

Identification of Species Adulteration in Raw Milk and Butter Using Polymerase Chain Reaction - Restriction Fragment Length Polymorphism

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Abstract: Species identification is crucial, especially in animal biodiversity protection, veterinary diagnostics and for food samples control in the food industry. This study was carried out to evaluate, Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) for detection of cow's milk and butter in buffalo's milk and butter, using universal primers. A total number of 100 milk and butter samples were collected from local market (50 of each) to apply this study. To evaluate the sensitivity of PCR-RFLP method, using model samples made from buffalo's milk containing defined percentages of cow's milk, which revealed the fitness of method till 5% adulteration. The obtained results revealed that the RFLP profiles of cytochrome b fragments displayed milk and milk products species-specific when digested separately with restriction endonuclease Hinf I revealed 150, 210 and 360bp in case of cow product while in buffalo product produced uncut fragment 360bp. 50% and 66% of the examined raw milk and butter samples, respectively were proven to be pure buffalo's. In conclusion, the PCR-RFLP assay is a reliable technique for products inspection to detect cow's genome in buffalo's milk and fat with a detection limit of 5%.

Key words: PCR-RFLP • Butter • Raw milk • Buffalo • Cow

INTRODUCTION

Milk and milk products are the main constituents of the human daily diet for all ages [1], but they are often prone to adulteration practices, especially milk and milk fat (butter), since milk is a fairly expensive raw material [2] and butter takes into account as a high valuable price product compared to other dairy products and both of them can be easily replaced in part by other dairy or non-dairy ingredients. [3]. Common adulterations of dairy products are the substitution of higher value milk by cheaper one [4]. So species identification of milk and dairy products is of great concern for Protection against species substitution or admixture in dairy products [5]. Cow's milk may be used for adulteration of buffalo's milk due to its lower cost and larger availability deriving from greater productions, compared to buffalos in the same

farm [6] but, this must be avoided due to many reasons including, health hazards as cow's milk protein is potential allergens for some people even at low concentrations and it was reported to be the main dairy product responsible for human adverse reactions [7]. Cow's milk also may be avoided due to religious, ethical or cultural objections [8] or due to governmental regulations [5]. Thus, the adulteration of buffalo's milk with cow's milk may be considered as a health risk in current food safety requirement.

Adulteration of milk fat (butter) has always been considered as a serious problem because of the economic advantages of partially replacing high-priced fats with low-priced one without any labeling of the product [9]. Several methods have been used for the identification of species of origin for milk and dairy products include chromatographic, electrophoretic and immunological

methods [2]. DNA based methods became a very popular methods for examination of food products, since they are considered as a reliable method. DNA is stable under high temperatures, pressures and chemical treatments used during processing of food products [10]. Milk can be easily used as a source of DNA, because it has a large amount of somatic cells (leukocytes and epithelial mammary cells) from mammary glands [5]. It could be successfully used for fast and sensitive species differentiation using the Polymerase Chain Reaction (PCR) which is the most widely used molecular technique due to its simplicity, sensitivity and reproducibility [11-13]. In the last few years, the PCR-RFLP have been widely used for species identification in dairy products [14-16]. PCR-RFLP has been proved to be as practical, highly repeatable and reliable technique and requires low amount of DNA, the quality of which has not shown to be crucial for generating PCR amplicons [17]. It does not require costly nucleotide sequencing and the whole experimental analysis, including scoring RFLP profiles, can be completed less than 4 hours [18].

The objective of the current study was to investigate the prevalence of the adulterated buffalo's milk and butter with cows' same products using PCR-RFLP analysis of cytochrome b gene and to evaluate its sensitivity in detecting cow's genome in buffalo's products using several restriction enzymes Hinf I, Hind III, Hae III and Bsa I.

MATERIALS AND METHODS

Sampling:

For Evaluation of the Applicability of the Test: 100 samples of buffaloes origin, including raw milk and butter (50 samples for each labeled as "buffalo milk and buffalo butter") were collected from local shops and markets in Sharkia Governorate, Egypt. The collected milk samples were stored at 4°C for one day or freshly, while butter samples stored at -20°C till used for extraction of DNA.

For Evaluation of the Sensitivity of PCR-RFLP for Detection of Cow's Milk in Buffalo's Raw Milk: The precision and sensitivity of the method for detecting cow's milk in buffalo's milk was evaluated at the following; Mixtures of cow's milk in buffalo's milk were prepared for further DNA extraction and PCR analysis. Different cow milk percentages 50%, 40%, 30%, 20%, 10%, 5%, 1% and 0.5% (v/v) were prepared in a final volume of 100 ml.

DNA Extraction: Milk samples (50 ml) were initially centrifuged at 1500 g for 15 min to collect somatic cells. The pellets were rinsed three times in 1 ml of Phosphate Buffered Saline (PBS) (lonza - bioWhittaker), centrifuged at 12,000 g for 5 min and finally resuspended in 200 ml of PBS. Total DNA isolation using 25 mg of buffalo butter or resuspended milk somatic cells were performed using G-spin™ Total DNA extraction kit (iNtRON biotechnology, Korea) as described in the instructions given by the manufacturer. The extracted DNA solution was stored in -20°C until further use.

Polymerase Chain Reaction Amplification: A 360 bp fragment of the cyt b gene of mitochondrial DNA extracted from milk and butter samples was amplified using universal primers- L14816 (5'-CCA TCC ACC ATC TCA GCA TGA TGA AA- 3') and H15173 (5'-CCC CTC AGA ATG ATA TTT GTC CTC A- 3'), as described by Kocher *et al.* [19]. Various combinations of primers and DNA of cow and buffalo origin were tested in a final volume of 25 µl containing 12.5 µl (1x) PCR master mix (iNtRON biotech-nology) 1 µl of each primer (10 pico mole) and 2 µl of DNA template. Amplification was performed in a T professional thermal cycler (Biometra, Germany) with the following cycling conditions: after an initial heat denaturation at 95°C for 11 minutes, 35 cycles were programmed as follows: 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 7 minutes. PCR products were separated by 2% agarose gel electrophoresis at 120 volt for 30 minutes and visualized by UV transillumination and analyzed using Gel Documentation System (Bio Doc Analyze, Biometra, Germany).

Restriction Enzymes Digestion: The PCR product of mitochondrial cytochrome b gene was subjected to 4 types of restriction enzyme digestion with 4 types of restriction enzymes, namely Hinf I, Hind III, Hae III and Bsa I. Five units of each enzyme were applied to 10 µl of amplified DNA in a final volume of 20 µl digestion mixture containing 1x reaction buffer. The digestion mixture was incubated for incubation time and incubation temperature was set according to the types of restriction enzyme (Hinf I and Hae III at 37°C/5 min., Bsa I at 37°C/10 min. and Hind III 37°C/20 min.). The digested products were separated by electrophoresis in a 2% agarose gel (Sigma) in TAE buffer and visualized by UV transillumination and analyzed using Gel Documentation System (Bio Doc Analyze, Biometra, Germany).

RESULTS AND DISCUSSION

There are several methods have been used for identification of species origin of dairy products. At first the European Union suggested a method based on detection of bovine proteins in dairy products (20). Recently, Nucleic acid based techniques (molecular biology techniques) have been used instead of protein for species identification in food especially that of animal origin. This is applied according to its stability at high temperatures, pressures, chemical treatments used during processing of food products and its conserved structure within all tissues of an individual [21]. The polymerase chain reaction (PCR) is the most widely used molecular technique [2] either multiplex PCR [5], PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) [16] and Real-Time PCR [22, 23].

The conventional techniques allow the qualitative detection of different species with a defined limit of detection. However, real time PCR generally offers greater sensitivity and specificity and is a quantitative method of identification of species [24]. Species specific PCR assay was found to be rapid and cost effective for identification of species due to specific detection of target sequence without the need to further sequencing or digestion of PCR products with restriction enzymes [25] but it can't be designed when species are very closely related [26] on the contrary, PCR -RFLP could differentiate closely related species [16].

In our work a method based on PCR-RFLP was established to identify cow's milk in buffalo's milk. The sizes of the amplicons were approximately 360bp (Fig. 1). The 360bp fragment of the cytochrome b gene was reported to be highly polymorphic [27] and could be used



Fig. 1: Electrophoretic analysis of PCR product amplified with cytochrome b gene, Lanes: M, 100 bp Plus DNA ladder (Fermentas); 1-14 random milk samples



Fig. 2: Electrophoretic analysis of PCR product amplified cytochrome b gene of random buffalo milk which treated with Hinf I; Lanes: M, 100 bp Plus DNA ladder (Fermentas); 1, 14 tested buffalo milk samples

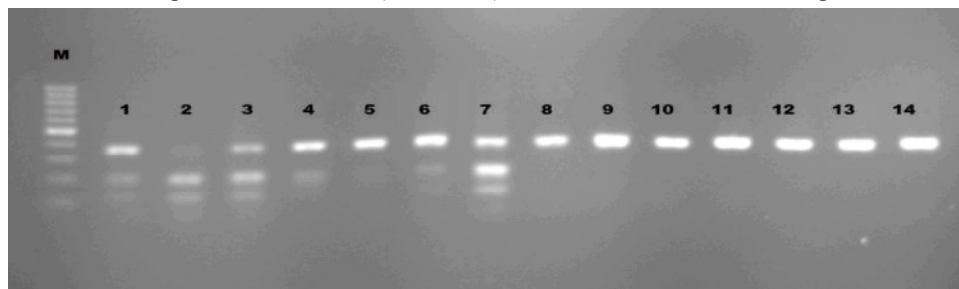


Fig. 3: Electrophoretic analysis of PCR product amplified cytochrome b gene of random buffalo butter which treated with Hinf I; Lanes: M, 100 bp Plus DNA ladder (Fermentas); 1, 14 tested buffalo butter samples

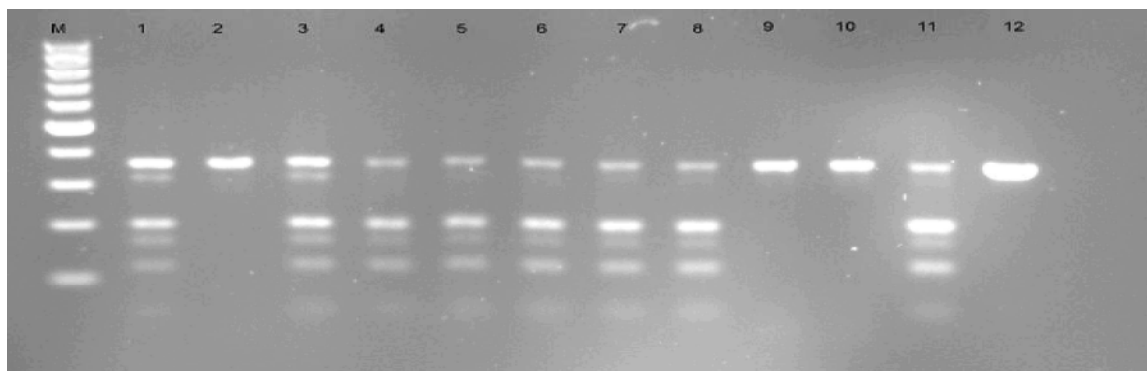


Fig. 4: Electrophoretic analysis of PCR product amplified cytochrome b gene of diluted bovine milk in buffalo milk which treated with Hinf I; Lanes: M, 100 bp Plus DNA ladder (Fermentas); 1, 11 cow milk; 2,12 buffalo milk; 3 (50%); 4 (40%); 5 (30%); 6 (20%); 7 (10%); 8 (5%); 9 (1%) and 10 (0.5%)

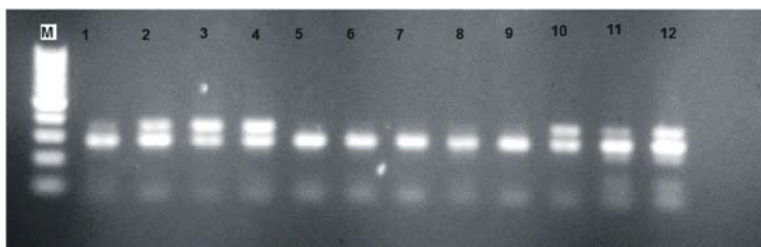


Fig. 5: Electrophoretic analysis of PCR product amplified cytochrome b gene of diluted bovine milk in buffalo milk which treated with Hae III; Lanes: M, 100 bp Plus DNA ladder (Fermentas); 1, 11 cow milk; 2,12 buffalo milk; 3 (50%); 4 (40%); 5 (30%); 6 (20%); 7 (10%); 8 (5%); 9 (1%) and 10 (0.5%)

Table 1: Prevalence of cow raw milk and butter in buffalo samples:

Dairy products	No. of samples	Normal samples		Adulterated samples	
		NO.	%	NO.	%
Raw milk	50	25	50%	25	50%
Butter	50	33	66%	17	34%

Table 2: Fragment length for cow, buffalo species after digestion of the PCR products with restriction enzymes:

Species	Amplicon bp	Restriction enzyme	Fragment length bp
Cow	360 bp	Hinf I	360 bp, 210 bp and 150 bp
Buffalo	360 bp	Hinf I	360 bp

to differentiate milk and butter samples in different species. When the amplicon was cleaved with restriction enzyme, the restriction map or RFLP profiles produced a difference between buffalo and cattle. The restriction enzymes used for the digestion were; Hinf I, Hind III, Hae III and Bsa I to digest the PCR amplification fragments for further differentiation of cow and buffalo. In case of Hinf I restriction enzyme in cow PCR product, It was digested into 150bp, 210bp and 360bp fragments (Table 2) while the cytochrome b amplicons of buffalo were not cleaved by Hinf I (Fig. 2, 3) in both milk and butter samples

respectively. Hae III; Hind III and Bsa I did not produce patterns that were easily distinguishable (Fig. 5). For the analysis of restriction profiles generated, only the typical major bands were taken into consideration. According to Jiang *et al.* [16] the RFLP profiles of cytochrome b generated by other restriction enzymes as MsapI and Pac I could possibly be used to differentiate between cow and buffalo raw milk.

Unexpectedly, results cleared that, out of the 50 tested raw milk samples, 25 samples contained cow's milk, while in case of butter samples only 17 samples contained

cow's butter (Table 1). These results suggest fraudulent practice can hurt consumer rights, both due to economics and related to the risk of milk consumption, which is not declared to be present. Furthermore, it is important to determine the type of milk (animal species) to ensure the authenticity of the product [28]. In referring to other works, Darwish *et al.* [29] detected cow's milk in 8/21 of buffalo raw milk samples. Others try to detect cow's milk, but in other types of milk or in other dairy products [30-33].

The possibility to detect the added quantities even they are small is important besides detecting adulteration itself, especially in consumers suffered from allergic reaction to cow milk proteins [6]. So in the present study, the authors tried to investigate the sensitivity of the corresponding method for detecting cow's milk in buffalo's one. We set up different concentrations of milk (50%, 40%, 30%, 20%, 10%, 5%, 1% and 0.5%). The results cleared that Buffalo milk containing 50%, 40%, 30%, 20%, 10%, 5% cow milk were detected, while it failed to detect the presence of cow milk at concentration 1% and 0.5% as shown in Fig. (4).

However, attempts to use PCR as a quantitative tool for food authentication are still very scarce. (PCR)-based methods used to detect very low amounts of cow milk, such as 0.5 % [29,34] or 0.1 % [35]. PCR helped to detect addition of 1% cow's milk in buffalo's milk [36] and 5% [37, 38]. In agreement with Cozzolino *et al.* [39] 5% detection limit was considered as sufficient for the proof of the undeclared milk component, whereas adulteration of milk by less than 5% lacks any economic effect. A very important point must be mentioned that, although the ability to detect lower levels of contaminating milk could be achieved, it is difficult to be established if a fraud is presumable or it just unintentional contamination might be supposed.

CONCLUSION

From the results obtained, it could be concluded that the PCR-RFLP method is potentially reliable technique for detection of different species. Moreover, this method can detect adulterated buffalo's milk mixed with cow's milk with a detection limit 5%. So it is suitable for routine testing of buffalo milk or dairy products of buffalo origin to help protect both producers and consumers from this fraud which is present as a common practice in markets as it was cleared through the results.

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