Universal Journal of Agricultural Research 11(1): 121-128, 2023 http://www.hrpub.org
DOI: 10.13189/ujar.2023.110111

Adulteration Test of Chicken DNA (*Gallus gallus*) by the Multiplex PCR Method Using a Specific Primer for Mitochondrial DNA CO1

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Received November 22, 2022; Revised January 12, 2023; Accepted February 1, 2023

Cite This Paper in the Following Citation Styles

***(a):** [1] Joni Kusnadi, Sinta Harfiyanti , "Adulteration Test of Chicken DNA (Gallus gallus) by the Multiplex PCR Method Using a Specific Primer for Mitochondrial DNA CO1," Universal Journal of Agricultural Research, Vol. 11, No. 1, pp. 121 - 128, 2023. DOI: 10.13189/ujar.2023.110111.*

***(b):** Joni Kusnadi, Sinta Harfiyanti (2023). Adulteration Test of Chicken DNA (Gallus gallus) by the Multiplex PCR Method Using a Specific Primer for Mitochondrial DNA CO1. Universal Journal of Agricultural Research, 11(1), 121 - 128. DOI: 10.13189/ujar.2023.110111.*

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Abstract Food adulteration cases continue to increase in line with the increasing public need for food. Multiplex PCR is one type of PCR method that is often used to detect adulteration in food. This study aims to confirm the performance of primers that have been designed based on the CO1 gene that specifically amplifies chicken DNA by a primer specificity and sensitivity test using single and multiplex PCR method. The results showed that the primer had specific properties because it is capable of amplifying the target DNA according to its size. The sensitivity test showed that the CO1 primers for chicken have a sensitivity of up to 10^{-3} ng/µl similar to the pig's D-loop primers, while the CO1 primers for horses have a sensitivity of up to 10^{-2} ng/ l similar to the Cyt b for dogs. Sampling test using five types of meatballs by the multiplex PCR method showed that the samples detected animal DNA that matched the respective raw materials for making it, while sampling using commercial meatballs showed that only three samples contained bovine DNA and it could be concluded that the other two samples had been adulterated with chicken meat.

Keywords Adulteration, DNA, Multiplex PCR, Primer, Chicken

1. Introduction

Adulteration or food counterfeiting can be described as a deliberate act to add, replace, alter, and misrepresent a food product and, food packaging and to provide incorrect information on labels for the purpose of deceiving consumers in order to obtain economic benefits [1]. Animal food products are one of the food products that often counterfeited. This is because the selling price of animal food products is relatively more expensive when compared to vegetable food products [2]. Chicken meat and its processed products are a good source of animal protein because it contains complete essential amino acids and has a relatively cheaper price compared to bovine or goat [3]. This not only causes an increase in public consumption of chicken meat, but also has the potential to increase counterfeiting of processed chicken products [3]. Cases of counterfeiting not only occur with the addition of non-halal animal meat to processed chicken or halal meat products, but also can be use as an adulterating material for a product, such as addition of chicken in meatball which is claimed as made from bovine [4].

Detection of counterfeiting in food products can be done with DNA-based detection [5]. DNA generally has stable properties at high temperatures and pressures. Besides that, DNA can also be found in almost all parts of the cell of organisms [6]. PCR is a molecular method for duplicating pieces of DNA up to millions of times in a relatively short time [7]. This multiplication is inseparable from the use of enzymes and a pair of primers that are specific to the target DNA [8]. Multiplex PCR is a PCR technique used to amplify various targets in one reaction [7] by using several

pairs of primers so the test process can run faster and can save energy and costs [9]. Primer design is one of the processes that must be considered in the multiplex PCR method, especially the annealing temperature of each primer so that cross-hybridization does not occur [10]. The use of mitochondrial DNA molecules as the basis for primer design is recommended compared to nuclear DNA, because the cell consist more mitochondrial DNA than nuclear DNA and it has high specificity for each species because it is only derived from the DNA of the female parent [11]. The genes most often used as markers of animal or meat species include cytochrome b (cyt b), 12S and 16S ribosomal RNA subunits and the displacement loop (D-loop) region [12].

This study aims to examine the adulteration and contamination of chicken DNA in processed meat products and to determine the specificity and sensitivity of the specific primer cytochrome c oxidase subunit 1 (CO1) as a result of the research design in identifying chicken DNA using the multiplex PCR method.

2. Materials and Methods

2.1. DNA Sample

In this study, the DNA samples used were fresh meat and processed meat products, namely meatballs. Fresh meat consists of chicken (*Gallus gallus*), dog (*Canis lupus familiaris*), pork (*Sus scrofa domesticus*) and horse (*Equus caballus*). The meatball samples consist of chicken meatballs, dog meatballs, pork meatballs, horse meatballs and a mixture of chicken, dog, pork and horse meatballs made by the researcher and commercial bovine meatball samples obtained from supermarkets and meatball shops in Malang, East Java, Indonesia.

2.2. Primer

The primers used in the study consist of the chicken CO1 gene primer designed by the researcher, the pig D-loop gene [13], the horse CO1 gene [14], the dog Cyt b gene [15] and the bovine 12S rRNA gene [16]. The primer specifications were showed in **Table 1**. The primer for chicken identification was designed during this study based on the cytochrome c oxidase subunit 1 (CO1) of mitochondrial DNA of chicken, whereas the design of other primers were obtained from some other papers.

2.3. Chicken Forward and Reverse Primer Design

The design of a specific primer for chickens (*Gallus gallus*) was conducted based on the nucleotide sequence of the CO1 gene in chickens contained in GenBank (NCBI with access number GenBank ARJ60440.1). The DNA sequence was downloaded in FASTA form and then aligned with other animal species using software Clustal X. Alignment was carried out using BioEdit software to obtain a conserved area.

Table 1. Primer specifications

Primer	Sequence Order (5` to 3`)	Number of Nitrogen Bases	Amplicon Size (bp)	Source
CO1 (Chicken)	Forward: 5'-C TTTACCTAATTTTCGGCAC -3'	20	306	Study
	Reverse: 5'-TTCTACGGTAGATGAGGCTA-3'	20		
D-loop (Pig)	Forward: 5'-TACTTCAGGACCATCTCACC-3'	20	835	Haunshi *et al.*, 2009
	Reverse: 5'-TATTCAGATTGTGGGCGTAT-3'	20		
CO1 (Horse)	Forward: 5'- CACCAGCCCTATCCCAATAT -3'	20	113	Hakiki, 2020
	Reverse: 5'- GAGAAGCATGGTAATGCCTG -3'	20		
Cyt b (Dog)	Forward: 5'-CCTTACTAGGAGTATGCTTG-3 '	20	101	Rahman *et al.*, 2014
	Reverse: 5'-TGGGTGACTGATGAAAAAAGA-3'	20		
12S rRNA (Bovine)	Forward: 5'-ACCGCGGTCATACGATTAAC-3 '	20	155	Cahyadi *et al.*, 2018
	Reverse: 5'-AGTGCGTCGGCTATTGTAGG-3 '	20		

2.4. DNA Isolation Using the Method of Chloroform:isoamyl alcohol

DNA isolation was initiated by weighing 20 mg of fresh meat and meatball samples and added with STE buffer (0.1M NaCl, 0.001M EDTA and 0.01M Tris-Cl). The samples were crushed using micropestle until completely destroyed, added 40 µl SDS 10% and 20 µl pro-K and vortexed for 20 seconds. The sample solution was incubated in a thermomixer at 55 ℃ overnight at 800 rpm.

Incubated samples were centrifuged for 10 minutes at a speed of 12000 rpm at a temperature of 29 ℃, separated between the supernatant and pellet solution. The supernatant containing crude DNA solution was then taken and placed in a new tube with a size of 1.5 ml and added 1x volume of Chloroform:Isoamyl alcohol (24:1) as much as 400 µl and 40 µl of 5M NaCl, then homogenized and centrifuged, on the supernatant formed by adding 1x volume of Chloroform:Isoamyl alcohol (24:1) as much as 300 µl. Then the mixture was inverted and centrifuged, separated as much as 200 µl of the supernatant formed into a new tube and added 600 µl of cold ethanol (temperature 4 ℃) and 40 µl of 5M NaCl, then the mixture incubated at -20 ℃ for 2.5 hours. After incubation, the samples were centrifuged at 12000 rpm at 4 ℃ for 5 minutes. The pellets obtained were tapped to release and added 70% ethanol, and the mixture was centrifuged at 12000 rpm at 4 ℃ for 5 minutes. The supernatant was discarded and the pellet was tapped so that it was released and dried using a thermomixer at 55 ℃, after drying add 50 µl of TE buffer pH 7,6. The samples were then stored at -4 ℃ for 15 minutes to measure the concentration and purity of the DNA obtained using nanodrops [17].

2.5. DNA Amplification Using Single PCR and Multiplex PCR Methods

The DNA amplification of the samples was carried out to test the performance of the specific primers that had been designed through specificity and sensitivity tests using the single PCR and multiplex PCR methods and then confirmed using electrophoresis. The primer specificity test was conducted to see the specific nature of a primer in amplifying the target DNA. The primer sensitivity test was carried out to determine how small the target DNA concentration could be detected by the primer. The concentrations of chicken DNA used were 10 ng/µl, 10-1 ng/µl, 10 -2 ng/µl, 10-3 ng/µl, 10-4 ng/µl, and 10-5 ng/µl.

PCR amplification was carried out using single PCR and multiplex PCR methods. In the single PCR, the PCR mix formulation used had a total volume of 10 µl, consist of My Taq Red Mix 5 µl, primer forward 0,5 µl, primer reverse 0,5 µl, nuclease free water 3 µl and DNA sample 1 µl. As for the multiplex PCR method, the PCR mix formulation used had a total volume of 21 µl, consist of 10 µl My Taq Red Mix, 0,5 µl forward primer, 0,5 µl reverse primer, 3 µl nuclease free water and DNA sample 1 µl. The PCR program used include hot start 95 ℃ 5 minutes, continued with 30 cycles of denaturation 94 ℃ 1 minute, annealing 52 ℃ 1 minute, extension 72 ℃ 1 minute, final extension 72 ℃ 7 minutes and cooling 4 ℃.

3. Results and Discussion

3.1. Chicken Forward and Reverse Primer Design

Primer design was manually done using Clustal X2 and BioEdit. The first step in the primer design process was to perform *multiple alignments* of the chicken CO1 gene sequence with other animal CO1 gene sequences. The chicken CO1 gene sequence (*Gallus gallus* access code GenBank KX987152.1) along 1550 bp was aligned with the dog CO1 gene (*Canis lupus familiaris* access code GenBank KJ522809.1) along 1544 bp, pig (*Sus scrofa domesticus* GenBank access code KJ789952.1) along 1544 bp and mice (*Rattus norvegicus* access code GenBank NC_001665.20) along 1544 bp. The primers designed at the time of the study consisted of forward primers and reverse primers. The forward primer attachment sites started from the sequence of the 55th base to the 75th base, while for the reverse primer attachment site starting from the 341st base sequence to the 361st base with the number of amplicons being 306 bp. Optimal primer length ranges from 18-30 bases [18], and the optimal GC percentage for the PCR process generally ranges from 40-60%. The low % GC content of the primers can cause the primers not to adhere effectively to the DNA template [19]. The optimal *melting* temperature ranges from 50-65 ℃, and the optimal annealing temperature ranges from 37-60 ℃ [18]. Based on **Table 2**, it can be seen that the primers that have been designed have met the criteria of a good primer, where the base length of the designed forward and reverse primers is 20 bp, the GC percentage is 40-45%, the annealing temperature is 51-53 ℃, the melting temperature is 56-58 ℃ and the length of targeted amplicons is 306 bp.

Table 2. Criteria for the specific primer design of the chicken CO1 gene (*Gallus gallus*)

Primer Type	Primer Sequence	Base Length (bp)	%GC	Ta (℃)	Tm (℃)	Number of Amplicons
Primer Forward	5'-CTTTACCTAATTTTCGGCAC-3'	20	40	51	56	306
Reverse Primer	5'-TTCTACGGTAGATGAGGCTA-3'	20	45	53	58	

3.2. PCR Optimization

The primer used during the research was optimized by the gradient PCR method to determine the most optimum annealing temperature, the annealing temperature used consisted of 5 different temperatures, the temperature was selected based on the calculated melting temperature (Tm) value, and the selected temperature range was 48 ℃, 50 ℃, 52 ℃, 54 ℃, and 56 ℃. The annealing temperature optimization with this gradient method has the aim of getting the optimal PCR product [20].

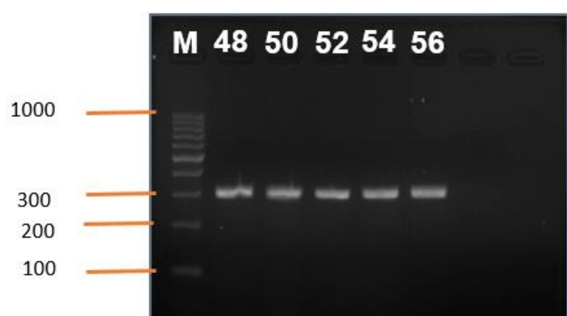

Figure 1. Visualization of PCR Annealing Temperature Optimization. M: 100 bp marker, 48-56: Chicken DNA band formed at annealing temperature of 48-56 ℃

Based on the results of visualization using 2% agarose (**Figure 1**), the chicken DNA bands were formed in all optimization temperature ranges with thick single bands. The temperature chosen as the temperature with optimal amplification for the PCR process was 52 ℃ because of the thick single band shape and the size that matched the target amount, which was 306 bp. The optimum temperature chosen based on the optimization results with the gradient method is a single DNA band that is clearly formed, not blurry or smeared and thick [21].

3.3. Primer Specificity Test for Single PCR Method

This specificity test was carried out to avoid primer identification errors and ensure that the primers that have been designed have properties that are specific to the target DNA. In this study, chicken DNA and other animal DNA including dogs, pigs and horses were used to test the specificity of the designed chicken CO1 primer.

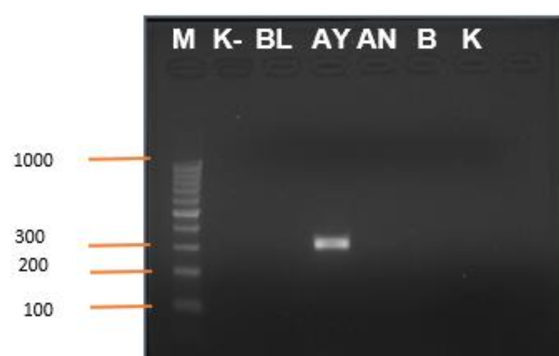

Figure 2. Visualization of Single PCR Method Specificity Test. M: 100 bp marker, K-: Negative control, BL: Blank, AY: chicken DNA, AN: dog DNA, B: pig DNA, K: horse DNA

The results of visualization using 2% agarose gel (**Figure 2**) showed that DNA bands were only formed in the column with the code AY which was the target DNA, and the target DNA was chicken DNA with a target length of 306 bp. The absence of the formation of DNA bands other than in the target DNA column indicates that the primers that have been designed have properties that are specific to the target DNA.

3.4. Primer Specificity Test for Multiplex PCR Method

In the specificity test using the multiplex PCR method, four types of animal DNA and different primers were used in the same reaction. The primers consisted of the CO1 gene primer to amplify chicken DNA 306 bp, the Cyt b gene primer to amplify dog DNA 103 bp, the gene D primer -loop to amplify 835 bp pig DNA and the CO1 gene primer to amplify 113 bp horse DNA.

Figure 3. Visualization of the Multiplex PCR Method Specificity Test. M: Marker 100 bp, K-: Negative Control, BL: Blank, 1: Chicken DNA, 2: Horse DNA, 3: Pig DNA, 4: Dog DNA, Mix 1&2: Mixed DNA (chicken, dog, pig and horse)

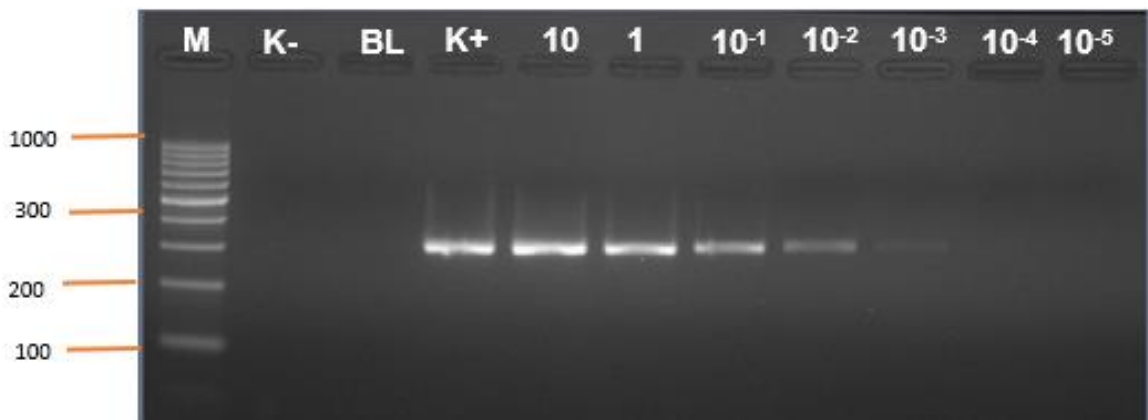

Figure 4. Visualization of Sensitivity Test of Single PCRM Method: Marker, K-: Negative control, BL: Blank, K+: Positive Control, 10-10 $^{-5}$: Chicken DNA dilutions

Based on the results of the primer specificity test using the multiplex PCR method (**Figure 3**), it can be seen that the primers used during the test have specific properties for the targeted DNA fragment. This is indicated by the formation of four DNA bands in the mix 1 and mix 2 columns. DNA bands that are formed in the mix 1 and mix 2 columns have different product sizes according to their respective target lengths. After the visualization process using the electrophoresis method, the target DNA bands will separate specifically which can be seen based on the thickness of the target DNA, and the thickness of the target DNA formed is based on the weight and amount of the targeted DNA [22].

3.5. Test the Sensitivity of the Single PCR Method

Primer sensitivity test is a test carried out to determine the smallest concentration limit that can be detected by a primer that has been designed. After the sensitivity test, the LOD *(Limit of Detection) will be known* or how sensitive the primer has been designed to detect the smallest concentration of the targeted template DNA [23]. PCR primer sensitivity testing was carried out using DNA from chicken meat, where chicken DNA with an initial concentration of 50 ng/µl was made in several dilution series, namely 10 ng/µl, 1 ng/µl, 10 $^{-1}$ ng/µl, 10 $^{-2}$ ng/ µl, 10 $^{-3}$ ng/µl, 10 $^{-4}$ ng/µl and 10 $^{-5}$ ng/µl.

Based on the visualization results of the chicken CO1 primer sensitivity test (**Figure 4**), it can be seen that a single band of chicken DNA is formed in the DNA column with a concentration of 10 ng/µl, 1 ng/µl, 10 $^{-1}$ ng/µl, 10 $^{-2}$ ng/µl, and 10 $^{-3}$ ng /µl, so it can be stated that the chicken CO1 primer has good sensitivity because it is able to detect sample DNA up to a concentration of 10 $^{-3}$ ng/µl, where the positive control used is chicken DNA with a concentration of 50 ng/µl and the negative control is ddH_2O. The single band formed is getting thinner or its intensity is getting lower as the DNA concentration decreases. The number of bands produced by each primer depends on the distribution of homologous sites in the genome [24].

3.6. Sensitivity test of multiplex PCR method

The sensitivity test of the multiplex PCR method was carried out using several series of dilutions with concentrations of 10 ng/µl, 1 ng/µl, 10 $^{-1}$ ng/µl, 10 $^{-2}$ ng/µl, 10 $^{-3}$ ng/µl, 10 $^{-4}$ ng/µl and 10 $^{-5}$ ng/µl. Based on the visualization results of the sensitivity test using the multiplex PCR method (**Figure 5**), it can be seen that each

primer used has a different sensitivity. In the chicken primer from the C01 gene with the target of 306 bp and the pig primer from the D-loop gene with the target DNA being 835 bp, a single band of DNA was formed in the DNA column with a concentration of 10 ng/µl, 1 ng/µl, 10^{-1} ng/µl, 10^{-2} ng/µl, and 10^{-3} ng/µl, while for dog primers from the Cyt b gene with a target of 101 bp and horse primers from the CO1 gene with a target DNA of 113 bp. Both types of primers were only able to detect sample DNA up to a concentration of 10^{-2} ng/µl. There are several factors that can affect the success of sensitivity testing with the multiplex PCR method including the number of primers used during the reaction, competition between primers during the reaction, the amount of DNA template used, the concentration and purity of the template DNA to be used in the reaction and the ingredients. Others are used during the reaction so that the reactions that occur in multiplex PCR are more complex [25].

3.7. Meatball Sample Sampling Test

The meatball samples consisted of four types of meat from different animal species, namely chicken, dog, pork and horse, which were processed with a composition of 50% animal meat (chicken, dog, pork and horse) and meatballs which were the result of a mixture of several types of meat (chicken, dogs, horses and pork) which were processed with a composition of 20% on each meat.

Based on the visualization results (**Figure 6**), in column A containing a sample of chicken meatballs a DNA band is formed which has a size of 306 bp; in column B which contains a sample of dog meatballs a DNA band is formed with a size of 101 bp; in column C which contains a sample of pork balls a DNA band is formed with a size of 835 bp; in column D which contains a sample of horse meatballs, one DNA band with a size of 113 bp is formed. So, it can be concluded that the primer is able to amplify the DNA of chickens, dogs, pigs, and horses specifically with the multiplex PCR method on meatball samples. While in column E which contains samples of meatballs made from a mixture of chicken, dog, pork and horse meat, four DNA bands of different sizes are formed which indicate that the four types of primers used during the sampling test with the multiplex PCR method were able to amplify the targeted DNA simultaneously. Specifically, the four DNA bands formed were chicken DNA with a size of 306 bp, dog DNA with a size of 101 bp, pig DNA with a size of 835 bp and horse DNA with a size of 113 bp. After visualization with agarose each sample DNA band will be formed according to the size of the intended product [26].

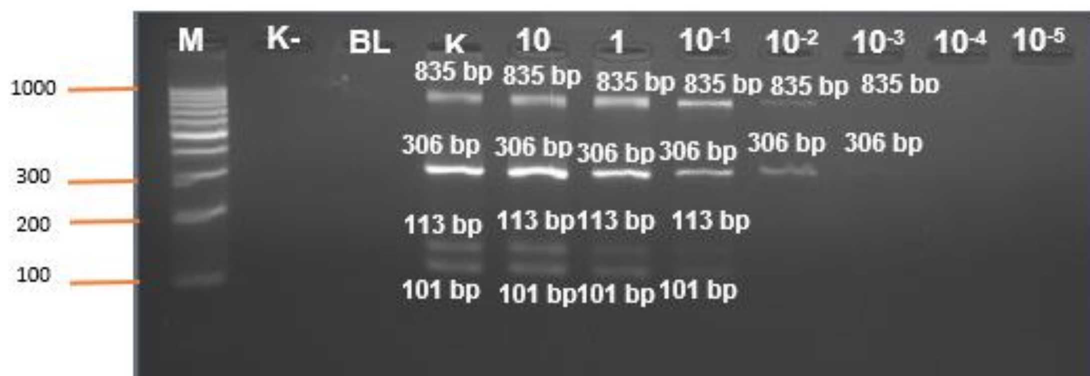

Figure 5. Visualization of the Multiplex PCR Method Sensitivity Test. M: Marker 100 bp, K-: Negative Control, BL: Blank, 10-10^{-5}: DNA dilutions (chicken, dog, pig and horse)

Figure 6. Visualization of Meatball Sampling Test with Multiplex PCR Method. M: Marker 100 bp, K-: Negative Control, BL: Blank, K+: DNA of chicken, dog, pork and horse, A: Chicken Meatballs, B: Horse meatballs, C: Pork meatballs, D: Dog meatballs, E: Meatballs mixed meats (chicken, dog, pork and horse)

Figure 7. Visualization of commercial bovine meatball sampling test. M: Marker 100 bp, K-: Negative Control, BL: Blank, K+: DNA of chicken, pork, and bovine, 1: bovine meatball brand A, 2: bovine meatball brand B, 3: bovine and chicken meatball brand C, 4: bovine meatball of stall A, 5: bovine meatball of stall C

3.8. Commercial Meatball Sample Sampling Test

Samples of commercial meatballs were taken based on 3 brands of bovine meatballs sold in supermarkets and 2 samples of meatballs sold by meatball traders in Malang, East Java, Indonesia. Four of the five samples of commercial meatballs used were meatballs which claimed to be made from bovine meatballs, while one of five commercial meatballs used was meatballs which are claimed to be made from a mixture of bovine and chicken meatballs. Sampling of commercial meatballs which are claimed to be bovine meatballs is carried out to authenticate the truth of the claims of these commercially sold bovine meatballs. In the positive control commercial bovine meatball sampling test used consisting of pork, chicken and bovine DNA, based on the visualization results in the positive control column three DNA bands of different sizes were formed. These bands represent chicken DNA (306 bp), pig DNA (835 bp) and bovine DNA (155 bp).

Based on the visualization results (**Figure 7**) in columns 1 and 2 containing bovine meatballs from brands A and B, a DNA band with the same size as the positive control bovine DNA was formed, which was 155 bp, which indicated that the sample brand A bovine meatballs contained bovine DNA, while in column 3 containing samples of brand C meatballs, two DNA bands with different sizes were formed, namely chicken DNA 306 bp and bovine DNA 155 bp which indicated that this meatball contained two DNA from different animal species, namely chicken and bovine and in columns 4 and 5 which contained bovine meatball samples from stalls A and B did not form bovine DNA bands with a size of 155 bp but formed 306 bp chicken DNA bands, so the claim that the meatballs were made from bovine was incorrect or inappropriate, because the meatball samples did not detect bovine DNA. The success of DNA identification in samples by the multiplex PCR method is influenced by the primers used during the sampling test, where the primers used function as a barrier to the amplified DNA fragments. Besides that the primer also gives a hydroxyl group (-OH) at the 3' end which will be required during the extension process [18].

4. Conclusions

CO1 primer designed to detect chicken DNA *(Gallus gallus)* has specific properties and is sensitive to target DNA based on testing with the Single PCR and PCR multiplex methods. Based on the sampling test on samples of meatballs using the multiplex PCR method using five types of meatballs, it shows that, in the meatball samples, animal DNA was detected that corresponded to the raw material for making it, while in the sampling test on commercial bovine meatballs from five samples of bovine meatballs, two of them did not contain bovine DNA and were falsified with chicken.

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