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Review Article

Detection of Porcine DNA in Cosmetic Products using Real-Time PCR Method: A Review of Method and Applications

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Abstract: For Muslim customers, the halal validity of cosmetics is a major concern. In cosmetics industry, one critical aspect is the contamination of porcine derivatives in halal cosmetic products. This review method discusses the application of real-time PCR (RT-PCR) for the detection of porcine DNA in cosmetic products, including the principle of the method, DNA extraction protocol, primers and probes design, and method application in various types of cosmetic products. DNA extraction from the cosmetic matrix samples requires optimization due to the complexity of the material and the possibility of DNA degradation during the cosmetic production process. The precise primers and probes design is also crucial to ensure specificity towards porcine DNA. The application of the RT-PCR method has been successful in various forms of cosmetics such as creams, lotions, lipsticks, and soaps, although each type of cosmetic product has unique challenges. Performance evaluation of the RT-PCR method showed good repeatability and reproducibility with coefficients of variation (CV) below 10% in numerous studies. The main challenges of the method include extraction of DNA due to the DNA degradation during the cosmetics production process, interference from cosmetic ingredients to RT-PCR analysis, and the need for method standardization. Solutions that have been developed such as the use of short gene targets, PCR additives to overcome inhibitors, and amplification techniques such as digital PCR to improve sensitivity. The recent development has led to the integration of protein-based methods and international standardization to support cosmetic halal certifications.

Keywords: porcine DNA, cosmetic, Real-Time PCR, detection, halal

1. INTRODUCTION

The rapid development of the global cosmetics industry has resulted in a vast array of cosmetic products with increasingly complex ingredients. In line with this progress, consumer demand for transparency of product compositions has also increased, particularly in relation to the authenticity of the halal material and components used in cosmetics products [1]. For Muslim consumers, who constitute a significant market segment, when making purchases, the halal status of cosmetics is an important consideration [2]. One of the main concerns is the possible contamination of porcine-derived in cosmetic formulations. There is a vast use of porcine-derived ingredients, such as collagen,

gelatine, and glycerine, in the cosmetic industry due to their beneficial physicochemical properties [3]. However, using porcine-derived ingredients will cause problems for Muslim and vegan consumers. Therefore, accurate detection of porcine-derived ingredients in cosmetic products is crucial, not only to satisfy consumer needs but also to guarantee the integrity of the halal label and adherence to international regulations [4].

The major challenge in the analysis of porcine-derived materials in cosmetic matrixes lies in the complexity of the product composition and the potential degradation of ingredients during the production process. Several analytical methods have been developed for the analysis of non-halal components in complex matrices, including cosmetics. These analytical methods are FTIR [5], ELISA [6], [7], SDS PAGE [8], [9], PCR [10], [11], LC-MS/MS [12], [13], [14]. Publications focusing on the development of analysis of pig derivatives in cosmetic products are still limited. However, DNA-based approaches using RT-PCR techniques have been widely used as a standard method in most countries to detect porcine DNA in pharmaceutical and food products [15]. PCR and RT-PCR methods have several advantages over other methods, namely sensitive, simple, fast and only require very small amounts of DNA samples [16].

Among various DNA-based techniques, Real-time PCR has been established that the preferred to be the method of choice for porcine DNA detection and quantification in various matrix samples, including cosmetics [17]. Real-time PCR has advantages in high sensitivity, its ability to perform quantitative analysis, and high-throughput analysis. This method does not require the laborious post-PCR analysis that is required for traditional PCR and may identify DNA targets in real-time during the amplification process [18]. Although Real-time PCR gives many advantages, its application for porcine DNA analysis in cosmetics has to encounter several challenges, such as complex DNA extraction from cosmetics matrix, primer and probe design that gives specificity, and PCR protocol optimization to overcome potential inhibitors in samples matrix[19].

A major challenge in DNA extraction from cosmetics lies in the presence of chemical ingredients such as oils, emulsifiers, and preservatives that can interfere with the DNA isolation process. Unlike food or meat samples, which typically have a simpler matrix, cosmetics contain a mixture of ingredients that require protocol modifications to remove inhibitors and ensure optimal DNA quality[20]. In addition, result interpretation and method standardization for the purpose of halal regulation and certification are important issues that require attention. The purpose of this review is to present a comprehensive overview of the application of real time PCR for porcine DNA analysis in cosmetic products. Aspects that will be discussed are the basic principles of the method, DNA extraction strategies from cosmetics matrix, primer and probe design, PCR protocols, as well as method application to various types of cosmetic products. Method performance evaluation, challenges encountered, and potential solutions will also be outlined. Furthermore, this review will discuss the recent developments and prospects of the application Real-time PCR in the context of halal cosmetics certification, and provide valuable insight for the researcher, industries, and regulators in an effort to guarantee halal cosmetic authenticity.

2. PRINCIPLE OF REAL-TIME PCR FOR PORCINE DNA ANALYSIS

Real-time PCR has become the preferred approach for porcine DNA detection and quantification in various matrixes, including cosmetic products. This technique combines target DNA amplification with real-time fluorescent detection, enables monitoring PCR process as it progresses, and makes post-PCR analysis unnecessary [21]. The schematic principle of the method is depicted in Figure 1.

Figure 1. The schematic principle RT-PCR [11]

2.1. The Fundamental Mechanism of Real-Time PCR

The fundamental mechanism of real-time PCR is similar to conventional PCR, involving a repeating cycle of DNA denaturation, primer annealing, and extension by polymerase DNA enzyme. The main difference lies in the use of fluorescent probes or dyes that allow real-time detection of PCR products. Fluorescent signal intensity increases proportionally with the amount of amplicon production [22]. The components of the real-time PCR amplification curve are exponential, linear, and plateau phases. The point at which fluorescent signal surpasses background noise is called the threshold cycle (Ct) or quantification cycle (Cq), which is inversely proportional to the initial target DNA amount. This Ct or Cq is then used as a basis for quantitative analysis reported as ΔCq or ΔΔCq values [23].

One of the key advantages of real-time PCR is its ability to provide both qualitative and quantitative results in a closed-tube system, significantly reducing the risk of contamination. Depending on the chemistry used, such as SYBR Green, TaqMan probes, or molecular beacons, the specificity and sensitivity of detection can vary. SYBR Green binds to any double-stranded DNA, making it suitable for general applications, while probe-based chemistries like TaqMan offer higher specificity due to sequence-specific hybridization and hydrolysis during amplification [24], [25]. The choice of chemistry and primer-probe design is critical in ensuring reliable quantification, particularly in complex samples or those with low DNA concentrations.

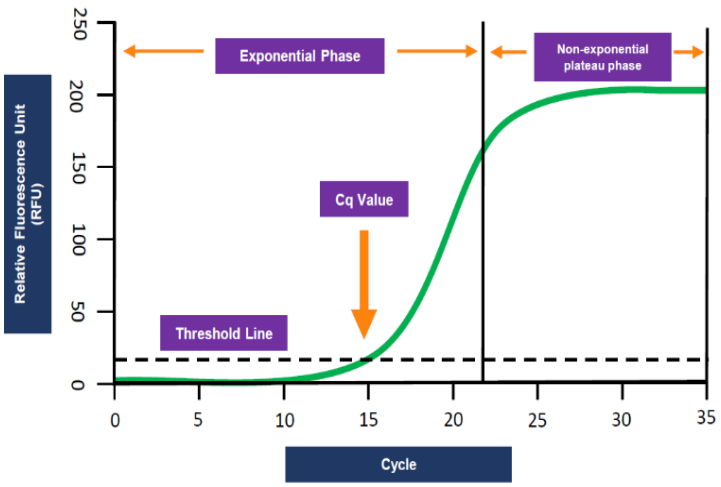

Figure 2. Amplification curve [11]

2.2. The types of Probe and Detection systems

For Real-time PCR, a number of fluorescence detection methods have been created, with their respective advantages and disadvantages. An intercalating dye called SYBR Green attaches to double-strand DNA in an unspecific manner. Although inexpensive and easy to use, SYBR Green can generate false positive signals that occur from the non-specific product or primer dimer [26]. Detection with TaqMan Probe using a specific oligonucleotide probe with reporter and quencher dyes. The activity of Taq polymerase 5' exonuclease will separate the reporter from the quencher, resulting in a fluorescent signal. In comparison with SYBR Green, TaqMan probes offer high specificity but are more expensive [27]. The system of Molecular Beacons uses hairpin probes with fluorescent dye and a quencher at its end. The hairpin structure opens when molecular beacon was attached to the target DNA, realising the fluorophore from the quencher and producing signals. Molecular Beacons are very specific but the design is more complex [28]. Scorpion primers are method that combines PCR primers with the Molecular Beacons probe. After the primer extension, the probe binds to the target sequences on the same strand, resulting in a fluorescent signal. This system offers faster reaction kinetics [29]. For DNA porcine analysis, TaqMan probes are often the choice due to their high specificity, although SYBR Green is also used in some studies due to its lower cost [30].

2.3. Quantitative and Qualitative Analysis

Porcine DNA can be analyzed both qualitatively and quantitatively using Real-time PCR. Qualitative analysis is based on the present or absent specific amplification, indicated by a characteristic amplification curve. Melt-curve analysis can give additional confirmation about the product specificity, especially when using SYBR Green dye [31]. Quantitative analysis uses a standard curve of the serial concentration of the DNA samples. The absolute quantification method determines the amounts of copies of the gene target, whereas relative quantification compares the amount of target DNA with the reference gene [32]. In the context of porcine DNA detection in cosmetics, a qualitative analysis is often sufficient to fulfill the halal certification purposes. Despite that, quantitative analysis can provide additional information on the degree of contamination, which is important for evaluating product safety and compliance with regulatory thresholds [33].

3. EXTRACTION AND PURIFICATION OF DNA FROM COSMETIC MATRIXES

Effective DNA extraction from cosmetic products is a critical step in the detection of porcine DNA using real-time PCR. The complexity of the cosmetic matrixes, which often contain various chemical and additive materials, makes the DNA extraction process challenging [34]. Isolation of DNA from fresh meat using different extraction methods always results in higher DNA concentration and quality compared to isolation of DNA from cosmetic products containing pork gelatin or its derivative [35]. DNA extraction from gelatine is more challenging than from fresh meat due to the low DNA content, degradation during the production process and strong interactions between DNA and the gelatine protein matrix, which significantly reduce the efficiency of DNA extraction and detection [36].

3.1. Extraction DNA method

For cosmetic matrixes, a number of DNA extraction techniques have been created and refined. Commercial kits, a commercial extraction DNA kit, such as DNeasy Blood and Tissue Kit (Qiagen)

or NucleoSpin Food Kit (Macherey-Nagel), are often used due to their simplicity and consistency. These commercial kits are commonly a silica column-based technology that binds DNA selectively [36]. Another DNA extraction method is Cetyltrimethylammonium Bromide (CTAB). CTAB is effective for samples containing polysaccharide and polyphenol compounds. CTAB will forms complexes with DNA, resulting in separation from other contaminants [37]. This method has been modified for various types of cosmetic samples [38]. The phenol-chloroform method is a conventional method that is still used for DNA extraction from samples that are difficult to extract, especially those rich in lipids. Phenol and chloroform separate DNA from the proteins and lipids [35]. The magnet-based method uses magnetic particles coated with silica to bind DNA. This method is efficient for small-volume samples and can be automated [39]. The selection of extraction method depends on the type of cosmetic product. For example, for lipid-based products such as lipstick, the phenol-chloroform method or specialized kit for fatty samples may be more effective than other methods [40]. Magnetic bead-based extraction, using silica-coated magnetic particles, is another efficient method, particularly suitable for low-volume and high-throughput applications. This technique offers automation compatibility and reduced reagent use [39].

3.2. Protocol Optimization for Different Types of Cosmetics

Optimization of DNA extraction protocols is critical due to the diverse physical and chemical properties of cosmetic products. Cosmetic products may exist in various physical forms, including solid, semi-solid, gel, oil-based, or liquid formulations, and typically contain complex mixtures of lipids, emulsifiers, surfactants, preservatives, and thickeners [41]. These components can significantly interfere with the efficiency of DNA isolation by inhibiting cell lysis or co-purifying with nucleic acids [10].

Some optimization strategies include: samples pre-treatment for solid products such as lipstick, mechanical homogenization or dissolution in organic solvent can improve extraction efficiency [38]. Buffer modification, the addition of detergents such as SDS or Triton X-100 can help to lyse cells and dissolve lipids [42]. The use of enzymes, such as proteinase K is often added to degrade protein and release DNA. For carbohydrate-rich samples, α-amylase enzyme can be helpful for DNA extraction. In addition of purification, for samples that are highly contaminated, additional purification steps such as alcohol precipitation of secondary column purification may be required [43].

3.3. Extracted DNA Quality and Quantity Evaluation

Evaluation of extracted DNA is important to ensure the success of PCR amplification. DNA quantification by measuring the concentration of DNA, performed using spectrophotometry (for example NanoDrop) or fluorometry (such as Qubit) methods. Absorbance ratios of A260/280 and A260/230 provide an indication of DNA purity. The measurement of DNA consentration is conducted at a peak absorption wavelength of 260nm, where an A260nm reading of 1 equates of 50μg/mL of double stranded DNA [44]. In the context of DNA integrity, gel electrophoresis can show a DNA degradation level. Degraded DNA may still be useable for amplifying short targets, but is not ideal for long targets [45].. Inhibitor assay, serial dilution of DNA samples and amplification of housekeeping genes can unveil the presence of PCR inhibitors. If required, additional cleaning steps such as dialysis or precipitation can be performed [43].

4. PRIMER AND PROBE DESIGN FOR PORCINE DNA ANALYSIS

The success of porcine DNA analysis using Real-time PCR is highly dependent on proper primer and probe design. The selection of appropriate target genes and specific nucleotide design are key factors in developing sensitive and selective methods.

4.1. Selection of Target Genes

Several genes have been used as targets for porcine DNA analysis in cosmetic products. One commonly utilized gene is Cytochrome B, a mitochondrial gene that is often chosen due to its high copy number in cells, which enhances detection sensitivity. Additionally, inter-species sequence variations allow for the design of specific primers [46]. Another gene, ATPase 8, also located in the mitochondria, has been proven effective for porcine DNA detection across various matrices, including cosmetics [47]. The Leptin gene has been widely used in studies because of its pig-specific sequence, making it a reliable marker [48]. Lastly, the Amelogenin gene, which plays a role in tooth enamel formation, has been used extensively for species identification in forensic and food analysis [49].

4.2. Design Criteria of Specific Primer and Probe

Effective primer and probe design must meet several criteria to ensure accuracy and efficiency. Specificity is crucial, as sequences must be unique to porcine DNA and should not cross-react with other species. In silico specificity examination using databases like BLAST is essential for verification [50]. Amplicon length is another important factor, with short amplicons (50–150 bp) preferred for Real-time PCR due to their higher amplification efficiency and compatibility with potentially degraded DNA in cosmetic products [51]. Thermodynamic characteristics also play a role, where the primer melting temperature (Tm) should range between 58–60°C, with a Tm difference of no more than 2°C between primers, and a recommended GC content of 40–60% [52]. Additionally, primer secondary structures should be minimized to avoid hairpins, self-dimers, or cross-dimers, which can be analyzed using tools like Primer3 or OLIGO [53]. Lastly, gene position selection is essential, ensuring primers target conserved regions unique to porcine DNA. For the TaqMan probe, its position should be close to one of the primers to enhance detection efficiency [51].

4.3. In Silico Validation and Experimental Testing

After primer and probe design, thorough validation is required to ensure accuracy and reliability. In silico analysis should be conducted using tools like Primer-BLAST to confirm primer and probe specificity toward porcine DNA and to check for any potential non-specific amplification [50]. Concentration optimization involves titrating primer and probe concentrations to determine the optimal conditions, with typical concentrations ranging from 300–900 nM for primers and 100–250 nM for probes [51]. Specificity validation is performed by testing the primer and probe against DNA from various species to ensure no cross-amplification occurs [19]. Sensitivity evaluation is carried out to determine the detection limit using a serial dilution of porcine DNA, with a standard curve used to assess amplification efficiency [33]. Lastly, reproducibility must be verified through repetitive trials to confirm the consistency of results [21].

5. REAL-TIME PCR PROTOCOL FOR PORCINE DNA ANALYSIS

The development of robust and sensitive real-time PCR protocols is a critical step in porcine DNA analysis for cosmetic products. The following table (Table 1) summarizes the critical steps involved in the real-time PCR-based analysis of porcine DNA, outlining key technical considerations that must be addressed at each stage to ensure accurate, specific, and reproducible results.

Table 1. Outlining the critical steps or protocol points in porcine DNA analysis using PCR (Polymerase Chain Reaction)

No.	Steps	Critical poin/ protocol	Explanation
1.	Sample Preparation	Use clean equipment and prevent cross-contamination	Sample contamination (especially with porcine DNA) can lead to false positives. Use disposable tools or thoroughly decontaminate instruments between samples.
2.	DNA Extraction	Choose an efficient DNA extraction method suitable for the sample matrix (e.g. food, gelatin, cosmetic)	Some matrices contain inhibitors or degraded DNA. Use validated kits or protocols that ensure high purity and sufficient yield of DNA for PCR amplification
3.	DNA Quantification & Quality Check	Use spectrophotometer or fluorometer; check A260/A280 ratio or run on agarose gel	DNA should be intact and free of inhibitors. Poor-quality DNA can cause PCR failure or inconsistent results.
4.	Primer Design/Selection	Use species-specific primers (e.g. D-loop or mitochondrial Cyt b genes for Sus scrofa)	Primers must be validated for specificity and efficiency. Cross-reactivity with non-target species leads to false positives.
5.	PCR Master Mix Preparation	Prepare in a DNA-free environment; use dedicated pipettes and filtered tips	PCR reagents are highly sensitive. Aseptic, contamination-free preparation ensures reliability. Include negative and positive controls in each run.
6.	Amplification (PCR or qPCR)	Optimize cycling conditions (annealing temp, extension time, etc.) for your primers and sample type	Sub-optimal conditions lead to poor sensitivity/specificity. For qPCR, ensure proper calibration and baseline setting.
7.	Controls (Positive/Negative/No Template)	Always run proper controls in each batch	High Ct values may indicate trace contamination or degradation. Set clear cutoffs for detection limits based on validation

5.1. Optimization of Reaction Condition

The optimization of reaction conditions covers several key aspects. Annealing temperature is crucial for primer specificity, with the optimum annealing temperature typically being around 5°C below the primer melting point. This can be determined more accurately using a temperature gradient. Cycle number is also an important factor, with 35–45 cycles commonly used. Too many cycles may lead to non-specific amplification, while too few cycles can reduce sensitivity [54]. Reagent concentration optimization is vital for ensuring specificity and efficiency in the reaction, with primers

generally used at concentrations of 100–900 nM, probes at 100–250 nM, and MgCl2 concentrations carefully adjusted to suit the reaction [51]. Finally, the reaction volume is typically set between 20–25 μL, with 1–5 μL reserved for template DNA. While lower total reaction volumes may improve sensitivity, they can also introduce variability in the results[52].

5.2. Positive and Negative Controls

Appropriate controls are essential for ensuring the validity of the results. Positive control involves using pristine porcine DNA with a known concentration to verify that the PCR system is performing correctly [30]. Negative control consists of a reaction with no template DNA to detect any contamination, and it is also important to include an extraction control, such as using water as a sample [43]. Internal amplification control (IAC) is used to detect PCR inhibition within the sample. The IAC may be a housekeeping gene or a synthetic sequence that is added to the reaction to monitor for inhibition during amplification [55]. Lastly, specificity control involves using DNA from non-target species to ensure there is no cross-amplification occurring [46].

5.3. Amplification and Melt Curve Analysis

The real-time PCR results interpretation involves analyzing the generated curve. The amplification curve shows a rise in fluorescence as PCR products accumulate, with the threshold cycle (Ct) being used in the quantification process. The value of Ct is inversely correlated with the quantity of initial target DNA [23]. The melting curve, especially with the SYBR Green system, helps differentiate specific from non-specific products. Specific products will have consistent melting peaks, which can be used to assess product specificity [56]. Finally, amplification efficiency is computed from the slope of the standard curve. The ideal amplification efficiency is 100%, corresponding to a slope value of -3.32, and efficiencies ranging from 90% to 110% are generally accepted [52].

5.4. Method Validation

Before a methodology can be used for routine analysis, a number of parameters must be tested to assess its performance and suitability for its intended use [57]. Halal determination is part of the qualitative analysis because Muslims are not allowed to use any items that include porcine components, even in trace amounts [58]. There are several parameters for the validation of qualitative real-time PCR methods, namely: specificity, sensitivity, PCR efficiency, linearity, and robustness [57]. Specificity is a quality of a technique that responds only to the characteristic/analyte of interest. Specificity examination needs to be performed both in silico and experimentally, utilizing the sequence of the target DNA and non-target [59]. For a real-time PCR technique that is developed by using the SYBR Green dye detection, an additional parameter, which is the melting temperature of the amplicon, needs to be taken into account [57]. The sensitivity of a real-time PCR method, expressed as the limit of detection (LOD), is the lowest concentration of analyte that can be reliably detected but not necessarily quantified [59]. The limit of detection value is determined by using the lowest dilution that shows detectable DNA target from 12 PCR replicates (LOD95%). This LOD95% value is expressed in DNA weight (ng) [57]. PCR efficiency is defined as the amplification rate that produces a theoretical slope of -3.32 with a 100% efficiency in each cycle. This parameter is calculated

by using a dilution series of a target DNA [57], [60]. The PCR reaction linearity is expressed as the correlation coefficient (R2) of the curve obtained by linear regression analysis. PCR linearity is established using the same methodology to assess PCR efficiency. This parameter is used to ensure accurate measurement over a range of concentrations [57]. Lastly, robustness analysis is an examination to measure real-time PCR's ability to be unaffected or provide a similar result toward minor and intentional modifications (e.g., a slight variation in annealing temperature) to the experimental procedure conditions [60]. This test is conducted to ensure consistent performance in routine analysis [57].

6. COSMETIC MATRIXES INFLUENCE ON DETECTION SENSITIVITY

The effectiveness of PCR amplification and DNA extraction may be negatively impacted by cosmetic matrixes. PCR inhibitors, such as colorants, preservatives, and surfactants in cosmetic products can inhibit PCR. Schrader et al. (2012) [43] recommend the use of PCR additives such as BSA or DMSO to overcome the inhibition. DNA degradation, cosmetic production processes like heating and the addition of chemicals can degrade the DNA. Mohamad et al. (2013) [36] suggested the use of short gene targets (<150 bp) to increase the success of amplification on degraded DNA. Low DNA concentration, several cosmetic products may contain a very low concentration of DNA. Floren et al. (2015) [61] used a pre-amplification technique to improve the sensitivity of the detection in samples with low concentrations of DNA.

7. METHOD PERFORMANCE EVALUATION

7.1. Limit of Detection and Quantification

Limit of detection (LOD) and limit of quantification (LOQ) are critical parameters in the evaluation of the method. LOD is defined as the lowest porcine DNA concentration that can be consistently detected. Zabidi et al. (2018) [62] reported a LOD value of 0.001 ng/μL to detect porcine DNA in cream cosmetic matrixes using real-time PCR. The lowest concentration that can be measured with reasonable precision and accuracy is known as the limit of quantification (LOQ). Although several studies have addressed the detection of porcine DNA in food products, the determination of LOQ in cosmetic matrices remains unexplored. The determination of LOD and LOQ is usually performed by using a porcine DNA dilution series in cosmetic matrixes [21].

7.2. Specification and Selectivity

The method's specificity towards porcine DNA and its selectivity against other species is critical. The specificity testing involves the analysis of samples containing DNA from various animal and plant species. Soares et al. (2013) [48] demonstrated high specificity of primers and probes that were created for the porcine cytochrome B gene, without cross amplifications with bovine, chicken, and goat DNA. Evaluation of the selectivity involves method testing on cosmetic samples that include a mixture of DNA from different species. Tanabe et al. (2017) [47] showed that their real-time PCR method could selectively detect porcine DNA in the complex mixture containing DNA from several animal species.

7.3. Repeatability and Reproducibility

Consistency of results within repetition and inter-laboratory tests is a crucial indicator of method reliability. This refers to the variation of results when analysis is repeated by the same operator, using the same instruments, and conducted in a short interval of time. Floren et al. (2015) [61] reported a coefficient of variation (CV) was below 5% for repeatability testing in porcine DNA detection using droplet digital PCR. Reproducibility: evaluate the variation of the results between laboratories or when a testing condition changes significantly. Amaral et al. (2019) [46] performed a collaborative study between laboratories and found a CV value of less than 10% for reproducibility in porcine DNA quantification using real-time PCR.

7.4. Accuracy and Precision

The method's accuracy is established by comparing the results obtained from the analysis with known values in reference materials. The impact of sample matrix effects, especially when the sample matrix is different from the reference material, must be considered. An accuracy value of ± 25%, at the PCR stage, should be acceptable throughout the dynamic range [19]. Precision is a critical parameter in validating PCR assays, representing the reproducibility and consistency of results under specific conditions. Precision is typically expressed through the relative standard deviation of repeatability should be ≤25% across the method's of dynamic range [19].

7.5. Robustness/Ruggedness

These evaluations examine the stability of the method against small variations in experimental conditions. Testing the impact of small variations within the method parameters, such as annealing temperature and reagent concentration. Cai et al. (2019) [63] demonstrated the robustness of the method with variations of ±1°C in annealing temperature and ±10% in primer concentration. Evaluate the stability of the method when performed by different analysts or using different instruments. The measurement response with slight changes shall not be more than ±35% in the reproducibility test for the response obtained under normal condition [18].

8. CHALLENGES AND SOLUTIONS

8.1. DNA Degradation within Cosmetic Production Processes

Cosmetic production processes such as heating, chemical substances addition, and homogenization may cause DNA degradation, reducing the amount and quality of target DNA that is available for amplification 20. Therefore, several solutions are needed including the usage of short gene targets, where shorter amplicons (<150 bp) have a higher probability of persist in DNA degradation. Ren et al. (2017) [64] demonstrated improvement in detection sensitivity using primers that amplified a 100 bp fragment in the cytochrome gene. In addition, another solution that can be done is by optimizing the extraction method. Yoshida et al. (2009) [42] developed a modified DNA extraction protocol by using guanidinium isothiocyanate (GITC) buffer that is effective for highly processed samples. Another option that can be considered is a whole genome amplification technology. Floren et al. (2015) [61] used multiple displacement amplification (MDA) before PCR to increase the amount of template DNA within the highly processed samples.

8.2. Cosmetic Materials Interference to PCR

The challenge that needs to be aware of is cosmetic materials such as colorants, preservatives, and surfactants that may inhibit the PCR resulting in false negative results [43]. From these challenges, alternative solutions can be chosen, namely, the usage of PCR additives, the inclusion of bovine serum albumin (BSA), or dimethyl sulfoxide (DMSO) to the PCR reaction can reduce the effect of PCR inhibitors. For additional DNA purification, a 2-steps purification method, combining silica-based extraction with ethanol precipitation, to remove PCR inhibitors from the complex matrices [10]. Last but not least, internal amplification control (IAC), the use of IAC helps identify samples with PCR inhibition. Soares et al. (2013) [48] integrated IAC within a real-time PCR assay to validate the negative results.

8.3. Sensitivity and Specificity

One of the main challenges is achieving high sensitivity and specificity of the method, especially for a low level of contamination detection in complex cosmetic matrixes [8]. For this reason, solutions that are offered such as: better primer and probe design by using advanced bioinformatic tools to design primers and probes that are highly specific. Tanabe et al. (2017) [47] used extensively in silico analysis to design porcine-specific primers with a minimal homology towards other species. Preamplification technique, Floren et al. (2015) [61] applied target-specific preamplification before real-time PCR to improve sensitivity of the detection in low DNA concentration samples. Digital PCR, Cai et al. (2019) [63] demonstrated sensitivity and precision improvements using droplet digital PCR, compared to the conventional real-time PCR for quantification of porcine DNA.

8.4. Method Standardization

The challenge faced is the lack of international standard method agreements for porcine DNA detection in cosmetics which inhibits inter-laboratories result comparison and consistent regulation implementations [34]. Therefore, the solution that needs to be considered is the development of the validated method, Amaral et al. (2019) [46] explained the importance of collaborative studies between laboratories to validate and standardize real-time PCR method in order to identify porcine DNA in various matrixes. Protocol harmonizations, an initiative such as Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) that have been proposed by Bustin et al. (2009) [52] gave guidance to standardize and report real-time PCR experiments. Using approved reference materials, the production and use of certified reference materials for porcine DNA in cosmetic matrixes can improve results homogeneity and comparability between laboratories [57].

9. RECENT DEVELOPMENTS

9.1. Usage of Digital PCR Technology

Digital PCR (dPCR) is emerging as a promising alternative to conventional real-time PCR. This is because digital PCR has high sensitivity. Cai et al. (2019) [63] demonstrated that droplet digital PCR (ddPCR) can detect and quantify porcine DNA much lower than conventional real-time PCR. Absolute quantification, Floren et al. (2015) [61] used ddPCR for absolute measurement of porcine DNA without the necessity for a standard curve, improving accuracy and reproducibility. Tolerance

to inhibitors, Morisset et al. (2013) [65] reported that ddPCR is more tolerant towards PCR inhibitors that are commonly found in complex matrixes.

9.2. Integration of Protein-Based Methods

Combining DNA and protein-based techniques provides a more comprehensive approach. Double confirmation, The mass spectrometry method provides sensitivity comparable to PCR and ELISA methods, so it has great promises as a different analytical method for identifying and authenticating meat [66]. Protein-specific detection, Yang et al. (2018) [67] developed an LC-MS/MS, this method allows comprehensive detection of gelatin speciation and adulteration as low as 0.1% (w/w) of unwanted porcine gelatin.

Furthermore, the use of liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) has advanced the detection of species-specific peptide markers for porcine gelatin, enabling untargeted proteomics approaches in halal authentication. Recent studies have demonstrated the successful identification of unique porcine peptide markers using LC-HRMS and bioinformatics-based database searches such as UniProt, which allow for confident differentiation between bovine, porcine, or dog in cosmetic matrices [68], [69], [70]. This integration of peptide-based authentication provides an alternative strategy when DNA integrity is compromised during processing.

9.3. Application of New Generation Sequencing Technologies (NGS)

The NGS offers a new approach to species detection, such as metagenomic analysis, Ripp et al. (2014) [71] used NGS to analyze the metagenomic of processed food products, detecting multiple species in a single analysis. As well as variant identification, Bertolini et al. (2015) [72] applied NGS to identify porcine-specific genetic variation, improving the specificity of the detection.

Moreover, NGS has also been employed for authentication and detection of animal DNA in complex matrices, where conventional PCR techniques may fall short due to DNA degradation or the presence of multiple species [73], [74], [75]. Additionally, targeted amplicon sequencing combined with bioinformatics filtering has been proven to enhance the sensitivity and resolution of species identification in highly processed foods, providing a robust tool for halal authentication and food safety surveillance [76], [77].

9.4. Development of Biosensors and Rapid Methods

Innovations in biosensor technology offer the potency for rapid testing in real-life settings. Such as CRISPR-based biosensors, Dai et al. (2019) [78] developed a CRISPR/Cas12a biosensor for rapid detection of porcine DNA, providing the results in less than an hour. Lateral flow assay, Ali et al. (2017) [19] designed a gold nanoparticles-based lateral flow strip for visual detection of porcine DNA, enabling on-site testing without sophisticated instruments.

9.5. Standardization and Harmonization of the Method

The development of standardized and harmonized methods for porcine DNA detection in cosmetics is essential to ensure the accuracy, reproducibility, and global acceptance of analytical

results, particularly in the context of halal assurance. Analytical approaches such as real-time PCR have been widely applied due to their high sensitivity and specificity; however, variations in DNA extraction protocols, target gene regions, primer design, and amplification conditions may lead to inconsistent results across laboratories [11], [25]. Establishing unified standards—similar to those developed by CEN/TC 327 for animal feed—can help minimize methodological discrepancies and enable reliable inter-laboratory validation [79]. Moreover, harmonized methods would support regulatory bodies and certification agencies in implementing consistent criteria for halal verification, product labeling, and import-export regulations, thereby strengthening consumer confidence and industry compliance worldwide [80].

9.6. Integration of Analytical Quality by Design (AQbD) in Porcine DNA Detection for Cosmetics

Recent advances in analytical chemistry have introduced the Analytical Quality by Design (AQbD) framework as a modern paradigm for developing and validating robust analytical methods [81]. In the context of porcine DNA detection in cosmetic products, AQbD offers a systematic, science- and risk-based approach to ensure method reliability across diverse cosmetic matrices. Unlike traditional validation approaches, AQbD begins with the definition of an Analytical Target Profile (ATP), followed by risk assessment to identify critical method parameters (CMPs) and critical quality attributes (CQAs). Tools such as Design of Experiments (DoE) are employed to optimize method performance within an acceptable Method Operable Design Region (MODR) [81], [82]. The application of AQbD in real-time PCR for porcine DNA detection enables better control over variability sources, enhances method transferability, and aligns well with international goals for method harmonization and proficiency testing [25], [83], [84]. As the demand for halal certification grows globally, implementing AQbD is increasingly important to support regulatory compliance and consumer trust. he following table presents the key elements of Analytical Quality by Design (AQbD) as applied to real-time PCR methods for porcine DNA detection in cosmetic products. The following table presents the key elements of Analytical Quality by Design (AQbD) as applied to real-time PCR methods for porcine DNA detection in cosmetic products (Table 2).

Table 2. AQbD Elements in Real Time PCR for Porcine DNA Detection in Cosmetics

AQbD Component	Application in Porcine DNA Detection
Analytical Target Profile (ATP)	Specific, sensitive, and reproducible detection of porcine DNA in cosmetics
Critical Quality Attributes (CQAs)	LOD, LOQ, specificity, PCR efficiency, R^2, reproducibility
Critical Method Parameters (CMPs)	DNA extraction protocol, primer/probe design, annealing temperature
Risk Assessment Tools	Fishbone diagram, FMEA (Failure Mode and Effects Analysis) for identifying risk-prone steps
Method Operable Design Region (MODR)	Acceptable range for robust performance
Control Strategy	Internal controls, SOPs, positive/negative controls, reference materials

10. CONCLUSION

Porcine DNA detections in cosmetic products using real-time PCR have been shown to be successful and trustworthy way method to ensure the halal authenticity of cosmetic products. This comprehensive review has covered many important aspects of the application of the method, from basic principles to current challenges and solutions. Real-time PCR provides excellent specificity and sensitivity for porcine DNA detection, has the capacity to identify contamination down to 0,001 ng/μL level in cream cosmetic matrixes. Proper selection of target genes, such as cytochrome B or ATPase 8, as well as specific primers and probes design are critical regarding the success of the detection. Extraction DNA protocol optimization from complex cosmetic matrixes is also a critical step to ensure adequate DNA quality and quantity for analysis. Method performance evaluation including parameters such as LOD, LOQ, specificity, repeatability, and reproducibility is essential to validate the reliability of the method. The main challenges in the application of the method are DNA degradation during cosmetic production processes, interference from cosmetic materials to PCR, and the need for high sensitivity for low-level contamination detection. Innovative solutions have been developed to overcome these challenges, namely the use of whole genome amplification technologies, additive PCR to reduce inhibition, and the application of digital PCR to improve sensitivity and precision. Method standardization and the use of reference-certified material are important to ensure consistency in results between laboratories and the effective implementation of regulations.

Although there has been significant progress in the application of real-time PCR for detection of porcine DNA in cosmetics, further studies are still needed to advance the method's, specificity, sensitivity, and applicability. The development of standardized protocols that can be implemented widely will greatly benefit the halal cosmetic industries, regulators, and consumers. With the advancement of molecular technologies and the increasing demands for transparency within cosmetic industries, real-time PCR is expected will remain the preferred method for porcine DNA detection, which are essential to guaranteeing the future integrity and halal label of cosmetic products.

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