Analysis of halal soft capsule gelatin with real-time polymerase chain reaction method

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INTRODUCTION

The government creates a halal certificate instrument to guarantee the halalness of a product to protect consumers from the circulation of non-halal products (Hasan, 2014). This is because not all products circulating in the market are guaranteed halal. Therefore, article 4 of Law no. 33 of 2014 concerning Halal Product Guarantee requires business actors to have a halal certificate and a halal logo (Anonymous, 2014). Products not including the halal logo and whose halal status is doubtful need to undergo a halal authentication process before being declared to contain non-halal ingredients. Therefore, product composition analysis regarding the presence or absence of non-halal components is necessary. The prospect of halal products, especially food, is developing rapidly. Currently, Indonesians consume halal products, reaching 1.8 billion people, and the current halal food market is predicted to be more than US 661 billion (Ali et al., 2012). Along with increasing public awareness of halal food, halal products are believed to grow exponentially (Rahman et al., 2014).

Approximately 10% of pharmaceutical preparations circulating in the community are capsules with gelatin's the main ingredient. Capsules are used for medicinal and preventive purposes, so most people must consume capsules. Capsule dosage forms provide various conveniences compared to other solid dosage forms of pharmaceutical products. Hence, manufacturers prefer to produce capsules for pork, beef, and fish skin and bones to meet potential market needs. Therefore, a sensitive and specific method for confirming the origin of gelatin is needed.

Gelatin is a protein produced from the partial hydrolysis of collagen tissue which can be extracted from the skin, connective tissue, and bones of farm animals, including fish and poultry, pigs, cattle, and fish (GMIA, 2012). Gelatin from pigs is the most widely used in the medicine and food industry, considering that more gelatin from pigs is produced internationally; this is because it is easier for raw materials from pig slaughter to be very large in quantity and ready to be used to create porcine gelatin compared to beef. The use of gelatin is increasingly diverse, but this needs to be matched by sufficient growth in domestic gelatin production so that until now, domestic needs have been met using imports. Based on data from the Central Statistics Agency (BPS) for 2020, the amount of gelatin imported into Indonesia reached 4808 tons worth 355 billion rupiahs. This statistical report shows how large the use of gelatin is in Indonesia, so it needs supervision regarding the certainty of halal gelatin (BPS, 2020).

Various kinds of gelatin and food products, both from imports and domestic industries, require clarity on the raw materials used, so the certainty of methods for detecting the raw materials used is urgently needed. Analysis of non-halal components such as porcine gelatin and pork in food products presents a certain complexity because these non-halal components are usually added/mixed in biochemically similar matrices. For example, (non-halal) pork added to meatballs is labeled as beef meatballs (halal meatballs) (Rohman et al., 2017), and porcine gelatin may be used in the manufacture of capsule shells, which are usually prepared from beef gelatin.

This study aimed to validate the quantitative PCR method for identifying DNA in gelatin-based products and to apply the confirmation method designed for samples of gelatin-based products on the market circulating in Yogyakarta to prove the halalness of these samples. The urgency of this research is related to the analysis method of pig DNA identification using qPCR with specific primers in various products to issue halal status. The negative results from this study still need to be confirmed using specific primers for bovine DNA to prove that the absence of porcine DNA indicates that bovine DNA is present in the product. The application

of this method is also very useful for ensuring the authenticity of the capsule shell, especially from cross-contamination and counterfeiting.

MATERIALS AND METHOD

Primer design

Primer design was carried out using the online software PrimerQuest Tool with access code AF034253 for DNA DLOOP (Table 8). The objective of primer design was to obtain a specific SS2 primer pair for the porcine DLOOP (Sus scrofa) sequence. The primer was chosen by considering several parameters of the primer design, namely having a length of 15-30 bp, 40-60% GC content, G and C nucleotides evenly distributed along the primer and primer Tm ranging from 50-60°C (Borah, 2011). Based on the *in silico* design of DNA primers, a pair of pig primers (SS2) were obtained which were used in this study, namely forward: 5'-ACTTCATGGAACTCATGATCCG-3', and reverse 5'-ATGTACGTTATGTCCCGTAACC-3'. This porcine primer has melting temperature characteristics of 58.20°C (F) and 57.95°C(R), GC content of 45.45% (F) and 40.00% (R) with an amplicon length of 103 base pairs (bp) (Salamah et al., 2021).

Preparation and isolation of DNA from gelatin and commercial capsule shell samples

The blended capsule shells were weighed approximately 250 mg, added with 800 μL of 70% ethanol, vortexed for 30 minutes, then incubated in a water bath at 65oC for 30 minutes with vortex every 15 minutes. Then each was added 800 μL lysis buffer and 20 μL proteinase K and homogenized, then incubated in a water bath at 65oC for 30 minutes with a vortex every 15 minutes. Add 10 μL of RNAse and incubate in a water bath at 38oC. Then phenol and 0.5x volume of MCHA each were added and shaken with a shaker for 40 minutes. The mixture was centrifuged for 30 minutes at 14500 rpm at room temperature.

Furthermore, the supernatant was transferred to a new Eppendorf, and 1x chloroform was added, then shaken for 15 minutes. Then the mixture was centrifuged for 10 minutes at 14500 rpm; the supernatant was transferred to a new Eppendrof and 0.1x volume of 3M Na-acetate pH 7.6 was added 2x volume of absolute cold ethanol was added, incubated at -80oC overnight. They were centrifuged for 5 minutes at 15000 rpm at 4oC. The remaining ethanol was air-dried in LAF for about 10 minutes, then added 40 μL of TE buffer and stored at -20oC for further analysis (Sambrook et al., 1989).

Instrumental analysis using qPCR

qPCR analysis was carried out using a 20 μL reaction mixture, as written in Table I, and the qPCR conditions followed the data in Table II.

Table I. qPCR reaction mixture using *Evagreen* with a total volume of 20 µL.

Formula	Volume (μL)
Evagreen	10
Primer Reverse SSB (5μM)	1.0
Primer Forward SSB (5μM)	1.0
DNA (50 ng)	2.0
NFW	Variety
Total Volume	20

Table II. Program the temperature of the qPCR reaction in one amplification cycle.

Phase	Condition
Initial Denaturation	95°C, 30 seconds
Denaturation	95°C, 5 seconds
annealing	52°C, 30 seconds
Extension	72°C, 10 seconds
Number of cycles	40 cycles

Primary specificity test

Using qPCR, a primer pair specificity test was carried out at the optimum attachment temperature to amplify 50 ng of bovine and porcine DNA. This test was also carried out on DNA isolated from pure bovine and porcine gelatin (Ponchel, 2006; BioRad, 2006).

Sensitivity test of the qPCR method on gelatin and capsule shells as a comparison

This was done by making eight DNA dilutions (1000; 200; 100; 10; 5; 1; 0.5; 0.01 pg/μL) either from pure bovine gelatin or from a comparison capsule shell with 100% bovine gelatin. Sensitivity tests were also performed on all comparison capsule shell formulas at the optimum sticking temperature (Soares et al., 2013).

Repeatability test

It was carried out by taking a point in the standard linear curve from the 100% bovine gelatin DNA dilution series as well as that produced by DNA from the comparison capsule (porcine-bovine gelatin mixture), which has a regression equation to measure the repeatability of the amplification response for four replications.

Analysis of commercial capsule shell samples

The samples tested came from 4 different soft capsule manufacturers, namely Market samples 1, 2, 3, and 4. The method used is the same for testing comparator capsule shells, starting from specificity, sensitivity, and repeatability tests.

RESULT AND DISCUSSION

Gelatin is a polypeptide resulting from the partial hydrolysis of collagen obtained from animals' skin, connective tissue, and bones, such as cattle, pigs, fish, and even insects (Abdelfadeel, 2012). The most dominant amino acids in the formation of the primary structure of gelatin are glycine, proline, and hydroxyproline, which are arranged repeatedly. In the process of gel formation, the structure of the gelatin undergoes a conformational change from the primary structure (random coil) to the secondary structure (α-helix) to return to the initial structure of collagen in the form of a triple-α-helix through the formation of intra- and inter-peptide hydrogen bonds, thus forming a woven/crosslink. This change in gelatin structure can complicate the DNA isolation step. Therefore in the gelatin DNA isolation technique, a preparation step is required by dissolving the gelatin in 1 mL of PBS-ethanol solution (1:1).

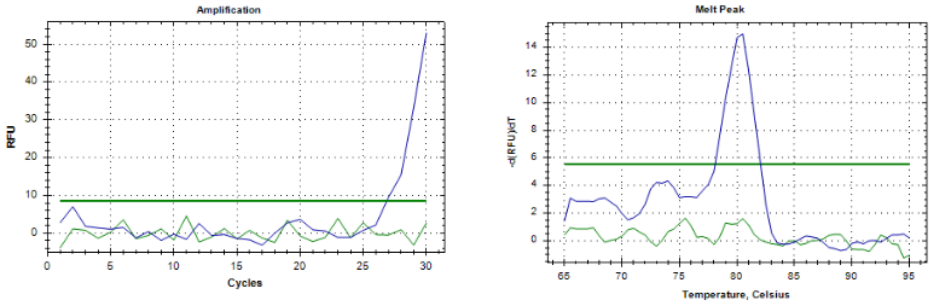
Phosphate Buffered Saline (PBS) used during the DNA extraction process aims to prevent changes in the random coil gelatin structure into a triple-α-helix because the salts contained in PBS cause ionic bonds to form. As a result, the possibility of forming hydrogen bonds is slight, so crosslinks do not occur. Gelatin is a lyophilic colloid that interacts strongly with the dispersion medium. It is very soluble in the dispersion medium, and gelatin is a colloid that dissolves easily in water to expand easily. This situation will complicate the isolation process. Therefore absolute ethanol is added to prevent the gelation of gelatin in water through a dehydration mechanism. Ethanol is a solvent with a lower polarity than water, resulting in competition in forming bonds between gelatin-water-ethanol. This situation causes the stability

of the colloidal gelatin to be damaged so that the gelatin does not expand and precipitate. After the sample was dissolved entirely in the preparation solution, added 1 mL of absolute ethanol was centrifuged for 3 minutes at 13000 rpm to precipitate the gelatin, then the supernatant was discarded. The resulting gelatin precipitate is then treated like a meat sample to isolate DNA, starting from the cell lysis stage to dissolving the DNA isolate in TE buffer (Erwanto et al., 2024).

The quantitative analysis of DNA isolates with a spectrophotometer (nanodrop) showed that a ratio of A260/A260, which was less than 1.8, indicated protein contamination. In contrast, a ratio of more than 1.8 indicated the presence of RNA contamination. The results of the quantitative analysis showed that the isolation included incomplete washing, so the DNA obtained was not pure. The Codex Alimentarius Commission (2010) states that high DNA purity allows for optimal amplification, affecting a PCR method's validity.

The results of the initial stages were carried out by testing the specificity of the primer at the optimum annealing temperature obtained in the previous test with several cycles of 30 cycles. Figure 1 shows that the specific primer where only the target DNA, porcine gelatin, gave a positive signal, while bovine gelatin was not amplified.

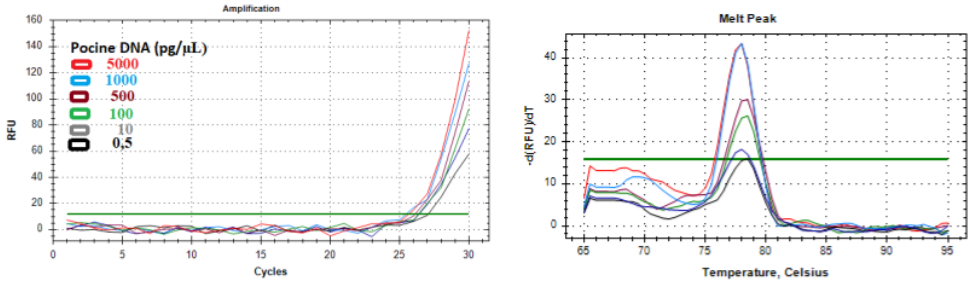
Cytochrome B primer pairs specific for pig DNA at an attachment temperature of 52.0°C were then tested for specificity against DNA isolates from pure gelatin, both bovine and porcine gelatin. In this test, 50 ng of bovine DNA was also used as a positive control. The amplification response of DNA isolates is shown in Figure 1. The resulting amplification only came from porcine gelatin DNA isolates (RFU = 50, Ct = 26.85, and Tm = 80.50), and bovine gelatin DNA isolates (positive control) did not experience amplification. This shows that the primer D-LOOP is specific only for porcine gelatin DNA with an annealing temperature of 52.0°C



A B

Figure 1. Amplification curve (A) and melt curve analysis (B) of CYTBWB2 primer on porcine gelatin DNA and bovine gelatin comparator DNA.

After obtaining a specific primer and optimum annealing temperature, it is followed by method validation consisting of a sensitivity, linearity, and repeatability test. The type of validation used is partial validation for quantitative methods. Method validation used several porcine gelatin DNA samples from the porcine gelatin DNA dilution series. DNA extracted from porcine gelatin with several types of composition, as well as DNA isolated from commercial soft capsules.



A B

Figure 2. Amplification curve (A) and melt curve analysis (B) in the sensitivity test for porcine gelatin DNA using primers.

Based on the results of the sensitivity test in Figure 2 shows that the Limit of Detection (LOD) value of porcine gelatin DNA in this method is at a concentration of 0.5 pg/µL. The next stage is the linearity test, as shown in Figure 3 and Table III. The linearity test was carried out by constructing a standard curve based on the amplification results of 7 dilution series of porcine gelatin DNA. The standard curve describes the linear relationship between the $\log^{10}$ concentration series of porcine gelatin DNA and Cq (cycle quantification) values. The standard curve of the serial amplification results of porcine gelatin DNA dilution using 7 points at 5000, 1000, 500, 100, 10, and 0.5 pg/µL. Making this standard curve also simultaneously calculates the efficiency value of the PCR amplification process.

The efficiency value can be influenced by several factors, including the presence of inhibitors in the PCR master mix, the purity of the reagents used, the inconsistency in the small volume pipetting process, which results in low accuracy, and is also influenced by the ability of the test which depends on the specificity of the primer and the length of the target sequence of Template DNA (Svec et al., 2015; Muhammed et al., 2015). An efficiency value that is too large indicates that the target DNA multiplied each cycle is disturbed by the presence of an inhibitor during the reaction. At the same time, an efficiency value that is too small indicates incomplete primer attachment, which results in exponentially less template amplification in each cycle.

Table III. Linearity test results of porcine gelatin DNA.

Concentration (pg/µL)	Cq
0,5	25,51
10	25,69
100	26,14
500	26,38
1000	26,43
5000	27,08

The linear regression equation was also obtained from the standard amplification reaction curve for porcine gelatin DNA isolates (Table III and Figure 3), with an R2 value of 0.962, a slope of 0.475, and a y-intercept of 26.168, so that the curve meets the criteria of fairly good linearity. The resulting efficiency value (E) (99.2%), so that the efficiency value obtained meets the acceptance requirements in the range of 90-110% (Bio-Rad, 2006; Anonymous, 2012). Linearity test results on porcine gelatin DNA with various concentrations also gave good results.

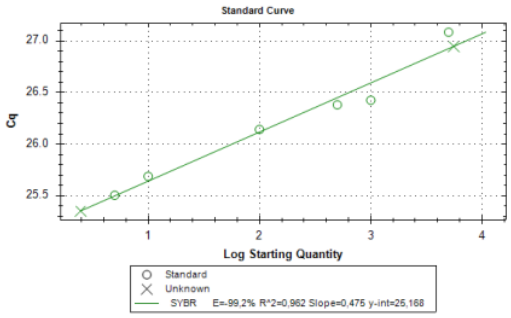
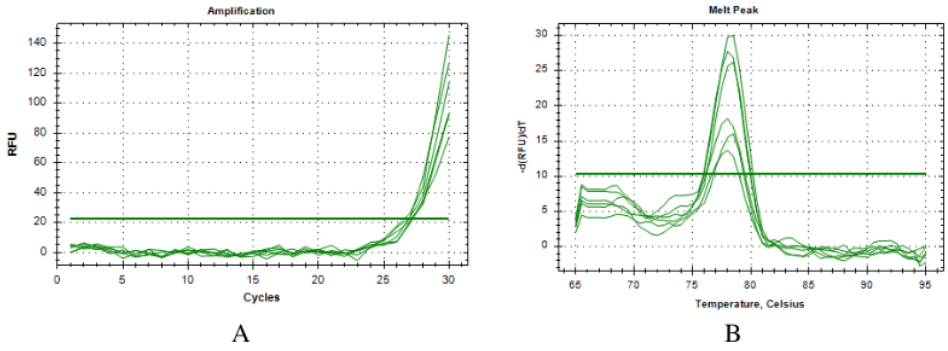


Figure 3. Standard curve of porcine gelatin DNA amplification results with various concentrations using primers

The following validation method is the repeatability test which is intended to measure the repeatability of the amplification results from bovine gelatin DNA at a concentration of 1000 pg/μL by calculating the average standard deviation (SD) and coefficient of variation (CV) values. Repeatability expresses the closeness of results between a series of measurements obtained from several homogeneous samples in a short time under predetermined conditions. The amplification data obtained can be seen in Figure 4. The amplification response repeatability test on porcine gelatin DNA isolates at a concentration of 1000 pg/μL resulted in a coefficient of variation (CV) of 0.85%. The CV value meets the recommended criteria for using the PCR method, namely CV≤25% (CAC, 2010). The resulting Ct (cycle threshold) data, average Ct, standard deviation, and coefficient of variation are shown in Table IV.



A B

Figure 4. DNA repeatability test results isolated from porcine gelatin at a concentration of 1000 pg/μL; amplification curve (A) and melt peak curve (B).

Table 4. DNA test results for porcine gelatin

Sample	Cq	Average Cq	SD	CV (%)
Porcine gelatin DNA concentration 1000 pg/μL	27.19 26.87 27.16 26.54 26.92 27.11	26.97	0.23	0.85

The market soft capsule DNA isolates were then amplified using cytochrome B primers at an attachment temperature of 52.0°C using qPCR. The amplification response is shown in Figure 5. The test results for soft market capsules gave a positive amplification response for each brand of capsule shell with the Ct and Tm values shown in Table 4. This indicates that the three commercial soft capsule samples contained porcine gelatin DNA. The Ct value of brand C capsule shells is the smallest because the resulting concentration is the highest among other brands, then brand B, and the largest Ct value is from brand A capsule shells. This result is consistent with the theory that the greater the amount of initial DNA amplified, the higher the Ct value. Small. The Tm value produced by the three soft capsule brands differs from the Tm value of porcine gelatin DNA (80.50). This is possible because the series of processes for making the capsule shells have resulted in a change in the percentage of GC bases in the DNA sequence, as previously described.

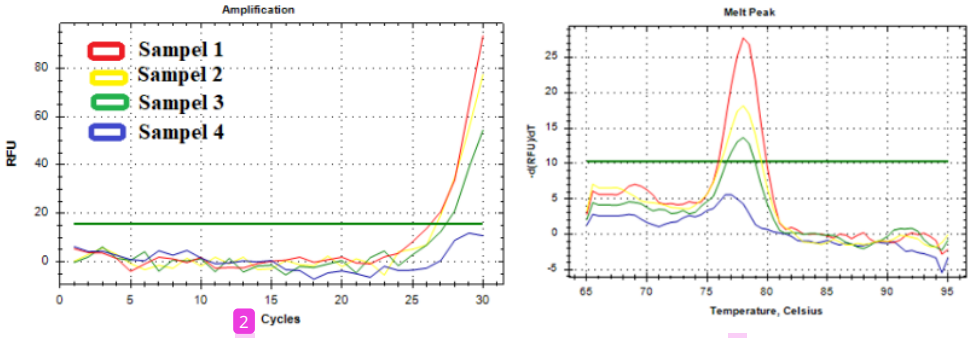


Figure 5. The results of DNA isolates from soft capsule market samples, Amplification (A), and Melting curve analysis (B)

The studies obtained D-loop primers (forward: 5'-ACTTCATGGAACTCATGATCCG-3' and reverse 5'-ATGTACGTTATGTCCCGTAACC-3') that are specific for porcine gelatin DNA, with an attachment temperature of 52.0°C. The qPCR method can still provide an amplification response at a concentration of 0.5 pg/μL bovine DNA using D-loop primers, so this value is used as the LOD (Limit of Detection) value. Analysis of the repeatability of the amplification response from porcine gelatin DNA isolates gave a sequential CV value of 0.85%, which met the recommended CV value for the PCR method, namely CV $\leq$ 25%.

CONCLUSION

The qPCR method using D-loop primers is specifically capable of amplifying porcine gelatin DNA up to a concentration of 0.5 pg/μL, with a CV value in the amplification response of porcine gelatin DNA isolates (1000 pg/μL) of 0.85% which meets the test criteria using the PCR. Three samples of commercial soft capsules tested gave a positive amplification response, meaning that the samples tested contained porcine DNA, and one negative sample, which probably had non-porcine gelatin.

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