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Optimisation and validation of end-point PCR for the detection of porcine DNA in anti-ageing cream products containing collagen

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ABSTRACT: Collagen is the protein-building block of the skin, muscles, bones, tendons, ligaments, and other connective tissues. It is commonly used as an anti-ageing agent in various cosmetic products, particularly anti-ageing creams. The most predominant collagen in the market is derived from pigs and cows. Based on Indonesian Law No. 33 of 2014 governing the halalness of products marketed in Indonesia, there is an emerging need to determine scientifically the halalness of products. Cosmetics containing porcine and its derivatives are classified as non-halal products. Therefore, there should be a robust method for detecting porcine-derived collagen content in anti-ageing creams. Polymerase chain reaction (PCR) can be an effective method for determining the animal source of products through DNA detection. This study aimed to determine the optimum DNA isolation and extraction conditions for the PCR detection of porcine DNA fragments. Incubation was performed twice for 30 min at 60°C and for each incubation, 5 mL of lysis buffer, and 25 L of proteinase K were used. Amplification was performed for 45 cycles and electrophoresis was conducted for 60 min at a voltage of 120 volts and a current of 100 mA. Validating the detection of porcine DNA in anti-ageing cream containing collagen using PCR resulted in a specific and robust method for detecting porcine DNA with a detection limit of 0.004 ng/µL. The repeatability of 10 repetitions consistently showed a band of 149 bp, with 0% false-positives and false-negatives. This method is valid for detecting pig DNA in anti-ageing creams containing collagen.

KEYWORDS: validation ; PCR, collagen; porcine DNA; anti-ageing cream.

1. INTRODUCTION

As the country with the largest Muslim population globally, Indonesia is a promising market in the world trade in halal products [1]. In recent years, the market has been dominated by halal food products, but currently, cosmetic products have also become one of the most significant contributors to the halal product trade [2]. This has attracted the world's attention from the perspectives of both business actors and the government [3]. Business actors compete to meet market needs, while the government is obligated to protect consumers by providing various regulations. To ensure the peace of the Muslim community in consuming halal products, the government issued Law No. 33 of 2014 concerning Halal Product Guarantee, which stipulates that all products that enter, circulate, and trade in Indonesian territory must have a halal certificate [4].

Collagen is a common ingredient added to cosmetics in creams, lotions, and face masks [5,6]. It is a protein found in many connective tissues in mammals, such as skin, bones, joints, and flesh [5,6]. The primary structure of collagen is an amino acid dominated by glycine (33%), proline and hydroxyproline (22%) [6]. The secondary system comprises chain amino acids coiled into a helix, with three amino acids for each spin, bonded to each other and forming a tight tertiary structure. Collagen can be classified into several types: type I (found in skin, flesh, and bones), type II (found in cartilage) and type III (found in skin and blood vessels). Collagen can be used in cosmetics, pharmaceutical products, and food because of its high biocompatibility, non-toxicity, biodegradability, and availability [6]. As we age, the collagen content in the skin decreases, which triggers the appearance of wrinkles and skin ageing. Thus, adding collagen to cosmetic products is expected to be anti-ageing. Collagen can be hydrolysed into molecules after breaking

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the chain of its chemical structure [6]. Smaller molecules are needed to facilitate absorption into the bloodstream.

Collagen can be obtained from beef, pork, or marine products [6]. However, the commercial collagen widely distributed today comes from pigs and cattle. The use of collagen derived from animal sources in cosmetic products is sensitive to adherents of several religions and beliefs in Indonesia. For followers of Islam, the use of materials derived from pork, or its derivatives is haram. Based on the istihlak process, physical or chemical changes in non-halal materials during the manufacturing process can change non-halal materials. However, the amino acid composition of collagen in cosmetic creams may remain intact or unchanged, even though it has gone through a heating process; thus, the pork component could still be detected in cosmetic products, and its status remains haram [7].

The method development not only aims to support ensuring the halal status of a product but also supports authentication and adulteration testing to ensure consumer satisfaction regarding product authenticity. Some methods have been applied to identify the animal sources of products such as immunosorbent kits, Real-Time Polymerase Chain Reaction (PCR), digital PCR, Gas Chromatography (GC) methods, and Fourier-Transform Infrared spectroscopy (FTIR spectroscopy) for analysis of lard. The challenges that arises from each method is how to carry out the suitable sample preparation and extraction since cosmetic samples usually contain various chemicals that may act as inhibitors during the testing process.

From all those reported methods, Porcine detection using End Point PCR has long been left behind since it was the first generation of PCR and required electrophoresis as a confirmation test. However, these methods are inexpensive, highly sensitive, specific, fast, and repeatable. In this study, a method for detecting Porcine in anti-ageing creams containing collagen using this PCR method was developed to determine the content of pork or its derivatives in the product. This study also brought up the concept of method validation to support the implementation of ISO 17025 by conducting various performance characteristics as proven that the Porcine detection method using the end point PCR is valid, sensitive, and reliable to identify authentic animal-sourced products.

2. RESULTS AND DISCUSSION

In this study, pork collagen isolation was carried out to obtain positive controls and ingredients to be added to anti-ageing cream samples. This experiment was conducted due to the difficulty of obtaining a positive control of pork collagen that will be used for method validation. Pork skin and bones are often not used by local slaughtering and turning them into waste. Waste in the form of skin is only used around 20-30%. Therefore, pork skin and bones were usually used in collagen isolation. Two different types of materials were used to compare materials that can produce collagen with the best quality.

Table 1. Isolation of pig collagen

Source	Raw Material Weight (g)	Description	Yield (%)
Skin	15.0017	Liquid form: slightly cloudy white Freeze-dried result: yellowish-white powder	8.78

From the isolation process, collagen derived from the skin yielded 8.78% (Table 1). Salt concentrations below 1.0 M can dissolve type I collagen from tissues, while those above 1.0 M can precipitate collagen type [8]. As a result, the collagen produced by this method tends to be unstable [8]. Acid solutions at low concentrations can break the intermolecular salt bonds and the Schiff base, causing the collagen fibres to expand and dissolve [8]. Acid solutions degrade collagen rapidly, resulting in low molecular weight collagen or hydrolysed collagen [8].

The Fourier Transform Infrared (FTIR) results (Table 2) showed that collagen was successfully isolated from pigskin, even though there was a shift in the absorption of amide I. The stretching vibration of the Amide area showed that -NH from the amide group was associated with hydrogen bonds (-OH) and hydroxyproline groups at wave numbers 3520–3400 [9]. Amide B showed asymmetric C-H stretching vibration at wave numbers 2962–2872. Amide I showed stretching vibrations of C=O at wave numbers 1680–1700, while amide II occurred because of the bending vibrations of the NH group with stretching vibrations of the CN groups at wave numbers 1480–1575, which showed α collagen chains. Amide III at wave numbers 1229–1301 represented the stretching CN and NH bending groups, which showed the triple-helix structure

of collagen [10]. Structural rearrangements of the protein [10,11] can also cause an absorption shift in amide. In addition, the change in the absorption of amide can be caused by a poor purification process, the effect of increasing temperature during storage and the distribution of collagen samples to be characterised by FTIR.

Table 2. Results of the identification of functional groups from the FTIR spectrum of porcine collagen

Wave number (cm^{-1})			Functional groups	Area
Skin		Literature		
1	2	[10,12]		
3451.00	3445.38	3520–3400	-NH stretching vibration of the amide group associated with hydrogen, OH, and hydroxyproline bonds	Amide A
2925.46	2925.31	2962–2872	Asymmetric C-H stretching vibration	Amide B
1745.40	1745.99	1680–1700	C=O stretching vibration	Amide I
1464.91	1464.71	1480–1575	NH bending vibration with C-N stretching vibration	Amide II
1239.08	1239.79	1229–1301	NH bending vibration with C-N stretching vibration	Amide III
-	-	900–1100	PO_4^{3-} stretching vibration	-
-	-			
-	-			
-	-			
-	-			
-	-	871	CO_3^{2-} bond	-
-	-			
-	-			
-	-	500–750	PO_4^{3-} stretching vibration symmetric and asymmetric	Amide IV–VII
722.02	722.63			
-	-			
-	-			
-	-			
-	-			

The results of the Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) showed that collagen was isolated from pigskin. As shown in Figure 1, the protein bands in the collagen derived from the skin show the presence of α1 and α2 chains at molecular weights of 184 kDa and 105 kDa, respectively. Type I collagen consists of α1 and α2 chains, each with a molecular weight of about 100 kDa [10]. Two chains can form a dimmer peptide chain (β chain), and three α chains can create a trimmer peptide chain (γ chain). The presence of two α chains indicates that the collagen produced is type I collagen [10]. Collagen isolated from pork skin can be used in the following validation process because it has better quality due to it harbours two chains as the specific character of type I collagen.

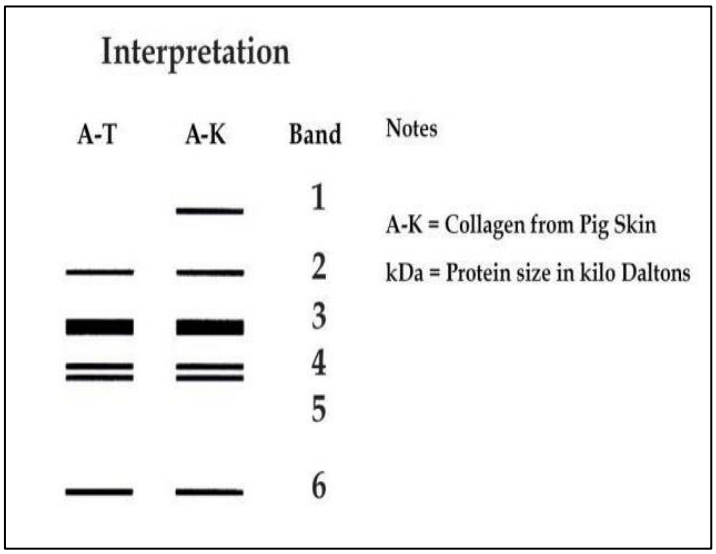

Figure 1. Results of the SDS-PAGE (b) interpretation of the protein bands in the polyacrylamide gel

2.1 Method optimisation

Based on the optimisation results shown in Table 3, 25 µL of the protease enzyme (according to the kit) was the optimal result in which the concentration value is similar to the use of 50 µL of protease enzyme. The requirement for a purity value—that is, the absorption ratio value at a wavelength of 260/280 nm—was 1.8 [13]. In the process of two times of incubation with 5 mL of lysis buffer, each process was more effective than one time of incubation with 10 mL of lysis buffer. Incubation with a lysis buffer is a fundamental step or critical component of nucleic acid extraction. In molecular biology experiments, a lysis buffer functions to disrupt the cell and enable the target molecules to leave the cells. In particular, the concentration of DNA solution extracted by two step lysis significantly increased the purity of DNA and resulted in similar DNA concentration compared to those in one step lysis protocol. This indicates that the second step of lysis is necessary to break the cells to collect DNA. In addition, using two times the lysis buffer could separate the components from the sample matrix, which has interference with the DNA isolation process.

Table 3. Concentration and purity of DNA from the optimisation of DNA isolation

Sample Treatment		DNA Concentration (ng/µL)	260/280 nm
Use of protease enzymes	10 µL	4.4	1.5
		4.1	1.41
	25 µL	**2.9**	**1.84**
		3.2	**1.80**
	50 µL	2.9	1.66
		2.7	1.73
Amount of lysis and incubation buffer	**2 x 5 mL**	**3.4**	**1.77**
		2.6	**1.84**
	1 x 10 mL	3.1	1.57
		3.4	1.6

In the amplification process, optimisation is carried out on the number of cycles of amplification, namely 30, 40, 45, and 50. By increasing the number of cycles in PCR amplification, it is expected that the detection limit will be lower. The PCR process is carried out in quantities cycles 35, 40, 45, and 50.

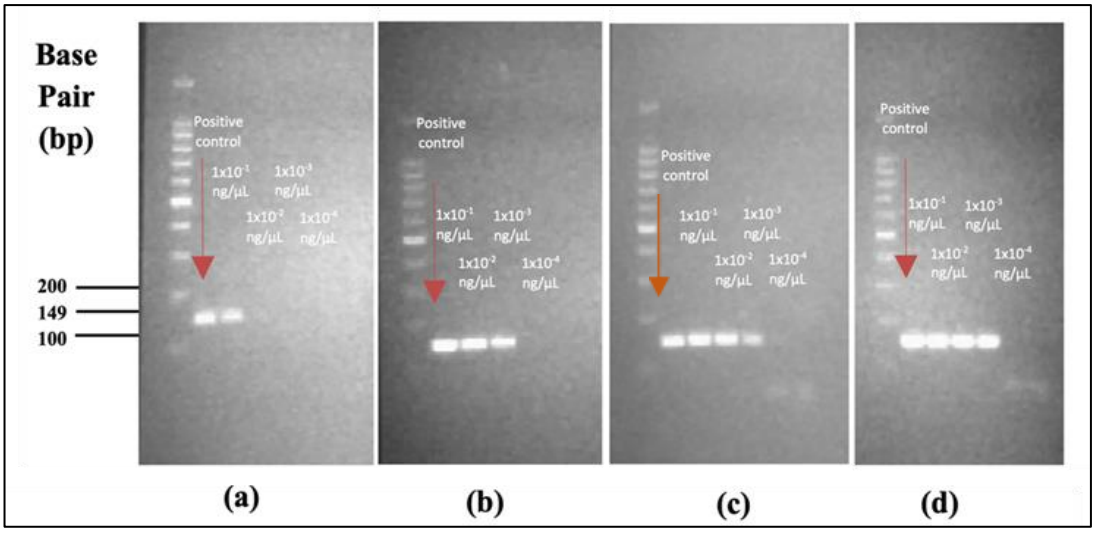

Figure 2. Electrophoresis results at the optimisation stage of PCR amplification cycles of (a) 35, (b) 40, (c) 45, and (d) 50.

The number of cycles of 45 gives optimum results (Figure 2). In the number of cycles, the Limit of Detection (LOD) value is the same as 50 cycles but with a shorter time. The LOD values in 35, 40, 45, and 50 cycles are 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} ng/µL, respectively. Thus, the reagent reacts with the analyte (the reaction is complete) in 45 cycles. The reaction occurs between the primer and DNA fragments in certain areas complementary to the primer, which is then amplified for 45 cycles. The reaction results are the formation of pork DNA fragments with a size of 149 bp.

The optimisation was also performed for the separation time during the electrophoresis stage. Optimisation was carried out at varying times namely 30, 45, and 60 minutes. The objective of this stage is to get a clearer visualisation of the DNA amplification results. The variations made in this process are time variations (30, 45, and 60 minutes) that the DNA split from the amplification results could be observed properly and clearly. In this way, the process of interpreting sample test results can be carried out easily.

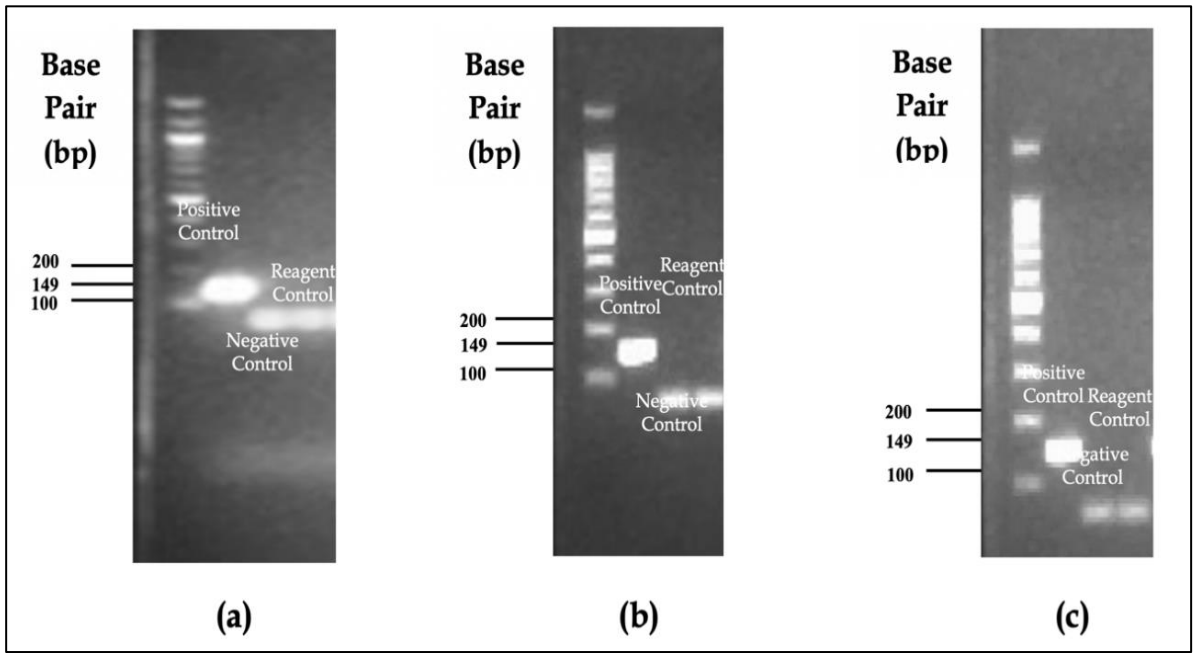

Figure 3. Results of the electrophoresis optimisation stage of the electrophoresis times of (a) 30, (b) 45, and (c) 60 min

Based on the optimisation results, the optimum separation time was 60 min (Figure 3). At that time, the separation of the bands on the DNA marker was visible, making it easier to identify the sample. If the electrophoresis time is more than 60 min, a larger agarose gel is needed so that the electrophoresis becomes less effective and efficient in terms of cost and time.

2.2 Specificity

The method specificity test was carried out to prove that the primer the Cyt B DNA fragment used as a pork-specific marker is not amplifying DNA from specific sources other than pork DNA as the target. Amplification results showed DNA amplification only occurred in samples containing pork collagen.

Figure 4 shows that the method reached 100% specificity from 10 repetitions. The specificity method detected only porcine DNA because the 149 bp band appeared only in the positive controls of porcine collagen and the samples with the addition of porcine collagen (spiked samples). Deliberately adding porcine collagen to the sample (spiked samples) at the same concentration as the positive control to check whether there are PCR inhibitors in the DNA sample. If the positive control in the sample is not amplified while the positive control is amplified, it can be indicated that there is an inhibitor in the sample and vice versa. When testing samples, inhibitor controls should be included to prove that negative results are not due to the presence of inhibitors.

To demonstrate method specificity, the use of non-target controls is important. Non-target controls must not be amplified by the primers used. The animal species that have been used as non-target controls [11,12] were cows, fish, chickens, and goats, with–negative results. There were no false positives or false negatives found.

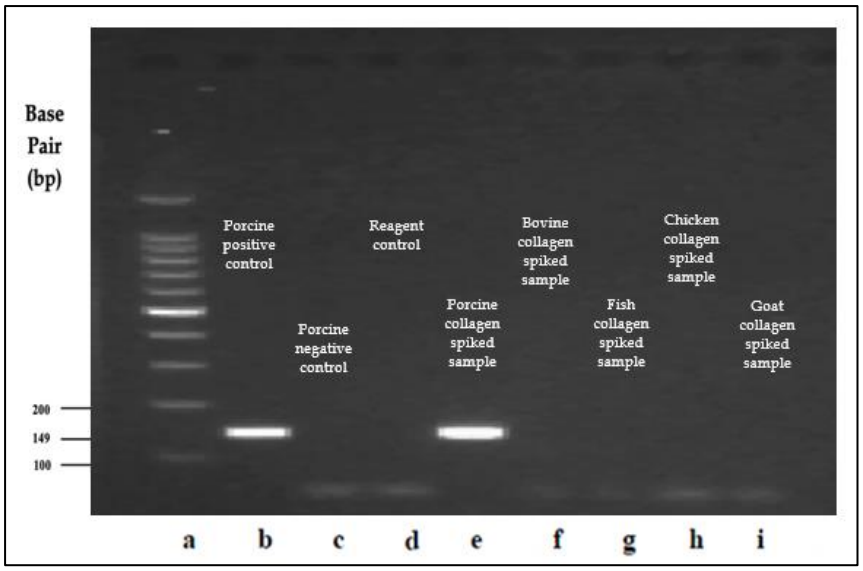

Figure 4. Specificity test results: (a) DNA marker, (b) porcine positive control, (c) negative control, (d) reagent control, (e) porcine positive target control-added sample, (f) bovine positive target control-added sample, (g) fish positive target control-added sample, (h) chicken positive target control-added sample, (i) goat positive target control-added sample.

These results indicate that the method used meets the requirements of measuring the analyte targeted explicitly in the presence of other components in the sample matrix. The DNA concentration of each sample used in the PCR process was five times the LOD concentration. This was done to anticipate the influence of the matrix at small analyte concentrations.

2.3. Detection limit

The determination of the detection limit was carried out to ensure the sensitivity test method. The detection limit is determined at the lowest concentration of the sample which gives results detecting pig DNA from 10 repetitions. The detection limit of the method was found to be 4 x 10-3 ng/µL, as shown in Figures 5–6 and Table 4. At this concentration, 100% of the replicates produced positive results. The detection limit was carried out at one sample concentration with 10 repetitions.

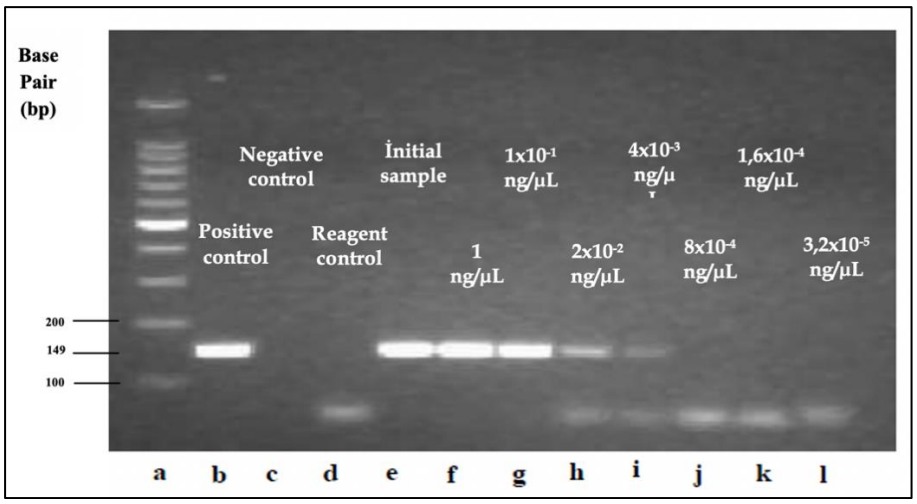

Figure 5. Electrophoresis results of determining the detection limit: (a) DNA marker, (b) positive control, (c) negative control, (d) reagent control, (e) initial sample concentration, (f) 1 ng/µL concentration, (g) concentration 1 x 10^{-1} ng/µL, (h) concentration 2 x 10^{-2} ng/µL, (i) concentration 4 x 10^{-3} ng/µL, (j) concentration 8 x 10^{-4} ng/µL, (k) concentration 1.6 x 10^{-4} ng/µL, (l) concentration 3.2 x 10^{-5} ng/µL

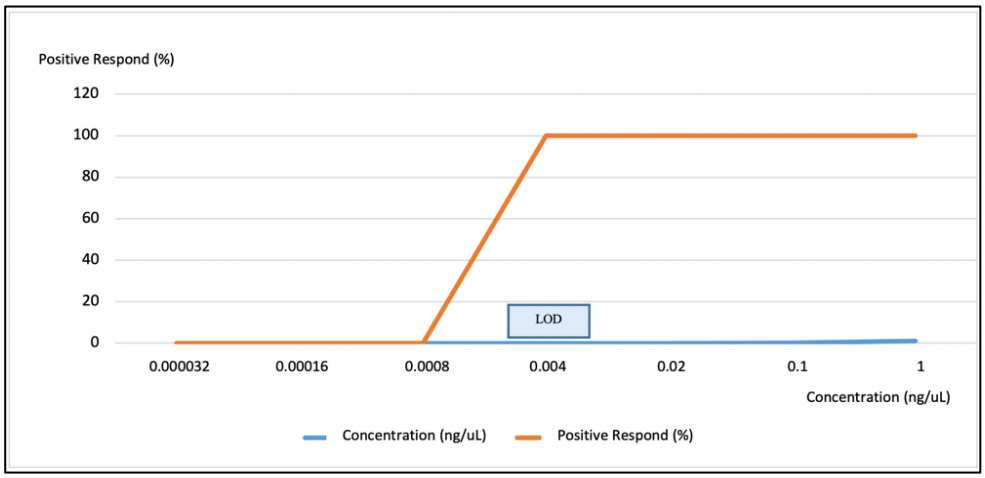

Figure 6. The curve for determining the detection limit concentration

Table 4. Determination of the detection limit concentration

Concentration (ng/µL)	Number of positive results/number of negative results
1	**10/0**
1 x 10^{-1}	**10/0**
2 x10^{-2}	**10/0**
4 x 10^{-3}	**10/0**
8 x 10^{-4}	0/0
1.6 x 10^{-4}	0/0
3.2 x 10^{-5}	0/0

2.4. Repeatability

The precision test carried out by the same analyst is called repeatability. The repeatability test was performed five times the LOD concentration to anticipate the influence of the matrix at a small concentration. The study results showed that the matrix did not affect the test at small concentrations. The precision test results indicated that the method could provide good repeatability at 10 repetitions. The value of the Relative Standard Deviation (RSD) DNA concentration obtained in the isolation process with 10 repetitions was 11.94% (Table 5). This result fulfills the requirement that it should be lower than 25% [8].

Table 5. DNA concentration in the precision test

Sample	DNA concentration (ng/µL)
1	4.4
2	5.4
3	4.8
4	4.8
5	3.7
6	3.9
7	4.3
8	4.7
9	4.2
10	5.2
SD	0.542
Average	4.54
RSD (%)	11.94

2.5. Robustness

In the robustness test, the consistency of the results was observed in 10 repetitions of the test (Figure 7), in which a porcine DNA fragment was produced at 149 bp in each replication. Based on the results, the validated method was quite robust, and different tools provided consistent results. A robustness test of the

method was performed to determine the effect of a slight change in test conditions on the test results [1]. This test was carried out using a PCR machine of the same brand with a different series.

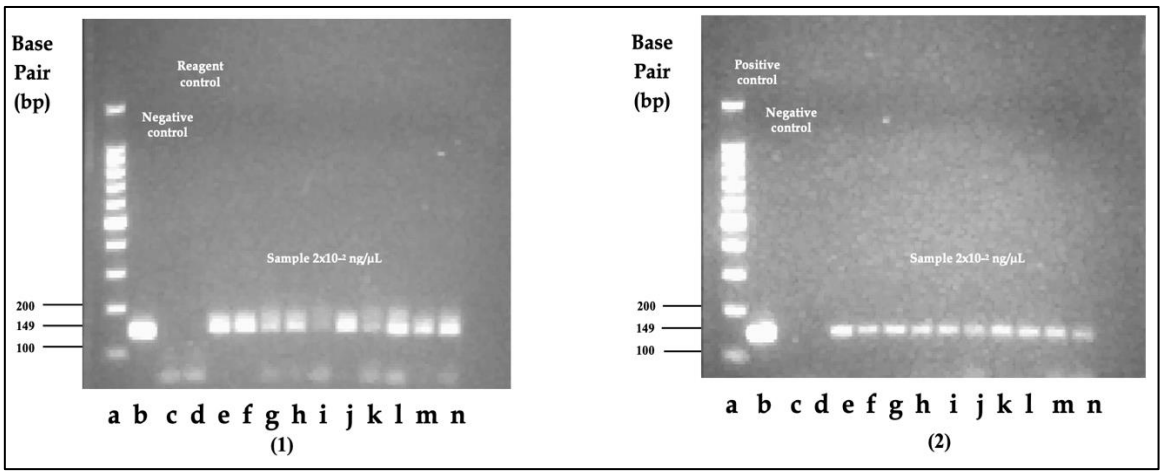

Figure 7. Electrophoresis results of the toughness test methods: (1) amplification with PCR 9700, (2) amplification with PCR Verity, (a) DNA markers, (b) positive control, (c) negative control, (d) reagent control, (e–n) sample at a concentration of 2 x 10^{-2} ng/µL

2.6. False positives and false negatives

The false-positive and false-negative test results were 0%. All supposedly positive samples produced positive results, and all negative samples showed negative results. Based on this, the method can provide reliable results. This test was carried out to determine the possibility of a result that should have been negative or a result that should have been positive due to the influence of the sample matrix or reagent used.

2.7. Sample testing

In this study, six anti-ageing creams in the market were tested, one of which was the sample used in validation. The test results showed that porcine in all tested samples were undetected. The six samples did not contain pork DNA or raw materials derived from pigs. Sample 1 is a negative sample used in the validation process (Figure 8). A sample will be declared as undetectable if there is no 149 bp band appearing on the gel indicating that there is no porcine in the sample or the porcine concentration is lower than the LOD. Cosmetics have quite complex chemical components and have gone through a manufacturing process that might cause the low porcine DNA concentrations in the samples.

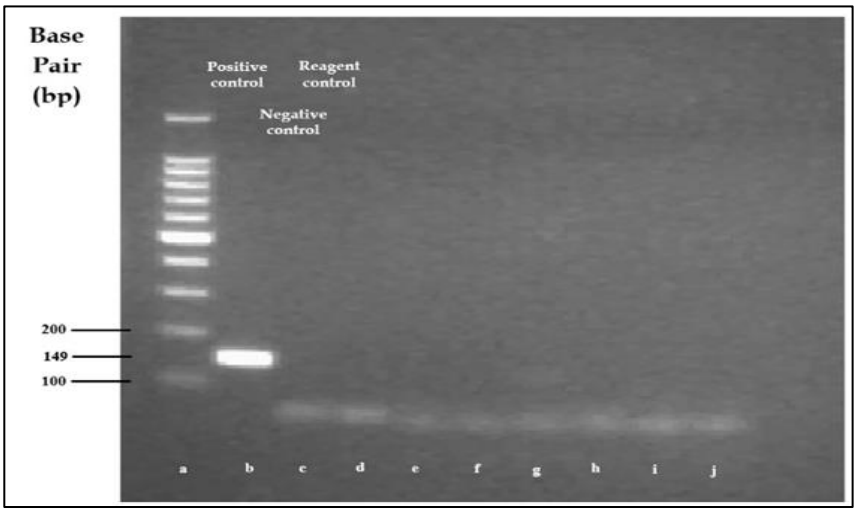

Figure 8. Electrophoresis results of the sample test: (a) DNA marker, (b) positive control, (c) negative control, (d) reagent control, (e) sample 1, (f) sample 2, (g) sample 3, (h) sample 4, (i) sample 5, (j) sample 6

3. CONCLUSION

The proposed method was proven valid with the existing optimisation conditions and validation parameters, including specificity, detection limit, repeatability, robustness, false positives, and false negatives. With a detection limit of 0.004 ng/µL, the proposed method was able to specifically detect porcine, consistently showing positive results from 10 replicates performed for both target (showing 149 bp) and non-target DNA. The results also proved to be robust, without any false positives or false negatives. The use of conventional PCR is based on the use of existing instruments in the laboratory. Therefore, conventional PCR can still be utilised and is not useless. Furthermore, this method can be an alternative for detecting porcine or other specific species in food products sensitively. In the future, it will be possible to develop methods with more sophisticated technology, depending on the type of sample to be tested. Nevertheless, conventional PCR can still be used as an inexpensive alternative method and it reinforces the choice of using the method in the laboratory when samples are abundant or when other tools are not available for several reasons.

4. MATERIALS AND METHODS

4.1 Sample preparation

Anti-ageing cream products containing collagen were purchased from an online store. The samples used in this research were skin radiant collagen double eye lift cream, moist full-cream collagen, ultimate anti-ageing cream, collagen night cream, collagen nutrition cream, collagen moisturiser cream, and collagen jelly pack.

4.2 Isolation of collagen from porcine skin

The reagents, materials, and environmental conditions were set to 4°C. This study did not require ethical approval because the porcine skin material used was taken from pigskin waste at a slaughterhouse. The porcine skin was rinsed in cold water. The hair, feathers, fat, and tissue were removed. The skin was rinsed in cold water and cut to approximately 1 cm x 1 cm. As much as 5 g of the sample was put into a 50 mL container with 30 mL of 0.5 M sodium acetate. The mixture was shaken for 1 min using a benchtop homogeniser. The supernatant was discarded, and this process was repeated seven times. The skin sample was rinsed with cold water, added to cold water, and then shaken for 1 min to remove the sodium acetate residue. The skin sample was then transferred to a new 50 mL tube, washed with 10 mL of 0.075 M sodium citrate, and homogenised for 1 min with a benchtop homogeniser. This time, 10 mL of sodium citrate was added to the skin sample, which was normalised again with a benchtop homogeniser for 1 min. This process was repeated seven times. The supernatant from each repetition was collected and centrifuged at 3,200 g for 10 min at 4°C. The supernatant was collected in a new centrifugation tube, transferred to a vial, and dried using a freeze dryer [13].

4.3 Fourier Transform Infrared (FTIR)

Several collagen samples were grounded with a KBr of approximately 150 mg until smooth. The sample was inserted into the sample holder on the FTIR device. The sample was measured in the medium-frequency region at wave numbers 4000–4500 cm-1. The spectrum obtained was identified by its functional group.

4.4 DNA extraction

In optimising the isolation process, 2 g of the sample was weighed into a 50 mL tube. Then, 25 µL of proteinase K solution and 5 mL of lysis buffer were added, vortexed for 2 min and incubated for 60 min at 60°C in a water bath shaker. The solution was centrifuged for 10 min at 3,000 g. The supernatant obtained was transferred to a new centrifugation tube, added with 5 mL of lysis buffer, and incubated again for 30 min at 60°C. The incubated solution was cooled to room temperature, put inside a freezer for approximately 15 minutes, and centrifuged for 5 minutes at 3,000 g. Afterward, 700 mL of the supernatant was transferred to a new 2 mL tube (solution a).

4.5 DNA isolation

The solution was added with 700 mL of chloroform, vortexed for 15 s, and centrifuged at 14,000 g for 20 min (solution b). After completion, 250 mL of the aqueous phase of the above solution was transferred to a new 2 mL tube containing 1 mL of PB buffer from Qiagen and vortexed for 15 s. This step was repeated by taking 250 L of the upper aqueous phase from solution b, transferring it to a new 2 mL tube containing 1 mL of PB buffer, and vortexing it for 15 s. Subsequently, the two replicates were mixed, filtered into a spin column in a 2 mL tube and centrifuged at 17,900 g for 2 min. Afterward, 500 mL of wash buffer was added to the column and centrifuged at 17,900 g for 2 min. The collected solution was discarded. The column was reinserted into the tube and centrifuged at 17,900 g to dry the membrane. Finally, the spin column was transferred to a new 1.5 mL tube and 30 L of elution buffer was added to the membrane, which was incubated for 5 min at room temperature. Then, the tube was centrifuged for 4 min and again at 17,900 g for 2 min. The DNA extraction process was performed in duplicate for each sample. The negative control was carried out by extracting a lysis buffer without a sample. The positive control was obtained by isolating porcine skin. The extracted DNA solution was stored at −20°C for further analysis. It was carried out with the same procedure as the sample for the positive control and the negative control.

4.6 Measurement of the concentration and purity of DNA isolates

The concentration and purity of the DNA isolates were measured using a DNA spectrophotometer. The DNA Nanodrop spectrophotometer was turned on and the 'nucleic acid' section was selected. The pedestal was cleaned with tissue paper, 2 µL of blank elution buffer was pipetted, and dripped onto it. The 'blank' part was pressed on the screen for blank measurements. Afterward, the pedestal was cleaned with tissue paper. A DNA isolate solution of 2 µL was pipetted and dripped onto the pedestal, 'measure' was clicked to display the spectrum, and the calculated concentration on the screen [14].

4.7 DNA amplification using PCR

The DNA amplification process using PCR began with the manufacturing of the master mix. The master mix consisted of 24.1 µL of nuclease-free water, 10 µL of 5x buffer solution without magnesium chloride, 3 µL of 25 mM magnesium chloride solution, 1 µL of dNTP Mix10 mM solution, 0.75 µL of 20 M porcine forward primer, 0.75 µL of 20 M porcine reverse primer solution, and 0.4 µL of Taq polymerase solution, which were homogenised by vortex and centrifuged. Then, 10 µL of extracted DNA was added, homogenised by vortex and centrifuged. Afterward, the sample was put into the PCR machine [15]. Table 6 shows the optimisation conditions of the PCR cycle.

Table 6. Optimisation of the PCR cycle

Step	Temperature and Time
Pre-denaturation	98°C for 3 s
Followed by 35, 40, 45, and 50 cycles	
Denaturation	98°C for 30 s
Annealing	60°C for 30 s
Extension	72°C for 30 s
Final Extension	72°C for 5 min

The primers used in this study were one pair of porcine primers from the Cyt B gene, namely F: 5'ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC C-3' and R: 5'CTA CGA GGT CTG TTC CGA TAT AAG G-3', which yielded a 149 bp fragment [15].

4.8 Analysis of PCR products using a gel electrophoresis system

The PCR products were analysed using a 2% agarose gel (Invitrogen) dye. The PCR products included the positive control, negative control, reagent control, and sample. Each 10 µL pipette was inserted into the prepared agarose well. Electrophoresis was performed on 2% agarose at a constant voltage of 120 volts and a current of 100 mA for 45 min. DNA visualisation on the gel was carried out using a documentation gel. The electrophoretic PCR product was inserted into the documentation gel and a band was formed using an ultraviolet lamp at a wavelength of 260 nm. If the sample DNA fragment was aligned

with the positive control of the porcine DNA fragment measuring 149 base pairs, then the test results would be declared positive for containing porcine DNA fragments [15].

4.9 Specificity

The specificity method was determined by comparing the results of the DNA amplification in the samples containing pig species with DNA from cattle, fish, chicken, and goat species. The tests for determining specificity were repeated 10 times at a concentration of five times the detection limit concentration [8,13]. The specificity rate can be calculated using the following formula:

$$Specificity = \frac{Negative\ Result}{Number\ of\ Negative\ Samples} \times 100\%$$

4.10 Detection limit

The detection limit was determined by repeating 10 times the detection of porcine DNA in anti-ageing cream samples spiked with 5% pork collagen accompanied by positive and negative controls [9]. The DNA isolated from the sample was diluted serially at 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} for each test [13].

4.11 Repeatability

The precision parameter was calculated from the RSD value of the DNA concentration isolated from the sample repeated 10 times, with an RSD value of 25% [8] [16].

4.12 Robustness

The robustness of the method was determined by comparing the results of the detection of porcine DNA in anti-ageing products using two thermocycler machines with different brands at 10 repetitions [8]. The method would be declared resilient if the test results showed data that were not significantly different. If the method were robust, the resulting band of the PCR product DNA fragments measuring 149 base pairs with a difference in conditions would be similar [15].

4.13 False-positive rate

All the negative sample tests and samples that should hbe negative but produced positive results (from 10 repetitions) were recorded and then calculated using the following formula [17]:

$$False\ positive\ rate = \frac{Fp}{tn+Fp} \times 100\%$$

Description:
Fp : False positive
tn : Number of negative tests

4.14 False-negative rate

All of the positive sample tests and samples that should be positive but produced negative results (from 10 repetitions) were recorded and then calculated using the following formula [17]:

$$False\ negative\ rate = \frac{Fn}{tp+Fn} \times 100\%$$

Description:
Fn : False negative
tp : Number of positive tests

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