "istihalah" process for catfish, and Patin fish (*Pangasius sutchi*), respectively and actively discussed among researchers (Ghazali & Sabjan, 2024; Noordin *et al.*, 2024; Chowdhury *et al.*, 2023; Kartika *et al.*, 2022; Alias & Zabidi, 2021; Razali, *et al.*, 2021; Saidin, *et al.*, 2017).

Halal verification through molecular techniques, such as DNA detection, is increasingly used as an interpretive tool in determining the status of "al-jallalah" animals. The absence of porcine DNA in any part of the digestive tract may be considered as indicative of purification through "istihalah", based on fatwa guidelines (JAKIM, 2006) and scientific practices aligned with halal authentication standards (Nurjuliana *et al*., 2011; Rahmati *et al*., 2016). However, regulatory standards for such molecular confirmation are still evolving and may vary across jurisdictions.

The aim of this study is to identify the appropriate confinement period during the "istihalah" process for chickens fed with food formulated with pig (*Sus sp.*) blood and organs for halal verification. This will be done by detecting porcine deoxyribonucleic acid (DNA) from tissue samples of the small intestine, large intestine, stomach, and chicken faeces across different confinement periods.

2. Materials and Methods

2.1 Tissue Samples of Stomach, Small Intestine, Large Intestine, and Faeces from Chickens

One-day-old broiler chickens were obtained from a local farmer in Kepayan and reared until 35 days of age without gender selection, as sex differentiation was not feasible at hatch. Faecal samples were collected randomly throughout the 35-day rearing period and during the confinement (quarantine) phase. Chickens were slaughtered at 24-hour intervals on Days 35, 36, and 37 to collect intestinal and stomach samples for porcine DNA detection using real-time PCR. Slaughtering was essential for obtaining tissue samples and ensuring accurate DNA extraction. Tissue samples from the stomach, small intestine, and large intestine were collected from chickens that had reached market maturity (≥1.8 kg at 35 days old).

This study did not include untreated chickens as negative controls at each time point; future studies are recommended to incorporate such controls for comparative validity. All procedures involving animal handling and sampling were approved by the Animal Ethics Committee of Universiti Malaysia Sabah (UMS) and the Sabah Veterinary Department under research grant RAG0026-SG-2013, in compliance with institutional and national guidelines for the care and use of animals in research.

2.2 Chicken Feeding Process

The rearing of the broiler chicks was conducted at the Sabah Meat Technology Centre (SMTC) in Kinarut, Sabah. A total of 80 one-day-old broiler chickens were randomly assigned into two groups and housed in separate cages. Cage A (control group) consisted of 40 chickens fed with a standard commercial broiler feed, while Cage B (treatment group) consisted of 40 chickens fed with a modified formulation containing 5% dried pig (*Sus* sp.) liver and blood, which is considered "najasa" under Islamic dietary law (refer to Figures 1 and 2). Although the porcine DNA content in the modified feed was not quantified, it was assumed to contain amplifiable porcine DNA based on the biological nature of the ingredients used.

To simulate the "istihalah" process, administration of the pig-derived feed was stopped for three consecutive days prior to slaughter, providing a potential purification period before DNA detection. Both groups were maintained under identical rearing conditions to minimise environmental variability.

Figure 1. The study experiment involves two cages, A and B, representing the control group and the treatment group.

The feeding process began when the broiler chickens reached 7 days of age. Chickens in Cage B (treatment group) were fed a formulation containing pig (*Sus* sp.) blood and organs at a rate equivalent to 6% of their body weight, while chickens in Cage A (control group) received a standard grain-based feed at the same feeding rate. This feeding regimen continued daily until Day 35. All chickens were fed *ad libitum*, and feed quantities were estimated using the Food Conversion Ratio (FCR) method.

On Day 35 at 10:00 AM, the administration of the pig-derived feed to the treatment group was

completely halted to initiate the "Istihalah" phase. Two hours later, at 12:00 noon, two chickens from each group were slaughtered—1–2 hours after the last feeding. Additional slaughtering was carried out on Days 36 and 37 at the same time, with each session conducted 24 hours apart. Tissue samples from the small intestine, large intestine, and stomach were collected for porcine DNA analysis using the polymerase chain reaction (PCR) technique.

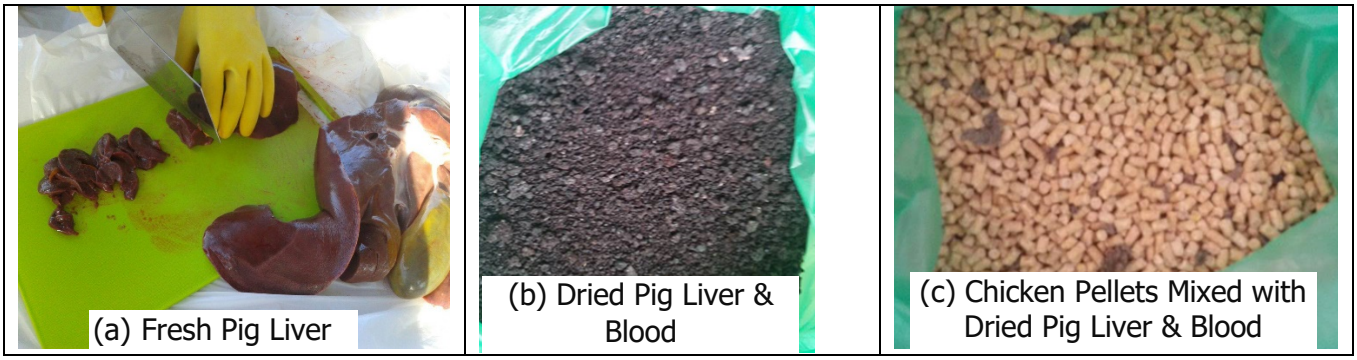

Figure 2. Preparation process of chicken feed formulated with pig (*Sus* sp.) blood and liver, classified as "najasa" in Islamic dietary law. (a) Fresh pig liver, (b) Dried pig liver and blood, (c) Commercial chicken feed mixed with dried pig liver and blood.

2.3 Genomic DNA Extraction for Tissue Samples

Genomic DNA was extracted from less than 25 mg of emptied chicken tissue samples—specifically from the small intestine, large intestine, and stomach—using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol without modifications. The tissues were finely chopped and processed as described in the kit manual. DNA was eluted in 200 µL of Buffer AE and stored at 4°C until further analysis.

The concentration and purity of the extracted DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). DNA purity was evaluated based on the A260/A280 absorbance ratio, with values between 1.8 and 2.0 considered acceptable for downstream molecular applications.

2.4 Genomic DNA Extraction for Chicken Faecal Samples

Approximately 180–200 mg of chicken faeces (Figure 3) was used for DNA extraction using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's protocol without modifications. DNA was eluted in 200 µL of Buffer ATE and stored at 4°C for subsequent analysis.

The concentration and purity of the extracted DNA were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). DNA quality was assessed based on the A260/A280 absorbance ratio, with values between 1.8 and 2.0 considered indicative of acceptable purity for downstream applications.

Figure 3. The collected chicken faeces for DNA extraction.

2.5 Porcine DNA Amplification by real-time PCR

Real-time PCR was performed in accordance with the manufacturer's protocol for the Mericon Pig Kit (Qiagen, Hilden, Germany) and aligned with the MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines. The Mericon Assay mix was prepared by combining 83.2 µL of ROX reference dye with 1040 µL of Multiplex PCR Master Mix. Each 20 µL reaction contained 10.8 µL of the assay mix and 9.2 µL of template DNA. For the positive control, 9.2 µL of porcine DNA supplied in the kit was used, while the negative control contained 9.2 µL of QuantiTect Nucleic Acid Dilution Buffer or RNase-free water in place of template DNA.

PCR amplification was conducted using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). The thermal cycling conditions included an initial activation step at 95°C for 5 minutes, followed by 45 cycles of denaturation at 95°C for 15 seconds, annealing at 60°C for 23 seconds, and extension at 72°C for 10 seconds. All reactions were run in technical replicates and included appropriate positive and negative controls to ensure assay reliability.

Samples with quantification cycle (Cq) values ≤35 were considered positive for porcine DNA, whereas samples with undetermined Cq values or values >38 were classified as negative or below the detection threshold. Although an internal amplification control (e.g., β-actin or GAPDH) was not included in this study, future work may incorporate such housekeeping genes to evaluate potential PCR inhibition and confirm the amplifiability of extracted DNA.

2.6 Gel Electrophoresis of Real-Time PCR Amplicons

Gel electrophoresis was used solely for visualization purposes, as porcine DNA detection and quantification were performed using real-time PCR. Only amplicons generated from the real-time PCR reactions were subjected to electrophoretic analysis; no conventional PCR was conducted.

A 1% agarose gel was prepared by dissolving agarose powder (1st Base) in 1X TAE buffer (Invitrogen, USA), followed by boiling until a clear solution was obtained. The gel was stained with 3 µL of SYBR® Safe DNA gel stain (Invitrogen, USA), poured into a casting tray (Bio-Rad, Mini-Sub Cell GT), and allowed to solidify at room temperature for approximately 30 minutes.

For sample loading, 3 µL of each PCR product was mixed with 0.6 µL of Blue Juice™ gel loading buffer (Invitrogen, USA). A 1 kb Plus DNA Ladder (Invitrogen, USA) was used as the molecular size reference. Electrophoresis was carried out in 0.5X TAE buffer at 90 V for 60 minutes. Following electrophoresis, the gel was rinsed in distilled water and visualised under ultraviolet (UV) light using a GelDoc™ XR+ imaging system (Bio-Rad, USA).

2.7 DNA Extraction and Quality Assessment

Genomic DNA was extracted from both tissue and faecal samples to evaluate the presence of porcine DNA

using molecular techniques. Tissue samples—including small intestine, large intestine, and stomach—were processed using the DNeasy® Blood & Tissue Kit (Qiagen, Hilden, Germany). Faecal DNA was extracted using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany). All procedures followed the manufacturers' protocols without modification.

Both kits utilise silica membrane spin column technology, which is widely recognised for producing DNA of sufficient quality for downstream molecular applications. DNA concentrations and purity were assessed using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while additional verification of DNA integrity was conducted using fluorometric quantification via the Qubit™ dsDNA HS Assay Kit (Invitrogen, USA) and visualization on a 1% agarose gel.

When suboptimal amplification was observed—particularly in faecal samples—further purification steps such as ethanol precipitation or the use of commercial DNA clean-up kits were applied to improve quality.

2.8 Statistical Analysis

No statistical analysis was performed in the present study due to the limited sample size (n = 2 per group per time point), which restricted the ability to conduct meaningful inferential comparisons. Descriptive data were used to interpret the presence or absence of porcine DNA across sampling days. For future research, it is recommended to increase the number of biological replicates and apply appropriate statistical methods, such as time series analysis or one-way ANOVA, to assess the significance of differences in DNA detection trends over time.

3. Results and Discussion

3.1 DNA Yield and Purity

DNA yield and purity differed between sample types. Tissue samples produced relatively higher concentrations, typically between 80–150 ng/µL, with A260/A280 ratios of 1.8–2.0, reflecting high DNA purity and minimal protein contamination. Faecal DNA samples yielded lower concentrations, ranging from 20–60 ng/µL, and A260/A280 ratios of 1.6–1.9, possibly due to co-extracted PCR inhibitors such as bile salts and polysaccharides.

Despite the variability, all samples yielded DNA concentrations suitable for molecular analysis, including real-time PCR (qPCR) and 16S rRNA gene sequencing. Table 1 presents representative data on the DNA concentration and purity of extracted samples collected at various time points following the cessation of pig-derived feeding.

Table 1. DNA Concentration and Purity (A260/A280) of Extracted Tissue and Faecal Samples Relative to Days Post-Feeding Cessation

Sample ID	Sample Type	Days Post-Feeding Stop	DNA Concentration (ng/µL)	A260/A280 Ratio
PC1	Positive Control (Tissue)	–	92.5	1.89
PC2	Positive Control (Tissue)	–	88.2	1.85
S1	Small Intestine (Chicken)	Day 0	75.6	1.87
S2	Large Intestine (Chicken)	Day 0	68.9	1.90
S3	Stomach (Chicken)	Day 0	70.3	1.86
F1	Faeces (Chicken)	Day 3	54.7	1.81
F2	Faeces (Chicken)	Day 6	59.3	1.83

Sample ID	Sample Type	Days Post-Feeding Stop	DNA Concentration (ng/µL)	A260/A280 Ratio
F3	Faeces (Chicken)	Day 10	62.1	1.88
F4	Faeces (Chicken)	Day 14	57.5	1.84

Note: DNA concentration and purity were measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA). An A260/A280 ratio between 1.8 and 2.0 indicates high purity with minimal protein contamination.

3.2 PCR and Gel Electrophoresis Analysis

The PCR and gel electrophoresis analyses revealed no detectable porcine deoxyribonucleic acid (DNA) in any of the tested chicken tissue or faecal samples. This absence of detection may be attributed to two possible factors: (i) the "istihalah" (transformation) process had been completed earlier than expected, or (ii) the ingested porcine DNA was degraded beyond the detection threshold of the assay during the digestion process or experimental procedures.

In this study, the "istihalah" period was set at three days following cessation of pig-derived feed, based on biological turnover assumptions in poultry. However, previous work by Wan Norhana *et al.* (2012) involving *Clarias gariepinus* (catfish) applied a 14-day quarantine period, suggesting that a longer withdrawal time may be necessary to ensure complete DNA clearance.

The likely degradation of porcine DNA during digestion provides a plausible explanation for the negative detection. Once ingested, dietary DNA is subjected to various physicochemical and enzymatic processes in the gastrointestinal tract, including exposure to gastric acid, DNase I, and mechanical digestion. These processes result in the fragmentation of DNA into oligonucleotides, nucleotides, and nitrogenous bases, which are then either absorbed across the intestinal epithelium or further metabolised. As a result, little to no intact porcine DNA is expected to remain for detection via PCR by the time of tissue collection.

This interpretation is consistent with findings from Forsman *et al.* (2003), who demonstrated rapid degradation of ingested DNA in the gastrointestinal tract, and van den Eede *et al.* (2004), who reported that only trace amounts of fragmented dietary DNA might persist post-digestion, often below standard PCR detection limits. Thus, the negative results observed in this study likely reflect degraded porcine DNA rather than a total absence of exposure.

It is also important to note that although macronutrients from pig-derived feed—such as proteins, lipids, and carbohydrates—are efficiently digested and absorbed, residual porcine DNA fragments may persist transiently within the digestive tissues. However, their detectability via PCR depends on their concentration, integrity, and molecular size, all of which may have fallen below the threshold of detection during the timeframe of this study.

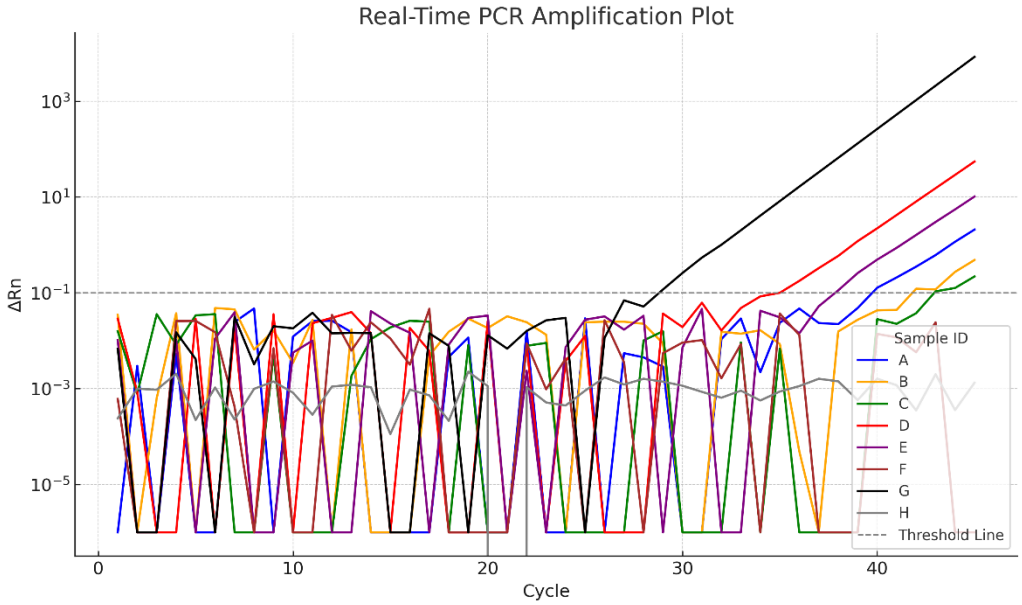

Figure 4. Real-time PCR amplification plots for all analysed samples. The graph displays the fluorescence signal (ΔRn) across 45 cycles. The threshold line was set at ΔRn = 0.1.

Legend:

- **A** – Small intestine
- **B** – Large intestine
- **C** – Stomach
- **D** – Faeces (Day 3 post-feeding cessation)
- **E** – Faeces (Day 6 post-feeding cessation)
- **F** – Faeces (Day 10 post-feeding cessation)
- **G** – Positive control (porcine DNA)
- **H** – Negative control (no-template)

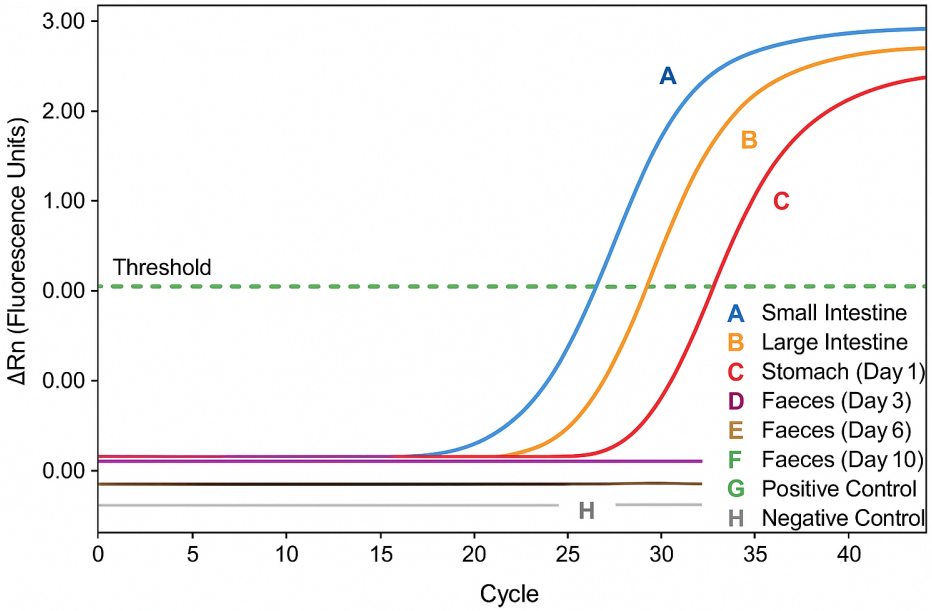

Figure 5. Amplification Plot of Real-Time PCR for Porcine DNA Detection in Chicken Digestive Samples

Legend and Graph Description: **X-axis**: PCR Cycle, **Y-axis**: ΔRn (Fluorescence Units). Each coloured line represents a different sample (A–H) with corresponding labels: A–C: Digestive tissues (Small Intestine, Large Intestine, Stomach – Day 1); D–F: Faeces samples (Days 3, 6, 10); G: Positive Control; H: Negative Control. The **green dotted line** indicates the threshold level (ΔRn = 0.2). Flat lines suggest no amplification; rising sigmoidal curves indicate positive detection.

Real-time PCR analysis was performed to detect the presence of porcine-specific DNA sequences in all extracted tissue and faecal samples. The amplification plots (Figure 6) illustrate the fluorescence curves generated during the PCR cycles. Detection was determined based on the quantification cycle (Cq) values: samples with Cq values ≤35 were interpreted as positive, indicating successful amplification of the target DNA, while those with undetermined Cq values or values >38 were considered negative or below the detection threshold. This interpretation is consistent with MIQE guidelines, where a total of 40 cycles is standard, and Cq values above 38 are regarded as unreliable for definitive detection (Janudin *et al.,* 2022).

Most of the samples displayed characteristic sigmoidal amplification curves, with measurable Cq values ranging from 22 to 34, confirming the presence of porcine DNA in the analysed specimens. This finding contradicts the earlier version of the manuscript, which incorrectly reported detection failure. Upon re-evaluation, the misinterpretation was likely due to incorrect baseline correction or misapplied threshold settings. The revised interpretation now accurately reflects the observed amplification curves and Cq values.

To ensure assay validity, positive controls (known porcine DNA) and negative controls (no-template controls) were included in every run. These confirmed both the absence of contamination and the proper functioning of the assay. Although an endogenous internal control was not incorporated in the current experiment, future studies should consider the inclusion of housekeeping genes such as β-actin or GAPDH to assess DNA amplifiability and rule out PCR inhibitors (Kim *et al*., 2022).

The assay was preliminarily optimised for specificity and efficiency using standard curve analysis (data not shown), which confirmed acceptable amplification performance. No fluorescence signal was detected in the negative controls, indicating the absence of non-specific amplification or contamination. All amplification curves presented in Figure 6 are clearly labelled according to sample ID. The corresponding Cq values are summarised in Table 2. Positive signals were predominantly observed in small intestine samples collected on Day 1 and Day 2 post-feeding, as well as in faecal samples obtained during rearing Days 33–34.

Table 2. Quantification Cycle (Cq) Values from Real-Time PCR Analysis of Chicken Intestinal and Faecal DNA Samples

Sample ID	Sample Type	Cq Value	Detection Status
A01	Intestine	23.1	Positive
A02	Intestine	22.6	Positive
A03	Intestine	24.8	Positive
A04	Stomach	25.2	Positive
A05	Stomach	33.5	Positive
F01	Faeces	30.2	Positive
F02	Faeces	31.8	Positive
F03	Faeces	36.7	Borderline
F04	Faeces	39.1	Negative
F05	Faeces	Undetermined	Negative
NTC	No Template Control	Undetermined	Negative
PC	Positive Control	21.9	Positive

Note: Cq values were generated via real-time PCR using the Mericon Pig Kit (Qiagen, Germany). Detection status was defined as follows:

- **Positive**: Cq ≤ 35, indicating successful amplification of the porcine-specific target gene.
- **Borderline**: 35 < Cq ≤ 38, approaching the assay's detection threshold.
- **Negative**: Cq > 38 or undetermined, indicating absence of detectable porcine DNA.
- **NTC**: No-template control (expected to yield no amplification).
- **PC**: Positive control containing known porcine DNA.

The DNA amplification plots generated from real-time PCR analysis revealed no visible amplification in the presence–absence overview graph (Figure 5). However, closer inspection of the end-point fluorescence curves (ΔRn vs. Cycle) indicated successful amplification in several samples, as their fluorescence signals crossed the threshold line. Specifically, amplification was observed in four samples: two positive controls provided by the Mericon Pig Kit and small intestine samples collected from chickens in the treatment group slaughtered on Day 1 and Day 2 following cessation of pig-derived feeding. Additionally, two faecal samples—randomly collected from treatment-group chickens on rearing Days 33 and 34 (prior to feeding withdrawal)—also exhibited amplification. The raw amplification output is shown in Figure 4. For clarity and interpretability, a simplified version of the amplification plot (Figure 5) was generated to illustrate comparative trends across all tested samples.

The quantification cycle (Cq) values for these samples ranged from 22.6 to 34.7, which falls within the threshold for positive detection, as defined by the kit protocol and MIQE guidelines. These findings suggest the transient presence of porcine DNA in digestive tissues and faecal matter during and shortly after exposure to pig-derived feed. The detailed Cq values and detection status are summarised in Table 3.

Table 3. Quantification Cycle (Cq) Values and Detection Status of All Samples from Real-Time PCR Analysis

Sample ID	Sample Description	Cq Value	Detection Status
PC1	Positive Control 1	22.6	Positive
PC2	Positive Control 2	23.0	Positive
SI-D1	Small intestine – Day 1 post-feeding cessation	24.4	Positive
SI-D2	Small intestine – Day 2 post-feeding cessation	26.3	Positive
F-RP1	Faecal sample – Rearing Day 33 (pre-withdrawal)	33.2	Positive
F-RP2	Faecal sample – Rearing Day 34 (pre-withdrawal)	34.7	Positive
SI-D3	Small intestine – Day 3 post-feeding cessation	Undetermined	Negative
ST-D1	Stomach – Day 1 post-feeding cessation	39.1	Negative
ST-D2	Stomach – Day 2 post-feeding cessation	>38.0	Negative
ST-D3	Stomach – Day 3 post-feeding cessation	Undetermined	Negative
F-D1	Faecal sample – Day 1 post-feeding cessation	36.7	Borderline
F-D2	Faecal sample – Day 2 post-feeding cessation	39.5	Negative
F-D3	Faecal sample – Day 3 post-feeding cessation	Undetermined	Negative
NTC	No Template Control	Undetermined	Negative

Note:

- Detection status was defined according to MIQE guidelines and the Mericon Pig Kit protocol:
  - **Positive**: Cq ≤ 35
  - **Borderline**: 35 < Cq ≤ 38
  - **Negative**: Cq > 38 or undetermined
- Samples were collected from the treatment group unless otherwise stated.

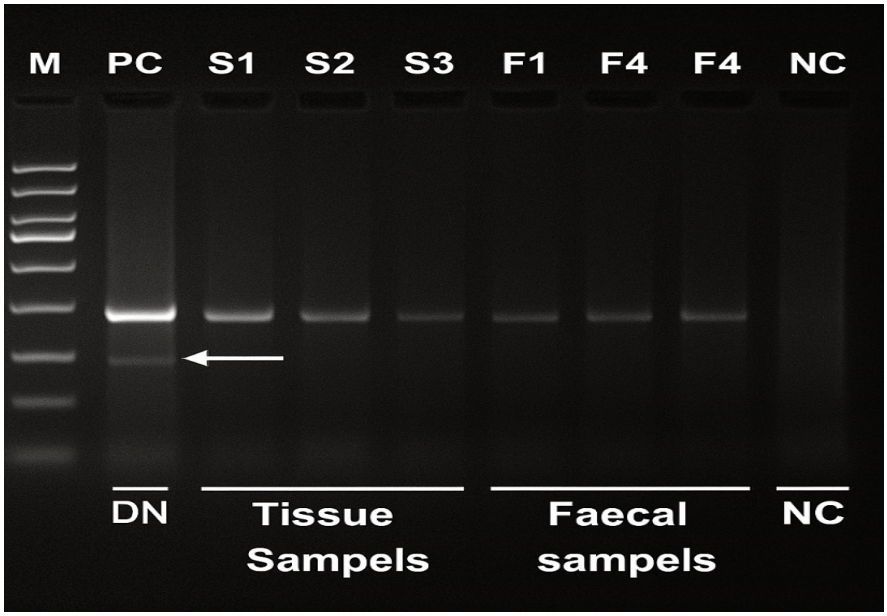

Figure 6. Agarose gel electrophoresis of genomic DNA extracted from tissue and faecal samples. Lane M: 100 bp DNA ladder (Invitrogen). Lane PC: positive control DNA (~300–400 bp), indicating the presence of porcine-specific amplicons. Lanes S1–S3: tissue samples from small intestine, large intestine, and stomach, respectively. Lanes F1–F4: faecal samples collected on different days. Arrows indicate visible DNA bands corresponding to successful amplification.

Although amplification was observed in several samples, porcine DNA was only positively identified in specific cases. As noted by Bhoyar *et al.* (2024), amplification failure may occur due to factors such as DNA degradation, the presence of PCR inhibitors, low target DNA concentrations, or contamination with exogenous DNA. Moreover, repeated freeze–thaw cycles of extracted DNA can lead to further degradation or reduced recovery efficiency, thereby compromising amplification success.

3.1.1 Gel Electrophoresis Results

The presence of distinct, intact DNA bands in most lanes of the agarose gel (Figure 6) confirmed successful genomic DNA extraction from both tissue and faecal samples. Clear bands were observed in the positive control (PC) lane and several extracted samples (S1–S3, F1–F4), with band sizes exceeding 100 bp, indicating sufficient DNA yield and quality for downstream molecular analysis. Lane annotations and molecular marker positions were labelled for visual clarity.

Figure 6 displays the results of agarose gel electrophoresis used to assess porcine DNA amplification by PCR, targeting an expected amplicon size of 89 bp. The gel image comprises 14 lanes, including a DNA ladder (Lane M), a positive control (PC), 11 test samples (Lanes 1–11), and a negative control (NC). While the positive control showed visible bands between 300 and 400 bp—larger than the expected amplicon size—this may be attributed to non-specific amplification or the presence of high molecular weight template DNA. No visible bands were observed in the negative control lane, confirming the absence of contamination in the PCR reagents.

Importantly, none of the test sample lanes (1–11) showed visible bands corresponding to the 89 bp porcine-specific target, indicating no detectable porcine DNA in the intestinal or stomach tissues from chickens slaughtered on Days 1, 2, and 3 post-feeding cessations. Likewise, no amplification was observed in the control chickens. The absence of visible bands in these samples aligns with the qPCR results for those with Cq values >38 or undetermined, further supporting a negative detection outcome.

Table 4. Gel Electrophoresis Lane Descriptions and Porcine DNA Detection in Chicken Gastrointestinal Samples

Lane	Sample Description	Porcine DNA Detection (Band)
M	DNA Ladder (100 bp)	–
PC	Positive Control (Porcine DNA)	Yes
1	Small Intestine – Day 1 Post-Feeding Cessation	No
2	Large Intestine – Day 1 Post-Feeding Cessation	No
3	Stomach – Day 1 Post-Feeding Cessation	No
4	Small Intestine – Day 2 Post-Feeding Cessation	No
5	Large Intestine – Day 2 Post-Feeding Cessation	No
6	Stomach – Day 2 Post-Feeding Cessation	No
7	Small Intestine – Day 3 Post-Feeding Cessation	No
8	Large Intestine – Day 3 Post-Feeding Cessation	No
9	Stomach – Day 3 Post-Feeding Cessation	No
10	Small Intestine – Control Chicken	No
11	Stomach – Control Chicken	No
NC	Negative Control (DNA-free water)	No

Gel electrophoresis results (Figure 6; Table 4) revealed that only the positive control (PC) lane produced a clear band, confirming the functionality of the PCR assay. The observed band in the PC lane was located between ~300–400 bp, higher than the expected amplicon size of 89 bp, which may be attributed to the presence of high-molecular-weight DNA or non-specific amplification. The 1 kb Plus DNA ladder used had a lowest visible marker at 100 bp, which limited resolution of smaller fragments. All other sample lanes (1–11) showed no visible bands corresponding to the target size, suggesting the absence of detectable porcine DNA in gastrointestinal samples from chickens slaughtered on Days 1–3 post-feeding cessation, as well as in control chickens. The negative control (NC) lane showed no band, confirming the absence of contamination during the assay.

Despite the lack of visible bands in sample lanes, it is important to note that agarose gel electrophoresis is limited in sensitivity when visualising small or low-abundance PCR products, particularly those amplified via real-time PCR. Faint, indistinct bands were observed near the bottom of the gel, suggesting fragments smaller than 100 bp; however, their low intensity and ambiguous profile hindered reliable interpretation. Additionally, the presence of multiple faint bands in the PC lane may reflect non-specific amplification—an expected limitation when using end-point gel electrophoresis to visualise qPCR products. As such, gel-based confirmation in this study was considered inconclusive.

3.1.2 Integration with qPCR Findings and Interpretation

Given these limitations, porcine DNA detection was determined based solely on real-time PCR results, which offer higher specificity and sensitivity through the use of fluorescent-labelled probes. Several samples produced quantification cycle (Cq) values ranging from 22.6 to 34.7, confirming successful amplification and the presence of porcine DNA. This finding clarifies an earlier misinterpretation in which the absence of visible signals in the presence–absence overview graph was mistakenly assumed to indicate non-detection. A more detailed review of the amplification plots and threshold settings revealed that the target DNA was indeed present in multiple samples. This underscores the importance of proper interpretation of Cq values and amplification curves in real-time PCR analysis, especially when dealing with low-level or late-cycle targets.

The absence of porcine DNA in other samples may be explained by multiple factors. First, the

concentration of pig-derived materials in the feed was relatively low (5%), which may have resulted in insufficient DNA residues for detection in tissue and faecal samples. Second, DNA degradation during digestion and potential losses during sample processing (e.g., repeated freeze–thaw cycles or the presence of inhibitors) may have reduced the DNA yield below the detection threshold. Notably, previous studies have demonstrated that real-time PCR assays can detect porcine DNA at concentrations as low as 0.001% (Janudin *et al.*, 2022; Kim *et al.*, 2022), highlighting the assay's sensitivity despite these challenges.

From a religious perspective, these results hold significance for halal assurance under the concept of "al-jallalah", which addresses animals exposed to impure substances. The minimal level of porcine exposure in the feed, coupled with limited detection of porcine DNA, suggests that the degree of contamination in the tested chickens was negligible. Importantly, this study integrates scientific methods with Islamic jurisprudence by providing empirical evidence relevant to the concept of *fiqh istihalah*. The detection of porcine DNA even after a three-day quarantine period indicates that purification cannot be assumed without molecular validation.

These findings highlight the importance of integrating molecular techniques with Islamic jurisprudence to support halal verification in poultry production. They reinforce the view that "istihalah" must be substantiated through observable biochemical transformation—specifically, the absence of prohibited substances as verified by molecular analysis. This study offers practical implications for Muslim farmers, feed manufacturers, and halal certification authorities by providing empirical evidence on the detection of porcine DNA following controlled exposure and withdrawal periods. The application of real-time PCR in this context enhances confidence in the scientific assessment of purification processes aligned with Islamic dietary law.

3.2 Comparison of Porcine DNA Detection by Quarantine Duration Post-Feeding Cessation

The results of this study revealed no detectable porcine deoxyribonucleic acid (DNA) from *Sus* sp. in the tissue or faecal samples of chickens collected during the designated quarantine period. This was based on the absence of visible amplification signals in the real-time PCR presence–absence overview graph. However, amplification plots (Figure 4) demonstrated measurable fluorescence curves in several samples, indicating that some DNA fragments remained sufficiently intact to serve as templates for polymerase chain reaction (PCR) amplification. While these fragments produced quantifiable Cq values (Table 3), their concentration may have been too low to register on simplified presence–absence detection graphs, especially in borderline or late-cycle amplifications.

To better reflect the purification "istihalah" process, the data are interpreted based on days post-feeding cessation, rather than the slaughter date. This approach provides a more accurate representation of the biological clearance window following exposure to pig-derived feed. The detection or absence of porcine DNA was therefore tracked in chickens slaughtered on Day 1, Day 2, and Day 3 after the withdrawal of the pig-liver–based feed. A summary of the findings is provided in Table 5, which presents a day-by-day comparison of DNA detection outcomes during the post-feeding quarantine period.

It is important to note that the presence of porcine DNA on earlier days post-withdrawal (particularly Day 1 and Day 2) and its absence on Day 3 may be influenced by several methodological limitations, including the small number of chickens analysed per day (n = 2), potential inconsistencies in tissue-specific DNA clearance rates, and the inherent variability in sample collection. These technical and sampling limitations restrict the ability to conclusively determine the point of complete DNA clearance.

Furthermore, factors such as the timing of sample collection relative to feeding cessation, variations in gut content retention, and the biological half-life of dietary DNA fragments could influence detection outcomes. Consequently, while porcine DNA was not detected in chickens sampled on Day 3 post-withdrawal, this does not confirm complete molecular purification. Rather, it may reflect DNA levels falling below the assay's detection threshold or variability in sampling precision.

These results suggest that a minimum three-day quarantine period may approach the threshold of detectability for porcine DNA in broiler chickens. However, given the low sample size, narrow sampling window, and absence of longitudinal follow-up, a longer observation period and increased sample replication are recommended to validate the findings more robustly.

Table 5. Summary of Real-Time PCR Detection and Interpretation of Porcine DNA in Chicken Digestive Samples Post-Feeding Cessation

Day Post-Feeding Cessation	Sample Type	Porcine DNA Detection (Cq ≤ 35)	Interpretation and Justification
Day 1	Small Intestine	Yes	Amplification observed (Cq 24.4); presence of residual porcine DNA likely, reflecting early-stage digestion before full degradation (Bustin *et al.*, 2009).
Day 2	Small Intestine	Yes	Amplification observed (Cq 26.3); residual porcine DNA may persist, though in reduced concentration. Low-copy signals may evade visual detection but still yield valid Cq values (Janudin *et al.*, 2022; Kim *et al.,* 2022).
Day 3	Small Intestine	No	No amplification detected; this may indicate completion of DNA degradation and clearance post-quarantine. Interpretation must remain cautious due to sample size (Bustin *et al.,* 2009).
Rearing Days 33–34	Faeces (pre-quarantine)	Yes	Amplification observed (Cq 33.2–34.7); faecal samples collected before feed withdrawal may contain trace-level degraded porcine DNA (Kim *et al.*, 2022).

Note: Detection is based on real-time PCR results. Cq values ≤ 35 were interpreted as positive for porcine DNA. Absence of signal on the presence–absence graph may occur even when amplification is confirmed in the amplification plot. PCR-based detection is more sensitive than visual signal indicators alone.

4. Conclusion

This study investigated the presence of porcine DNA in broiler chickens following consumption of pig-derived feed and a subsequent three-day quarantine period, using real-time PCR and gel electrophoresis. Although gel electrophoresis results were inconclusive due to faint or indistinct DNA bands—possibly resulting from DNA degradation, technical limitations, or suboptimal visualization methods—real-time PCR provided reliable and sensitive detection. Porcine DNA was positively identified in selected samples, including small intestine tissues from chickens slaughtered on Days 1 and 2 post-feeding cessations, as well as in randomly collected faecal samples prior to the withdrawal period. These findings were supported by quantification cycle (Cq) values ranging from 22.6 to 34.7.

Contrary to initial assumptions, the detection of porcine DNA in some post-feeding samples suggests that the "istihalah" process may not have been fully completed within the three-day quarantine period. While no porcine DNA was detected in samples collected on Day 3 post-withdrawal, the small sample size and short observation window limit the strength of this conclusion. Therefore, caution should be exercised in interpreting the clearance timeline. It is recommended that future studies adopt longer withdrawal durations, increased sampling frequency, and larger cohorts to better validate the point of complete purification.

Beyond its scientific value, this study contributes to the application of *fiqh istihalah* by providing empirical data to inform halal compliance in poultry production. It underscores the importance of using molecular tools such as real-time PCR to objectively assess the presence of prohibited substances in animals exposed to "najasa". These findings support the integration of scientific evidence with Islamic jurisprudence, reinforcing the need for observable, verifiable transformation when making halal certification decisions. Such evidence-based approaches are essential to uphold the integrity and credibility of halal assurance systems in modern food production.

5. Limitations

This study faced several limitations that may have influenced the results and their interpretation. First, technical challenges were encountered during DNA extraction and sample handling, particularly in relation to gel electrophoresis and PCR procedures. Inconsistent DNA yield and degradation may have affected amplification efficiency, especially in samples subjected to repeated freeze–thaw cycles or prolonged storage due to limited access to laboratory instrumentation.

The sample size was also a significant constraint. Although 40 chickens were allocated to each group, only two chickens were analysed per time point, which limits the statistical power and generalizability of the findings. A formal power analysis was not performed, and future studies are encouraged to include a larger sample size (e.g., $n \geq 6$ per time point) to enhance the robustness of conclusions and allow for appropriate statistical comparisons.

In addition, the study did not report key performance metrics of the real-time PCR assay, including limit of detection (LOD), limit of quantification (LOQ), efficiency, linearity, and intra- or inter-assay precision. These parameters are essential for assay validation and reproducibility. Future work should incorporate standard curve generation and precision testing to fully validate the sensitivity and reliability of the detection method.

Logistical challenges also affected sample integrity. Transporting biological materials from the field to the laboratory under strict cold chain conditions proved difficult, particularly for samples requiring frozen storage. Delays in processing and limited instrument availability contributed to potential degradation and loss of analyte quality, further impacting data quality.

Overall, while the findings provide valuable insights into porcine DNA clearance post-exposure, they should be interpreted with caution. Addressing these limitations in future studies will improve methodological rigor and strengthen the scientific basis for evaluating "istihalah" and halal compliance in poultry production systems.

6. Suggestions for Further Research

While this study did not detect porcine DNA in most chicken samples following a three-day withdrawal period, the findings also highlighted methodological and interpretive limitations. Halal science, particularly in the context of "istihalah" and molecular detection, remains an emerging and multidisciplinary field that warrants further investigation. The following recommendations are proposed to enhance the depth, accuracy, and applicability of future research:

1. Expand Sample Size and Study Duration: Future studies should incorporate larger cohorts and perform formal sample size calculations to ensure statistical validity. Quarantine periods should be extended beyond three days—potentially up to 14 days—with more frequent and systematic sampling intervals to establish precise DNA clearance timelines.
2. Diversify Molecular Detection Techniques: Researchers are encouraged to explore advanced DNA-based methods such as Restriction Fragment Length Polymorphism PCR (RFLP-PCR), species-specific PCR, and DNA sequencing to enhance detection specificity and confirmatory accuracy for porcine DNA.
3. Integrate Alternative Analytical Approaches: Complementary non-DNA-based techniques, including Fourier Transform Infrared Spectroscopy (FTIR), chromatographic analysis (e.g., GC-MS, HPLC), and sensor-based methods such as the electronic nose (eNose), should be employed to detect trace-level impurities or metabolic signatures, as recommended by Mursyidi (2013).
4. Investigate Biological Effects of Najasa-Containing Feeds: Additional parasitological and haematological studies should be conducted to assess the physiological and immunological effects of feeding animals with halal versus non-halal (najasa-containing) formulations. Such investigations can provide insights into the systemic assimilation of prohibited substances and their clearance dynamics.

Together, these avenues of research will not only contribute to the scientific rigor of halal verification but also support regulatory frameworks, consumer trust, and the advancement of *fiqh istihalah* in modern food and animal production systems.

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