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Standardization of DNA extraction method of milk samples and development of PCR-based method of adulteration detection in milk

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Abstract

Milk and dairy products are generally nutritious foods that are widely consumed by the public and are an essential component of the diet for specific consumer categories, especially youngsters and pregnant women. Due to its significant value and popularity, fraud in the dairy business has become widespread. Species identification using DNA-based techniques is especially helpful for the analysis of commercial dairy products because all dairy products contain DNA derived from physical animal cells, which is stable and recoverable for polymerase chain response (PCR) analysis even after thermal treatment and other forms of processing. The most common way to identify differences in dairy products is grounded on PCR, given its low limits of discovery, strong particularity, and high perceptivity. In this work DNA extraction was done from cow, goat, buffalo, and sheep by using PCR amplification with two primer UMP and cytochrome B successfully.

Keywords: mtDNA, Cytochrome B, Milk adulteration, DNA extraction, Polymerase chain reaction.

Introduction

Milk, in its natural state, is highly nutritious. It contains moderate levels of protein, fat, carbs, vitamins, and minerals in a readily digestible form. Milk is essential to people of all ages because of its nutritional worth. Milk composition is influenced by a variety of factors, including the breed of the cow, the stage of lactation, the feed, the time of year, and many others. Some component interactions, though, are extremely reliable and may be used to identify milk compositional changes.

The Food and Safety Standards Authority of India (FSSAI) defines food adulteration as the addition or removal of any ingredient from food in such a manner that the food's natural makeup and quality are altered. If the quality of food products is lowered by the addition of any particular ingredient that is harmful to health or by the removal of a nutritional component, the food product is considered to be thinned. Due to price variations and seasonal availability, which make this attractive to farmers and producers, milk, milk-based products, and milk derivatives constitute a significant group of food items with high nutritional value and widespread consumption by a sizable segment of consumers[1]. The replacement of milk by dairy products of lower commercial value is one of the most prevalent issues encountered in the marketing of dairy products[2]. The majority of these unreported substitutions are made by mixing cow's milk with dairy products from buffalo, sheep, and goats. The addition of cow's milk to these items, however, is not the only way that these adulterations happen. For example, goat milk is frequently used in place of sheep milk in dairy products because sheep milk costs more[3]. Furthermore, there are mixed herds of goats and sheep, which may lead to unintentional or dishonest substitutions of sheep milk for goat milk, and vice versa[4]. Unintentional substitutions can also happen when different species are processed on the same production line. Species identification of dairy products is critical because some milk proteins cause frequent fatal adverse reactions (disinclinations). In addition, it permits the detection of contamination in the form of the bargaining of a lower priced sort of milk for one of a higher grade, which is especially beneficial for individuals who dislike cow milk. Conversely, drinking milk or dairy products from contaminated sources tainted with cow milk may induce negative responses in people who may have an aversion to it. The inclusion of varying amounts of cow milk during the production of buffalo dairy products, which amounts to fraud by product contamination, may be permitted due to the increasing use of buffalo milk derivatives, seasonality, and lesser added value when compared to dairy products from cows.

Milk and dairy products are mostly nutritious foods that are widely consumed by the general public and play a significant role in the diets of certain demographic groups, especially youngsters and expectant moms. Because of its high value and demand, fraud in the dairy industry has become a widespread issue and a genuine concern for many customers and governments, particularly in the wake of the melamine controversy. In terms of other food products, the labelling of dairy products is a critical problem since the information provided must correspond to the product's attributes, particularly in terms of the components utilized and manufacturing methods. The discovery of mislabelled and/or sub-standard dairy products is of the highest importance for both financial and public health considerations. Furthermore,

the milk with a different introduction of milk from non-declared species may pose health risks pertaining to the presence of allergens, as well as ethical retaliation because of religious practices or specific decisions that prohibit the consumption of milk from certain species.

In milk and dairy goods, there is currently a wide variety of methods used to identify species, including immunoassay, chromatography, and electrophoresis. The immunoassay approach may be used to differentiate milk from similar species, such as water buffalo, taurine cattle, sheep, and goats, but it cannot recognize heat-treated material. The chromatographic method may be used to identify variations in the proportion of fatty acids, but it requires a significant amount of work. Species identification has relied on molecular techniques in recent years, and they have shown to be trustworthy, precise, and effective. PCR is the most commonly used molecular method for determining the species of food. The DNA-based technique for identifying species is particularly helpful for analysing commercial dairy products since they all include DNA obtained from physical beast cells, and DNA is stable and can be recovered for polymerase chain response (PCR) analysis even after thermal treatment and other forms of processing. The most popular method for identifying differences in dairy products is PCR because of its low limits of discovery, high particularity, and high perceptivity. Its unique benefit for increasing target sequences from mtDNA is the large dupe count per cell, which provides it with the web advantage of reduced sequence variation over nDNA. It is a dependable, quick, and responsive approach.

The circular chromosome configuration found inside the cellular organelles known as mitochondria is referred to as mitochondrial DNA. The site of the cell's energy production and other metabolic activities is the mitochondria, which are found in the cytoplasm. Seeds receive mitochondria and, consequently, mitochondrial DNA, from their moms. The genome of mitochondrial DNA is quite tiny. It has a double-stranded, circular structure. It is haploid, has 37 genes, and these genes are critical to the functioning of mitochondria. It has 13 that are in charge of ATP synthesis via oxidative phosphorylation. ATP is produced using oxygen and sugar. The mitochondrial genome ranges in size from 16 to 18 kbp, which translates to 16,569 base pairs. There are 37 genes, 2 tRNAs, 22 rRNAs, and 13 proteins in the mtDNA. The sperm cell just contributes its nuclease, which consists of 23 chromosomes, throughout the fertilization process. Since egg cells provide their mitochondria during fertilization, mitochondrial DNA is solely maternal.

Species-specific data from mtDNA's cytb has previously been employed in forensic analyses. As a result, cytb analysis has been demonstrated to be helpful in identifying the biological makeup of casework samples. Sometimes, the kind of food sample discovered in the victim's stomach might be extremely significant. It is possible to use this data to ascertain the postmortem interval and the location of death.

The COI gene has gained popularity because it is simple to amplify using a common primer set and offers greater sequence variation between species than at the intraspecies level. As a result, DNA barcoding based on the COI gene is a viable alternative method of species identification that can be standardized to produce consistent results from a variety of sources.

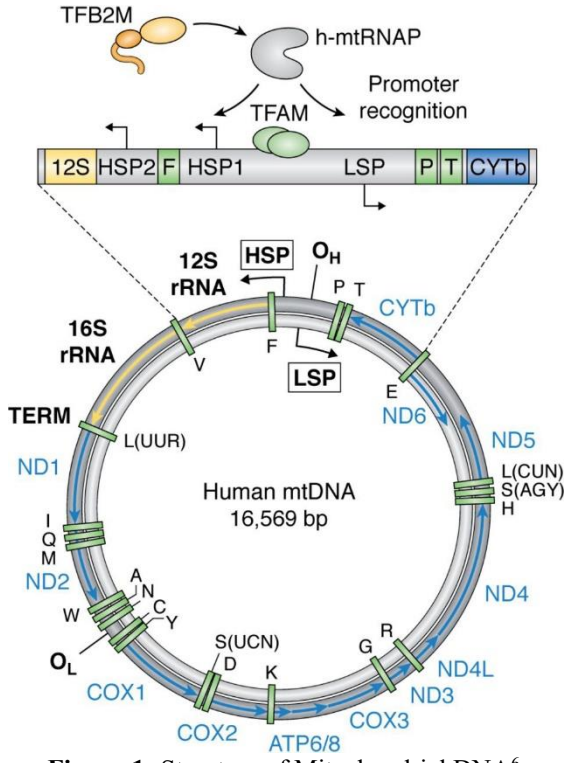

Figure-1: Structure of Mitochondrial DNA[6].

Materials and Methods

Sample collection: Milk samples of cow (*Bos taurus*), buffalo (*Babuls bubalis)* sheep (*Ovis aries*), and goat (*Capra hircus*) were collected from different dairies of Bilaspur.

Material required: Electrophoresis set up, PCR Machine, Incubator, Centrifuge Machine, Eppendorf tube (1.5mL & 2 mL), Micro Pipette, pH Meter, Microwave, UV trance illuminator, Scissor, Cotton / Tissue, Cello tape, Marker pen, Glass rod, Reagent bottle, Hand gloves, Measuring cylinder, Parafilm, Spatula, Vertex, Centrifuge tube

Chemical required: EDTA (ethylene diamine tetra acetic acid), Tris, Distilled water, SDS (sodium dodecyl sulphate), Sucrose, Isopropanol, TE buffer, Agarose powder, Et Br (ethidium di bromide), Bromophenol blue, NH_2PO_4, N_2HPO_4.

Reagent preparation: PBS Buffer 1 M-$NaH_2PO_4.H_2O$ – 8.9µL, $Na_2HPO_4.2H_2O$–6.9µL, NaCl – 5.844 gm, Distilled water – 50 mL, pH – 7.4, 10% SDS- SDS – 10gm, Distilled water – 90mL, pH–8.8, Mitochondrial extraction buffer- NaCl 0.1M – 8.33µL,

Tris Cl 50Mm-1.66µL, EDTA 5Mm -500µL, Distilled water – 100mL, pH–7, Homogenization buffer - Sucrose 0.25M – 4.278g, Tris 30Mm - 1000µL, EDTA - 1000µL, pH–7.5. TAE Buffer- Tris (24.2gm)+EDTA(2.72gm) + GAA(5.71ML), pH - 8.5, 50X. 50X Agarose gel-TAE (1mL) + Agarose (0.4gm) + Distilled water (50mL), Heat it, Loading dye-Sucrose (4gm) + Bromophenol blue (25gm) + Distilled water (10mL), Protocol for PCR reaction mixture (for 200µL Master Mixture)-Buffer 10X-15µL, $MgCl_2$-4µL, Taq Polymerase-1µL, DNTPs-12µL, Forward Primer-4µL, Reverse Primer-4 µL, MiliQ Water-115 µL, Template DNA-2 µL.

DNA extraction: In centrifuge tubes, collect a 10 mL milk sample. Next, spin it for 10 minutes at room temperature at 5000 RPM. Use a 1000-microliter PBS buffer to pallet wash and get rid of milk fat. At room temperature, transfer into 2 mL Eppendorf and quick spin for 2 minutes at 10000 RPM. At room temperature, centrifuge it for 10 minutes at 5000 RPM. Centrifuge the mixture for three minutes at room temperature at 5000rpm, and then transfer the supernatant to a new tube after discarding it. Add 500 microliters of homogenization buffer and 5 microliters of lysozyme. Put it in a 37-degree Celsius incubator for 30 minutes. It was just centrifuged for 10 minutes at room temperature at 10,000 rpm. The mixture should be incubated at a temperature of 65°C for 30 minutes after adding 200 µL of mitochondrial extraction buffer and 50 µL of SDS. At room temperature, centrifuge the pellet wash at 10000 rpm for 5 minutes using 500µL of isopropanol. Centrifuge the sample once more at room temperature for 5 minutes at 10,000 rpm after washing it with 500µl of isopropanol. Leave the sample out to dry after discarding the supernatant. To dissolve the DNA pellet, add 50µL of triple-distilled water or TE Buffer. Heat the 50X agarose gel in the microwave after it has been prepared. Mix in a drop of EtBr. Pour in gel casting try and let it cool. Gently take the comb out of the casting try. Maintain it in an electrophoresis chamber with TAE buffer. Mix 8 µL of the sample with one drop of bromophenol dye. Put the mixture into the wells. Turn on the power and let the sample run. Imagine the sample in the UV Transilluminator.

PCR amplification: For Checking PCR amplification of mitochondrial DNA isolated from Different Milk sample a set of reported UMP (Table-1) for mt –cyt b gene was used.

Table-1: Primer used for PCR amplification.

Primer	Primer sequence	Amplicon size
UMP	Forward: 5'-CCATCCAACAT CTCACATATGAAA3' Reverse: 5'-CCCCTCGAATGA TATTTGTCCTCA3'	Forward- 415 Reverse – 512
Cytochrome B	Forward: 5'-TACGCAATCCT ACGATCAATTCC3' Reverse: 5'-GGTTGTCCTCCA ATTCATGTTAG3'	Forward – 528 Reverse - 387

Using around 25 ng of genomic DNA, the Polymerase Chain Reaction was carried out in 25 chain reaction volumes in microliters. The reaction mixture contained 2.5 mM $MgCl_2$, 50 pmol primer (P-1), 200 µM each of dATP, dCTP, dGTP, dTTP, and 1(U) Taq polymerase (Sigma) in the right Taq buffer. The first denaturation was carried out under the following conditions: 2 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 5 seconds, annealing at 55°C for 30 seconds, and extension at 72°C. Next, there was a 2-minute extension at 72°C. Electrophoresis was used to separate the PCR products on 2% agarose gels (with ethidium Bromide staining) at 90 volts for 30 minutes, alongside a 100 bp DNA ladder.

Results and Discussions

In this work DNA extraction was done from cow, buffalo, goat and sheep successfully. PCR amplification was done using UMP (Universal Mammal Primer) and *Cytochrome B* primers successfully. Further we can perform Real-Time PCR and RFLP-PCR for quantitative and qualitative analysis.

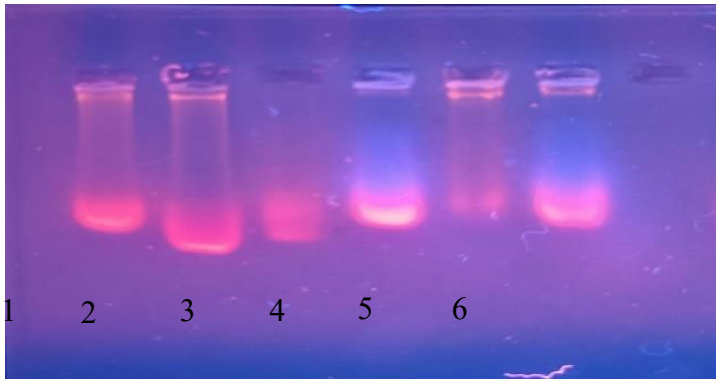

Figure-2: mtDNA separation in Electrophoresis at 0.4% agarose, Lane 1 – Cow, Lane 2 – Buffalo, Lane 3 – Goat, Lane 4 – Sheep, Lane 5 & 6 – Mix.

Figure-3: PCR product separation in 2% agarose gel, Lane 1 to 5 – UMP PCR Product Lane 6 – Marker, Lane 7 to 8- Cytochrome B PCR Product.

Conclusion

Due to their high specificity, sensitivity, ease of use, and relatively affordable cost of analysis, DNA-based techniques have been essential in identifying the species of dairy products. The techniques presently used to identify milk species involve producing conserved mtDNA or mtDNA primer for PCR amplification. A single-step PCR test has been created for this study to specifically identify affordable cow goat milk in sheep and goat milk. This method can be used to differentiate and quantify the DNA samples of buffalo, goat, and sheep. The mitochondrial gene (cytochrome B oxidase and UMP) is highly efficient in differentiating cow, buffalo, goat, and sheep milk, as well as in identifying cow DNA in samples from other species.

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