International Food Research Journal 32(3): 800 - 807 (June 2025)
Journal homepage: *http://www.ifrj.upm.edu.my*

Development and validation of TaqMan qPCR assay for porcine DNA detection in gelatine-based foods for halal authentication

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Article history

Received:
28 June 2024
Received in revised form:
24 May 2025
Accepted:
3 June 2025

Keywords

qPCR,
TaqMan probes,
porcine,
food,
LOD,
LOQ

Abstract

Porcine-derived gelatine in food products is a critical concern for halal and kosher consumers, necessitating accurate and reliable detection methods. The present work aimed to develop and validate a TaqMan probe-based real-time PCR (qPCR) assay for detecting porcine DNA in gelatine-based ice creams, cakes, and pastries. After careful optimisation of the qPCR system, DNA was extracted using commercial kits, and analysed for specificity, sensitivity, and stability under various processing conditions. The assay demonstrated high sensitivity with a detection limit of 0.05 ng/µL, and confirmed the presence of porcine DNA with 99 - 100% sequence similarity. A pilot survey of 25 halal-branded commercial samples revealed no detectable porcine DNA. The present work demonstrated qPCR assay as a reliable method for routine halal authentication of gelatine-based food products, ensuring compliance with dietary regulations.

Abbreviations

PCR: polymerase chain reaction; DNA: deoxyribonucleic acid; *Cytb*: cytochrome b; bp: base pair; and LOD: limit of detection.

DOI
https://doi.org/10.47836/ifrj.32.3.14

Introduction

Muslim halal and Jewish kosher laws require food products to be free from porcine derivatives such as porcine and lard (Sultana *et al.,* 2023; 2024). It is projected that by 2025, the global food trade's share of halal food will have increased to 30% from its present 20% (Ali *et al.,* 2018). Thus, there is a clear connection between public health, religious and economic behaviours, and authenticity difficulties. The contamination of food with porcine ingredients can result from cross-contamination or improper food handling practices. More significantly, one of the most common fraudulent activities in the food industry involves hiding the presence of porcine substances on product labels. In such instances, manufacturers may deliberately substitute costly ingredients, such as beef, with more affordable porcine alternatives to increase profits while concealing this substitution to avoid losing consumers from specific communities (Nhari *et al.,* 2019). Among various ingredients, gelatine is an essential ingredient, and a mixture of polypeptides made up of partial hydrolysis of collagen from animal skin and hides (Karim and Bhat, 2009). The gelatine market is growing at a compound annual growth rate (CAGR) of around 6.6%. By 2028, this expansion is expected to drive the market value to approximately USD 5.1 billion (Globenewswire, 2024). The extensive application of gelatine in the food and beverage sector is attributed to its outstanding gelling properties, making it a popular choice as a thickener,

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stabiliser, and emulsifier (Mohamad *et al.,* 2018). Gelatine has been widely used in ice creams, cakes, pastries, and candies (Sudjadi *et al.,* 2016).

About 90% of commercial gelatine comes from porcine origins, as porcine gelatine is less costly, and gives superior quality than bovine gelatine (Kuramata *et al.,* 2022). Porcine is prohibited in halal and kosher consumer products. Porcine contamination in food products can take place at any stage of the production process, whether during processing or after manufacturing (Mohamad *et al.,* 2013). Therefore, it is crucial to know the source of gelatine in food products (Sudjadi *et al.,* 2016). Consequently, it is crucial to detect even trace amounts of porcine, highlighting the necessity for a highly sensitive and specific method for porcine identification.

Various analytical methods have been developed to feed the need to ensure halal authentication, including immunological methods (Tukiran *et al.,* 2016), chromatography-chemometrics methods (Zhang *et al.,* 2022) combined with supervised pattern recognition strategies (Forooghi *et al.,* 2023), and TaqMan probe-based qPCR (Sultana *et al.,* 2020) for the determination of gelatines in various matrices, even from food products. Only fluorescent label probe-based qPCR offers qualitative and quantitative estimation among these various techniques (Sultana *et al.,* 2020).

The most popular dessert alternatives include cakes, pastries, and ice creams. Due to grinding, intense heat treatments, pressure changes, and freezing temperatures, most constituents in processed foods undergo significant transformations (Al-Shaibany *et al.,* 2022). Due to their complex structure and high processing conditions, these foods are challenging to analyse. Undeclared components are often detected in processed foods, and proper detection techniques are required (Kamandi *et al.,* 2022).

Due to its high sensitivity, specificity, and fast detection capabilities, real-time PCR has been widely utilised by researchers for species identification. This technique represents a significant advancement in molecular diagnostics, particularly in detecting and tracing DNA origin (El Sheikha *et al.,* 2017). Over the years, various qPCR assays have been documented for the authentication of porcine species in food products (Sudjadi *et al.,* 2016) and pharmaceutical capsule shells (Nikzad *et al.,* 2017; Mahamad *et al.,* 2023). However, no qPCR methods are currently available to detect porcine simultaneously in gelatine-based ice creams, cakes, and pastries. Furthermore, most documented assays are based on a single target and long amplicon sizes (243 - 672 bp) that are easily broken down under extensive food processing atmospheres (Ali *et al.,* 2012). Therefore, the present work aimed to document a concise amplicon length (106 - 146 bp) qPCR system for detecting porcine DNA in various processed food products.

Materials and methods

Sample collection and preparation

Pure porcine gelatine powders were used as a reference standard for optimising the qPCR system (Sigma-Aldrich, Missouri, USA). Twenty-five commercial food samples under the halal brand, comprising pastilles (n = 5), cakes (n = 10), and ice creams (n = 10), were acquired from various locations located around Selangor, Malaysia.

DNA extraction

Pure gelatine samples (pork gelatine, 100%, w/w) and food products were prepared using a column-based DNeasy Mericon Food Kit (Ping-Tung, Taiwan). The DNA extraction was performed under aseptic conditions to ensure good-quality DNA following the manufacturer's protocol (Mohamad *et al.,* 2016). A NanoDrop™ UV-VIS Spectrophotometer (2000/2000c) was employed to determine the concentration and purity of the isolated DNA (Berlin, Germany). The extracted DNA samples were stored at -20°C until further analyses.

Repeatability test

The repeatability test took one point on the standard linear curve from the 100% porcine gelatine DNA dilution series, taken from the commercial food samples adulterated with 10, 1, 0.1, 0.01, and finally, 0.001% of DNA samples, and was conducted on three different days.

Design of primers and probes

The primers and probes utilised in the present work were taken from our previous work on pig mitochondrial *cytb* gene (Table 1; Sultana *et al.*, 2020). Integrated DNA Technologies (IDT), Singapore, supplied all the designed primers and probes. The porcine probe was labelled with ROX at the 5' end, and TAO-IOWA BLACK RQ at the 3' end. Eukaryotic 18S rRNA universal primers and probes

Table 1. Concentration and sequences of primers and probes.

Species	Target gene	Sequence (5′ - 3′)	Amplicon size (bp)	Final concentration (nM)	Reference
Porcine	Cytb	Forward: TATCCCTTATATCGGAACAGACCTC	146	300	Sultana *et al*. (2023)
		Reverse: GCAGGAATAGGAGATGTACGG		300	
		Probe: ROX-CCTGCCATTCATCATTACCGCCC- TAO-IOWA BLACK RQ		200	
Eukaryotic	18S rRNA	Forward: GGTAGT GACGAAAAATAACAATACAGGAC	141	250	Hossain *et al*. (2017)
		Reversed: ATACGCTATTGGAGCTGGAATTAC C		250	
		Probe: FAM-AAGTGGACTCATTCCAATTACAGGGCCT- ZEN/IOWA BLACK FQ		125	

were used as endogenous control (EC). The EC probe was labelled with fluorescent reporter dye FAM at the 5’ end, and ZAN/IOWA BLACK FQ at the 3' end.

Multiplex real-time PCR

TaqMan probe-based qPCR assay was performed in a 20 μL reaction volume in a Quant Studio 12 K flex real-time PCR system (Applied Biosystems, Foster City, CA). The amplification was performed using an initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 18 sec, and annealing and extraction at 59°C for the 60 sec (Table 2). The reaction mixture contained 10 μL 1× GoTaq Probe qPCR Master Mix (Promega, Madison, USA), 0.5 μL primer, 0.3 μL probe, 1 μL (20 ng/μL) total DNA template, and the rest of the reaction volume was adjusted with the required quantity of distilled water.

Table 2. qPCR amplification cycle.

Phase	Condition
Initial denaturation	95°C, 30 sec
Denaturation	95°C, 10 sec
Annealing	59°C, 60 sec
Extension	72°C, 10 sec
Number of cycle	45

Specificity test

To determine the specificity of the primer pair, reference PCR systems were evaluated for 12 non-target animal DNA types (*e.g.*, beef, buffalo, goat, chicken, dog, lamb, cat, duck, donkey, rabbit, deer, and horse).

Limit of detection

To determine the limit of detection (LOD), the TaqMan probe-based qPCR assay was calibrated with serially diluted DNA extracted from pure gelatine and gelatine-adulterated food samples. The DNA mixture was ten-fold serially diluted, and the concentrations of the diluted DNA samples were 10, 1, 0.1, 0.01, and 0.001 ng. The TaqMan qPCR of each dilution was assayed in three replicates on three separate days.

Generation of standard curve

A standard curve was constructed to determine the qPCR efficiency and quantify the PCR targets. The concentration of the DNA was adjusted to 10 ng/μL. After that, it was ten-fold serially diluted to 1, 0.1, 0.01, and 0.001 ng/μL. Next, 1 μL of each diluted DNA was added to the final reaction mixture (20 μL). The standard curve was constructed by plotting the Ct values of the target species against the logarithm ($\log_{10}$) of the DNA concentrations (Khairil Mokhtar *et al.,* 2020). Afterwards, the standard curve was constructed to form a linear graph, and the assay efficiency was calculated using Eq. 1 (Mohamad *et al.,* 2018):

$$E\ (\%) = [10^{(-1/\text{slope})} - 1] \times 100 \quad \text{(Eq. 1)}$$

The acceptance range of qPCR efficiency was between 90 and 110%, corresponding to a regression slope between -3.1 and -3.6, while the R^2 value was ≥ 0.98 (Rojas *et al.,* 2010; Hossain *et al.,* 2017). The quantity of porcine DNA from unknown specimens was determined based on respective Ct values according to Sultana *et al.* (2020) using Eq. 2:

$$Ct = m\log[\ ] + C \quad \text{(Eq. 2)}$$

where, m = slope, and C = intercept.

Results and discussion

Assessment of DNA quality

Pure gelatine samples, raw animal meats, ice creams, cakes, and pastries were all used to harvest DNA. The concentration of extracted DNA was 59 - 76 ng/μL for pure gelatine, 96 - 126 ng/μL for raw animal meats (non-target), and 10 - 27 ng/μL for food product samples. The absorbance ratio of 260/280 nm extracted DNA sample yielded 1.7 - 2.0 purity that reflected the suitability of the extracted DNA (Sultana *et al.*, 2018a; 2020). The nature of the food product samples, which included various chemicals and ingredients, could be the reason for the low concentration of DNA obtained from the products.

Development of qPCR model

Porcine primers and probes were carefully evaluated for mismatch sequence and melting temperature (Tm) (Sultana *et al.,* 2020). The chosen primers exhibited Tm values of 58°C, and annealed at 59°C. The Ct values of the qPCR for porcine were 19.32 ± 0.5.

Limit of detection

The limit of detection (LOD) is essential for any quantitative assay. The LOD was determined by analysing serially diluted DNA extracts. Ten-fold serially diluted DNA was used, and the concentrations of the diluted DNA samples of the target species were 10, 0.1, 0.01, and 0.001 ng/µL. The amplification curve reflected the corresponding Ct values from higher to lower concentrations of each DNA sample (Figure 1). In Table 3, the diluted DNA sample, resulting Ct values, and the corresponding relative standard deviations (RSD) are tabulated. The developed qPCR could quantify and detect up to 0.001 ng/µL of DNA from raw gelatine and gelatine-mixed food products. Previously, Sultana *et al.* (2020) detected 0.005 ng/µL DNA from raw gelatine, desserts, and dietary supplements.

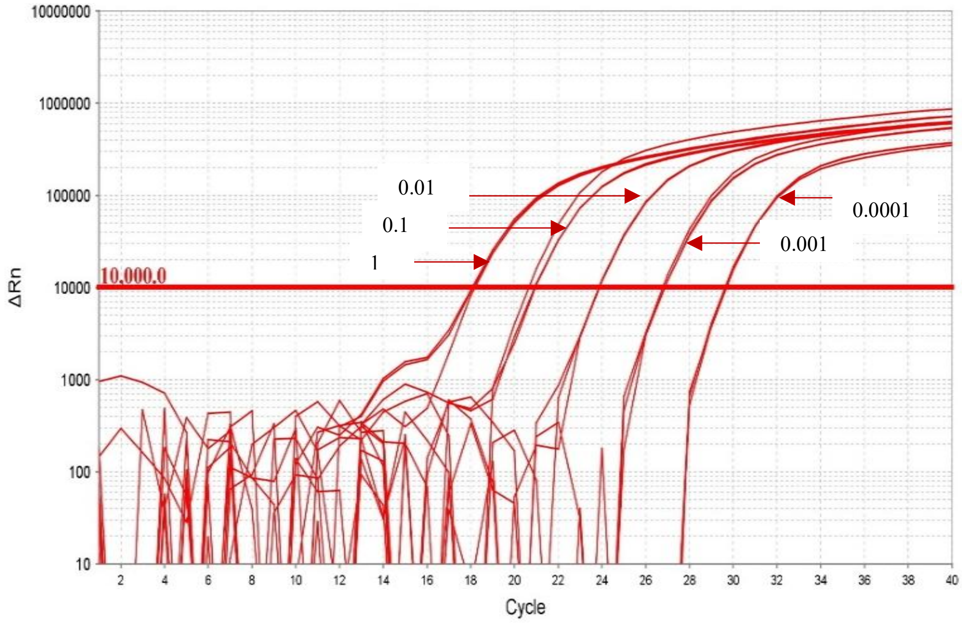

Figure 1. TaqMan probe-based amplification curve for porcine primer.

Table 3. Determination of LOD from Ct value of porcine species obtained from ten-fold serially diluted raw porcine gelatine DNA.

DNA concentration (ng)	Porcine Mean Ct value ± SD
10	17.89 ± 0.02
1	20.65 ± 0.05
0.1	24.03 ± 0.01
0.01	27.02 ± 0.04
0.001	28.97 ± 0.02

Similarly, Hossain *et al*. (2017) documented a tetraplex qPCR assay for buffalo, bovine, and porcine with a sensitivity of about 0.003 ng/µL. The variation of LOD could have been due to the difference between species to species, and some other factors such as processing conditions, degree of decomposition, and background matrices (Hossain *et al.*, 2017; Sultana *et al.,* 2020). With increased specificity, the porcine species could be found and quantified in raw gelatine samples using the TaqMan qPCR test that was established.

Target quantification and qPCR efficiency

The standard curve was used to quantify target DNA, and for this purpose, ten-fold serially diluted DNA was used to get 10, 0.1, 0.01, and 0.001 ng of total DNA in the reaction mixture. The qPCR assay was performed using each of these diluted DNA samples, and four different standard curves were constructed by plotting the Ct value against the logarithmic concentration of DNA (Figure 2). The standard curve was created using the R programming language's ggplot2 tool for improved comparison and display.

An excellent linear regression curve was found for the standard curves, as was reflected by its respective regression coefficient (R^2) in the range of 0.99 for porcine. The calculated PCR efficiency was 110% for the porcine, and the corresponding slope of each standard curve was -3.18. Hossain *et al.* (2017) previously reported PCR efficiencies of 108.73, 107.82, 94.68, and 104.03% for cow, buffalo, pig, and IAC, respectively. Similarly, Cheng *et al.* (2014) found an efficiency for duck, chicken, and porcine species of 104.38, 91.75, and 97.46%, respectively.

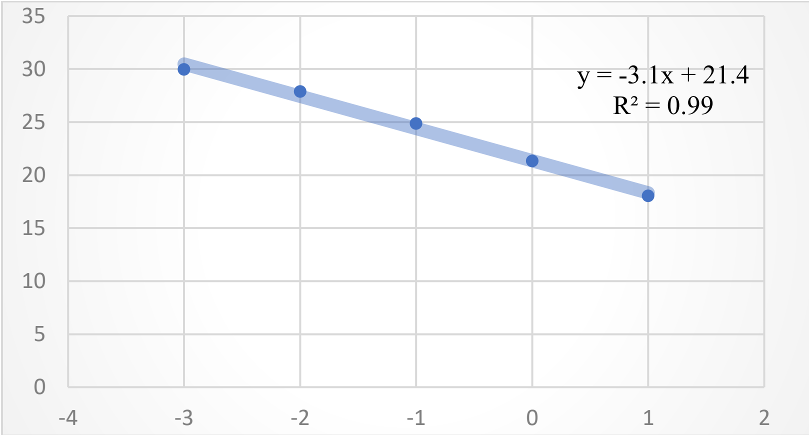

Figure 2. Standard curve for porcine primer generated using ggplot2 package of R programming language.

As a result, the obtained regression coefficient, PCR efficiency, and its associated slope aligned with the reports that had been previously published.

Analysis of porcine derivatives in ice creams, cakes, and pastries

Cakes, pastries, and ice creams are popular and delectable desserts that people consume worldwide. These food items are prepared using cocoa powder, emulsifiers, milk, sugars, and a whipping agent such as gelatine. Although gelatine is only 0.8 - 1.4% in the above-mentioned products, there is a high potential for porcine gelatine adulteration (Sultana *et al.,* 2018b). Since porcine gelatine is more widely available, and costs less, it is primarily the cause of adulteration. As a result, there is always a risk against halal adherence (Schrieber and Gareis, 2007; Sultana *et al.,* 2020). Consequently, any animal DNA found in those items indirectly confirmed that those products' used gelatine/gelling ingredients of animal origin (Tanabe *et al.,* 2007; Demirhan *et al.,* 2012; Yilmaz *et al.,* 2013; Amqizal *et al.,* 2017). In the present work, we developed and used a TaqMan probe-based qPCR assay to detect and quantify 25 halal-branded products: ice creams (10), cakes (10), and pastilles (5); purchased from various supermarkets in Malaysia. The experimental results demonstrated that all the tested food products were negative for porcine derivatives, and labelled accurately.

Conclusion

In the present work, a novel and highly sensitive qPCR system was successfully developed to detect porcine DNA in gelatine-based ice creams, cakes, and pastries. The shorter amplicon size (141 bp) provided improved stability under diverse conditions, and the assay demonstrated an exceptional sensitivity of 0.001 ng/µL, with a high correlation coefficient (R^2 = 0.99). These findings established the method as a robust tool for routine halal authentication of processed food products, ensuring compliance with dietary regulations. Furthermore, internal amplification control efficiently eliminated false-positive PCR amplifications, improving test reliability. Future applications of this assay could extend to testing broader categories of processed foods and beverages that may involve gelatine or animal-derived components. It is also recommended to adapt the assay to detect other sources of contamination, such as bovine or non-halal fish gelatines, to enhance its utility in verifying the authenticity of halal and kosher products. Looking ahead, integrating this qPCR-based detection system with portable or automated platforms could revolutionise on-site testing capabilities, enabling quicker results for food manufacturers and regulatory bodies. Moreover, adopting cutting-edge techniques such as CRISPR-Cas systems or next-generation sequencing could further augment the precision and scalability of porcine DNA detection. These advancements could ensure greater consumer trust, and elevate global standards for halal authentication practices.

Acknowledgement

The present work was financially supported by a research grant (grant no.: 6300256-14001) awarded to Prof. Ts. Dr. Azrina Azlan, Department of Nutrition, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

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