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Molecular Detection of Pork Adulteration: A Study based on Dairy Products in Sri Lanka

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Abstract:- Milk is considered as the 'ideal food' because of its abundant nutrients that are essential for both infants and adults. Milk and other dairy products can be easily adulterated throughout the world due to the demand and supply gap, low purchasing capability of customer, perishable nature of milk and lack of monitoring tests. Detection of bovine and porcine species in commercialized dairy products are required for health safety concerns and for some religious practices. There are successfully applied immunological, chromatographic and electrophoretic methods to identify lipids and proteins in dairy products, in the food industry. Conventional multiplex polymerase chain reaction is more convenient among these methods when the food goes under higher process. In this study isolation of DNA was performed with DNeasy Mericon Food Kit by QIAGEN from milk powder, fresh milk, cheese, yoghurt and pork, beef flesh for positive samples. A conventional duplex polymerase chain reaction (PCR) assay was performed targeting a 289 bp porcine and 251 bp bovine region from mitochondrial DNA to simultaneously detect both porcine and bovine DNA in above mentioned dairy products. The PCR products were analyzed on a 2.0% Agarose gel. The positive band observed in one fresh milk sample may due to unintentional contamination or a human error. None of the other dairy products were not shown pork adulteration. In this study conventional duplex PCR methodology proved to be a reliable and sensitive tool for detecting porcine and bovine DNA fragments (longer than 100 bp) present in milk powder, fresh milk, cheese and yoghurt. The proposed methodology is an easy-to-follow, inexpensive, reliable method used for monitoring dairy products.

Keywords:- *Dairy, Pork DNA, Bovine DNA, Adulteration, PCR, Gel Electrophoresis.*

I. INTRODUCTION

1.1 Food adulteration.

Food adulteration is a global concern that has been investigated for potential food safety and public health concern in recent years [1]. The developing countries are at a higher risk due to lack of monitoring and policies. Food fraud for economic gain is prevalent throughout history. Economically motivated adulteration, a subset of food fraud, was defined in 2009 by the Food and Drug Administration (FDA) as "the fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production", and can often affect public safety through the unknown addition of allergens, toxins, and hygienic risks [2].

1.2 Importance of dairy products consumption.

Milk is one of the best sources of protein, carbohydrate, fat, vitamins and minerals as it contains 87% of water, 3.3% of proteins, 3.9% of fats, 5% of lactose and 0.7% of ash. Milk helps the body to build proteins, supply vitamins, bone-forming minerals and lactose for energy supply, and milk fat. Besides, it provides certain fatty acids and all the essential amino acids [3]. There are various dairy products that are produced using milk such as milk powder, cheese, butter and ghee. Production and consumption of dairy products vary in different nations and countries. The demand for dairy products and the rate of production is found to be increasing day by day all over the world [2].

1.3 Dairy products adulteration.

Milk and other dairy products are in a higher position on the list of food items being at the risk of adulteration. Therefore, it is necessary to make regulatory standards and detection methods to test dairy product adulteration. It can be adulterated either accidentally or intentionally during the production and processing of dairy products [3].

Dairy product adulteration had become a major concern after the breakthrough of melamine contamination in Chinese infant milk products in 2008. Prior to that, in 1850 swill milk scandal has been reported and it killed 8000 infants in New York [1]. In 2006, the food and agricultural organization of Sudan had reported that more than 95% of samples from 100 random samples, were adulterated with water, 35.5% for starch and none of the milk sample has total solids according to standard values [4] [5]. In the year 2019, there was a scandalous rumor in Sri Lanka, that the imported milk powder is adulterated with other fats (animal or vegetable).

Adulteration of pork into dairy products intentionally to enhance the taste, softness and smell, targeting the market is utterly illegal. Considering health issues, ingredients that are not indicated on the label of the product may provoke various allergic reactions of the consumers. Moreover, when considering religious aspects, it is known that people in certain religious groups such as Islam are not allowed to consume some specific foods including pork [4].

ISSN No:-2456-2165

1.4 Detection of adulterations in dairy products.

There are successfully applied immunological, chromatographic and electrophoretic methods to identify lipids and proteins in dairy products inthe food industry. But, they may not be practical due to the poor stability of the target analytes under high temperatures or pressures and chemical treatments during the production and processing of dairy products. Therefore, DNA molecules have received much attention to be identified based on the polymerase chain reaction (PCR) as they are more thermally stable than lipids and proteins. Hence, conventional multiplex PCR can be used as a qualitative method to detect pork adulterations in dairy products. It allows co-amplification of different regions of a specific fragment or a single gene, using different pairs of primers in the same reaction mix. In this study, the duplex PCR technique is proposed to identify pork DNA in a single PCR assay in milk powder, yoghurt, cheese and fresh milk [6].

1.5 Importance of detecting pork adulterations in dairy products in Sri Lanka.

Dairy products such as milk powder, fresh milk, cheese and yoghurt have a higher demand in Sri Lanka. Before the adoption of an open economy in 1977, Sri Lanka was 80% self-sufficient in satisfying the milk requirement. Currently, Sri Lanka is around 40% self-sufficient. Therefore, about 96 million kg of milk powder and other dairy products were imported to Sri Lanka in 2020. Adulteration of pork fat in these imported dairy products have been rumored throughout these years. The most recent case was reported in 2019 as milk powder can be adulterated with plant and pork fat. It made a controversial discussion among Sri Lankans as Milk powder is the most common dairy consumption way in Sri Lanka. Therefore, the detection of adulteration in dairy products (milk powder, fresh milk, yoghurt and cheese) is a necessity. By comparing the DNA of these dairy products with the pork DNA, pork adulteration can be detected [7].

II. MATERIALS AND METHODOLOGY

3.1 Materials.

3.1.1 General materials.

Analytical grade chemicals were used for general assays and molecular grade chemicals were used for Polymerase Chain Reaction (PCR). The DNA was extracted using Qiagen (Germany) (DNeasyMericon Food Kit, REF 69514).

3.1.2. Primers.

The oligonucleotides primers (Table 3.1) were synthesized and purchased by Integrated DNA Technologies, Singapore.

TABLE 3.1. Primers used in PCR amplification.

Primer name	Sequence	Sequence length
PRK-CYTB-F2	5'-ATC GGA ACA GAC CTC GTAGAA T-3'	289bp
PRK-CYTB-R2	5'-TCT CCTAGTAGGTCTGGT GAG AA-3'	
Cattle F	5'-CAT CGG CAC AAA TTTAGT CG-3'	259bp
Cattle R	5'-GAG CTA GA ATTA GTA AGA GGG CC-3'	

3.2 Methodology

3.2.1 Sample collection.

As authenticated positive samples, raw pork and beef meat samples were obtained from the Veterinary Research Institute of Peradeniya, Sri Lanka and stored at -20ºC. The dairy products (six milk powder samples, five from each fresh milk, cheese and yoghurt samples) were collected from the local supermarkets and stored under optimum temperatures.

3.2.2 Pretreatments.

Sample pre-treatment was carried out only for fresh milk. Fresh milk samples were centrifuged under 13000RPM for 20min and the pellet was used to extract DNA.

3.2.3 Extraction of DNA: DNeasymericon Food Kit: Qiagen.

3.2.3.1 Raw pork and beef samples (positive samples): 200mg Standard Protocol:

Raw pork and beef were homogenized using motor and pestles. DNA from the homogenized samples were extracted according to the Qiagen 200mg standard protocol of DNeasymericon food kit (50) (REF 69514). The resulting DNA samples were stored at -20˚C.

3.2.3.2 Yoghurt, milk powder, fresh milk and cheese: 200mg small fragment protocol:

The DNA was extracted according to the Qiagen 200mg small fragment protocol of DNeasymericon food kit (50) (REF 69514) with some modifications such as the amount of Proteinase K and centrifugation time.

3.2.4 Confirmation of DNA.

3.2.4.1 UV Spectrometry:

Positive samples (raw pork and beef DNA) and DNA from other dairy products were quantificationally analyzed using UV spectrometry (Aligent Technologies - USA). The absorbance was measured at 260/280 nm.

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3.2.4.3 Amplification of DNA using Polymerase Chain Reaction:

The PCR amplification was done using conventional duplex PCR to simultaneous detection of bovine and porcine DNA using species specific oligonucleotide primers. The DNA bands were compared and source of dairy samples was detected.

Duplex PCR was performed based on the following master mix for a total volume of 15 µL using 7.5 µL of 2X ceygen master mix, 0.6 µL of bovine and porcine species-specific forward and reverse oligonucleotide primers (5µmol) each, 0.6 µL of Taq polymerase (2 units / µL), 0.5 – 3 µL template DNA and balanced with PCR water.

3.2.5.1 PCR Amplification:

The PCRs were carried out in Verity 96 well thermal cycler- applied biosystems by Thermo Fisher Scientific, under the following programs: initial denaturation at 94°C for 5min, followed by 35 cycles at 94ºC for 30S, 59ºC for 30S, 72°C for 60S, and the final extension was carried out at 72°C for 8min.

3.2.6 Visualization of DNA

3.2.6.1 Agarose gel electrophoresis:

The gel was run at 65V, 80mA, 150W for 2.5 hrs. The amount of the PCR products loaded into each gel was varying from each other. PCR products were visualized via the Bio Rad gel documentation system, Image lab 5.1 software.

III. RESULTS

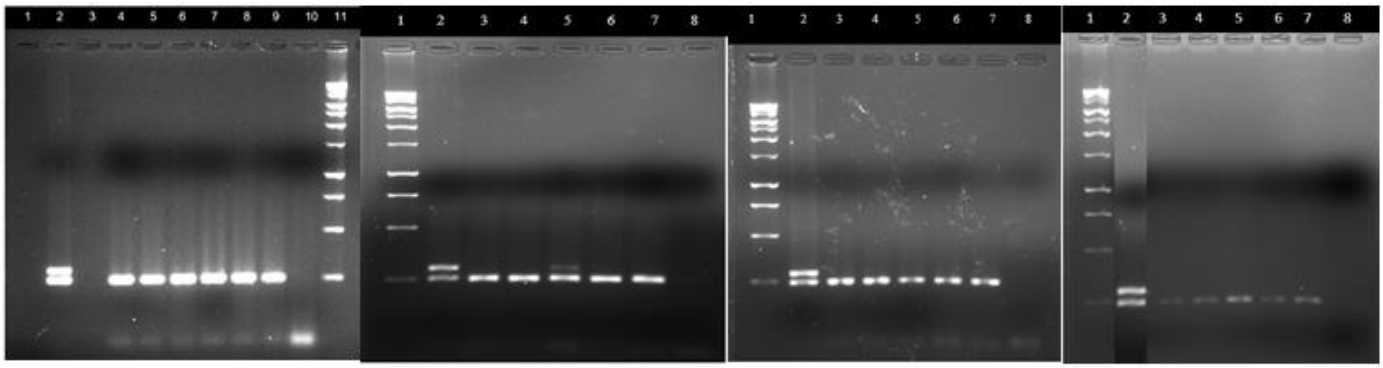

Figure 4.1. Agarose gel electrophoresis image for milk powder samples. Well 1- blank, Well 2- positive control (Upper band- 289bp, Lower band- 251bp), Well 3- blank, Well 4- MP1, Well 5- MP2, Well 6- MP3, Well 7- MP4, Well 8- MP5, Well 9- MP6, Well 10- negative control and Well 11- 1kb ladder.

Figure 4.2.Agarose gel electrophoresis image of fresh milk DNA samples. Well 1- 1kb ladder, Well 2- positive control, Well 3- FM1, Well 4- FM2, Well 5- FM3, Well 6- FM4, Well 7- FM5 and Well 8- negative control.

Figure 4.3. Agarose gel electrophoresis image of yoghurt samples. Well 1- 1kb ladder, Well 2- positive control, Well 3- Y1,Well 4- Y2, Well 5-Y3, Well 6-Y4, Well 7- Y5 and Well 8- negative control.

Figure 4.4. Agarose gel electrophoresis image of cheese samples. Well 1- 1kb ladder, Well 2- positive control, Well 3- C1, Well 4- C2, Well 5- C3, Well 6- C4, Well 7- C5 and Well 8- negative control.

IV. DISCUSSION

Detecting whether dairy products have been adulterated with pork is vitally important for religious communities (Muslims and Hindus) and the vegetarian community. However, after going through the processing procedure, DNA got degraded and only a very small amount remains with the products [8] [9]. Therefore, PCR based methods are ideal for the identification of DNA present in products. As described in the methodology, the DNeasy Maricon food kit was used for the extraction of DNA from the dairy samples, raw beef and pork samples. It is suitable for DNA extraction from highly processed foods, where high degradation of DNA can be expected and it is cost-effective [10] [11] [12].

The DNeasymericon Food Kit uses modified cetyltrimethylammonium bromide (CTAB) extraction. The nonionic detergent CTAB may complex with nucleic acids (low-salt conditions) or complex with inhibitors, such as polysaccharides, proteins, and plant metabolites. The optimized protocols use CTAB in combination with Proteinase K to first digest compact tissue and to subsequently precipitate proteins with simultaneous precipitation of other cellular and food derived inhibitors. Inhibitors are precipitated by centrifugation, while the extracted DNA remains in the solution. In the subsequent chloroform extraction, any remaining CTAB-protein, CTAB-debris, or CTAB-polysaccharide complex that has not been precipitated is removed along with the other lipophilic inhibitors into the organic phase. Only the aqueous phase which contains the DNA and significantly depleted inhibitors are processed further. This phase is mixed with binding buffer (to adjust binding conditions) and applied to the silica membrane Columns. The obtained DNA is ready for use in a downstream PCR assay. For the dairy samples, 200mg small fragment protocol was used with the modifications, as it was optimized for the highly processed food. For the raw pork and beef samples, 200mg standard protocol was used as it was designed for the DNA extraction from raw material [13] [14] [15] [16].

ISSN No:-2456-2165

Fresh milk samples were pretreated by 20 min centrifugation to collect the cattle's udder cells to the bottom of the tube, which had not solubilized.

To visualize the PCR products, a 2% gel was used since the amplicon sizes of the primers used are within a small range (Bovine-251bp and Porcine 289bp). Therefore, precise separation of bands needs to be taken place to identify the contaminations of samples. The band separation is much more precise in a 2% gel because the pore size is smaller in concentrated gels [17] [18] [19] [20] [21].

The errors in the PCR process may occur due to the contaminated reagents. Thus, fresh working stocks had been used under proper conditions to avoid contaminations throughout the experiment. During the process, only primer dimers had formed due to the self-annealing of primers in the negative control. It confirms that there was no contamination had occurred during the process [22].When the stringency of the PCR products are excessively low, it produces nonspecific PCR bands with variable lengths. This results in a ladder effect on agarose gel [23]. The results do not indicate ladder effect and exhibits a clear illustration of the PCR bands. Furthermore, the disappearance of PCR products can be happened due to the degradation of DNA or presence of PCR inhibitors [24].

According to Figure 4.1, Figure 4.3 and Figure 4.4, there is no pork adulterations in the selected six milk powder products, five yoghurt and five cheese samples respectively. All the samples shows the 259bp band confirming that the samples contain cattle DNA only. The excess primers can be seen as primer dimers, at the bottom of the figure in all the sample lanes and in the positive and negative controls. The bands appear to be dumbbell-shaped in Figure 4.3 and 4.4 due to the voltage error. Hence, it was reduced to 65V from 70V at the next electrophoresis and the bands appeared more clearly in Figure 4.2.

According to Figure 4.2, FM3 is adulterated with pork DNA as it clearly shows a 289bp band in the lane, when all the other samples shows negative for porcine. Therefore, for further confirmation of the presence of pork DNA in the FM3 sample, another PCR was carried out with a newly purchased FM3 sample. In there, FM3 does not indicate pork DNA. Therefore, it can be concluded that the FM3 was adulterated with pork DNA may due to unintentional contamination or a human error occurred by ourselves. None of the other dairy products were shown pork adulteration.

A low intensity of the bands can be observed in Figure 4.4. This can be due to insufficient DNA concentration, insufficient or uneven staining or diffusion of the gel [17].

The smears in DNA bands can be occur due to improper electrophoresis conditions, gel shift effect, poorly formed gel wells or high salt concentration in samples [23] [25].

V. CONCLUSION

DNA in dairy food products can be easily detected using conventional duplex PCR approaches due to its high sensitivity and reliability.

Porcine adulteration in dairy products were accurately detected via simple, low cost, duplex PCR method.

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