

## Original Article

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# Development and Validation of a PCR Based Method for Species Identification of Cow and Buffalo Milk

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## Abstract

**Background and Objective:** Food fraud through the substitution of high value milk with lower cost cow milk represents a significant challenge in the dairy supply chain. Reliable analytical methods capable of accurately determining the species origin of milk are therefore essential for ensuring product authenticity and consumer protection. The aim of this study is to develop and validate a PCR based method for the species identification of cow and buffalo milk.

**Materials and Methods:** Raw milk samples from cow and buffalo were collected from private farms. Genomic DNA was successfully extracted using the CTAB method in combination with a commercial extraction kit. Species specific primers were designed using bioinformatic analyses, and PCR amplification conditions were optimized to ensure efficient amplification of the target fragments. The developed method was validated by evaluating key performance parameters including specificity, sensitivity, limit of detection, repeatability, and robustness under varying experimental conditions.

**Results:** The developed PCR assay generated clear species specific amplicons of 118 bp for cow milk and 142 bp for buffalo milk, with no cross reactivity with non target species. Cross reactivity tests confirmed the absence of amplification with non target DNA, indicating strong discriminatory power of the assay. The method also showed satisfactory sensitivity, repeatability, and robustness across different reaction conditions.

**Conclusion:** Overall, the validated PCR based method provides a rapid, sensitive, and reliable approach for the identification of cow and buffalo milk species origin. This method can serve as a practical tool for authenticity verification and quality control within the dairy industry.

**Keywords** PCR, Species identification, Cow milk, Buffalo milk, Food authenticity, Dairy authentication

## 1. Introduction

Food fraud has become an increasingly important concern for global food systems due to its potential impacts on consumer trust, market integrity, and public health. Economically motivated adulteration involves the in-

tentional substitution, addition, or misrepresentation of food ingredients for financial gain, and dairy products are among the commodities most frequently affected by such practices [1,2]. Milk authentication has therefore gained significant attention in recent years as part of broader



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efforts to ensure transparency and safety in food supply chains.

Among dairy products, milk from different animal species often differs substantially in both nutritional composition and economic value. Cow milk is the most widely produced and consumed milk globally due to its high production volume and relatively low cost. In contrast, buffalo milk possesses distinctive physicochemical properties, including higher concentrations of fat, protein, calcium, and total solids compared with cow milk. These characteristics make buffalo milk particularly suitable for the production of high value dairy products and contribute to its higher commercial price in many markets [3,4]. As a result, fraudulent substitution or undeclared mixing of buffalo milk with cow milk has been reported in various dairy products.

Species adulteration in milk not only deceives consumers but also violates labeling regulations and may compromise product quality and nutritional characteristics. Furthermore, accurate identification of milk species is essential for regulatory monitoring, food authentication, and quality control within the dairy industry [5]. Reliable analytical methods are therefore required to detect species substitution and to verify the authenticity of milk and dairy products.

Several analytical approaches have been developed to determine the species origin of milk. Traditional methods include techniques based on physicochemical characteristics, protein electrophoresis, chromatographic analysis of lipids, and spectroscopic methods. Although these approaches can provide useful information, they are often influenced by technological processing conditions such as heating, fermentation, or enzymatic treatments, which may alter protein structures and reduce the reliability of the analysis [6]. Consequently, molecular techniques have gained increasing importance in food authentication studies.

DNA based methods are considered highly reliable tools for species identification in food products because DNA molecules remain relatively stable under various processing conditions and contain species specific genetic information. Compared with protein based methods, DNA analysis offers improved specificity and sensitivity for detecting biological origins in complex food matrices [7]. Among these techniques, the polymerase chain reaction (PCR) has become one of the most widely applied molecular tools for species identification due to its rapidity, high sensitivity, reproducibility, and relatively low cost [8].

Species specific PCR assays commonly target mitochondrial DNA sequences such as cytochrome b, 12S rRNA, or 16S rRNA. Mitochondrial DNA markers are

particularly suitable for species authentication because they occur in multiple copies per cell and exhibit sufficient sequence variation among different species while remaining conserved within the same species [9]. These characteristics enable the reliable amplification of target DNA fragments even when only small amounts of genetic material are present.

Recent studies have successfully applied PCR based methods, including multiplex PCR and real time PCR, for the identification of animal species in milk and dairy products. These approaches have demonstrated high sensitivity and specificity in distinguishing milk from different species, including cow, buffalo, goat, and sheep [10–12]. Advances in molecular techniques such as quantitative PCR and digital PCR have further improved detection accuracy and the ability to identify trace levels of adulteration in dairy matrices [13].

Despite these advances, variations in primer design, amplification conditions, and analytical validation parameters highlight the need for further development and validation of reliable PCR based assays for specific applications in dairy authentication. In particular, the differentiation between cow and buffalo milk remains an important analytical challenge in regions where both types of milk are widely produced and utilized.

Therefore, the aim of the present study is to develop and validate a PCR based method for the identification of the species origin of cow and buffalo milk. The analytical performance of the developed method was evaluated by assessing key validation parameters including specificity, sensitivity, limit of detection, repeatability, and robustness. The results of this study are expected to contribute to the development of reliable molecular tools for dairy authentication and support regulatory monitoring and quality control in the dairy industry.

## Materials and Methods

**Sample collection:** In the present study, raw cow and buffalo milk samples were obtained from various private dairy farms and local milk collection centers. To ensure sample integrity, all specimens were collected in sterile containers and transported to the laboratory under chilled conditions ( $4^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ ). Samples were analyzed within 24 hours of collection to minimize potential DNA degradation. Additionally, milk samples from goat and sheep were included as non-target controls to evaluate the analytical specificity of the developed PCR-based method. Detailed characteristics of the samples, including their origin and storage conditions, are summarized in Table 1.

**DNA extraction:** Genomic DNA was extracted from

**Table 1.** Characteristics and sources of the milk samples used for method validation.

| Sample Group           | Number of Samples (n) | Source / Origin     | Storage Conditions   |
|------------------------|-----------------------|---------------------|----------------------|
| <b>Target Species</b>  |                       |                     |                      |
| Raw Cow Milk           | 10                    | Private dairy farms | 4 °C (analyzed <24h) |
| Raw Buffalo Milk       | 10                    | Local farms         | 4 °C (analyzed <24h) |
| <b>Control Species</b> |                       |                     |                      |
| Goat Milk              | 4                     | Local farms         | 4 °C (analyzed <24h) |
| Sheep Milk             | 4                     | Local farms         | 4 °C (analyzed <24h) |

milk samples using a commercial food grade DNA extraction kit according to the manufacturer's instructions. Briefly, milk samples were centrifuged at 10,000 rpm for 10 min to obtain a cellular pellet. The fat layer and supernatant were carefully removed, and the pellet was used for DNA extraction.

The quality and quantity of the extracted DNA were assessed using a NanoDrop spectrophotometer by measuring absorbance at 260 and 280 nm. DNA integrity was further evaluated by electrophoresis on a 1% agarose gel. Additionally, PCR amplification using universal 18S rRNA primers was carried out to verify the amplifiability and suitability of the extracted DNA for downstream PCR analyses. Extracted DNA samples were stored at -20 °C until PCR analysis.

**Primer design:** Species specific primers were designed to identify the species origin of cow and buffalo milk based on mitochondrial gene regions. Target gene sequences were retrieved from the NCBI database and analyzed using BioEdit and Primer BLAST software. Primer design criteria included species specificity, melting temperature ( $T_m$ ), GC content, and the absence of secondary structures or primer dimer formation. The sequences of the primers used in this study are presented in Table 1.

**PCR amplification:** PCR reactions were performed in a final volume of 25  $\mu$ L. The reaction mixture consisted of 12.5  $\mu$ L of PCR Master Mix, 1  $\mu$ L of each primer (10 pmol), 2  $\mu$ L of template DNA, and nuclease free distilled water to reach the final volume.

The amplification reactions were carried out in a thermal cycler using the cycling conditions summarized in Tables 1 and 2. Each PCR program included an initial denaturation step at 95 °C for 3 min, followed by 35 cycles consisting of denaturation at 95 °C for 15 s, primer annealing for 15 s at the optimized temperature, and extension at 72 °C for 15 s. Two annealing temperatures were applied depending on the primer set: 67 °C and 64 °C, respectively (Tables 2 and 3). A final extension step was performed at 72 °C for 5 min to ensure complete amplification of the

PCR products.

All PCR reactions included a negative control (no template control) to monitor possible contamination.

**Agarose gel electrophoresis:** PCR products were separated by electrophoresis on a 1.5% agarose gel containing a safe DNA staining dye. Electrophoresis was conducted at 100 V for 45 min. Amplified DNA fragments were visualized and documented using a gel documentation system. A 100 bp DNA ladder was used as a molecular size marker.

**Table 2.** Thermal cycling conditions used in the PCR reaction for species identification of cow milk.

| PCR program Temp (°C) |    |          |
|-----------------------|----|----------|
|                       |    | Time     |
| Initial denaturation  | 95 | 3 (min)  |
| Denaturation          | 95 | 15 (sec) |
| Annealing             | 67 | 15 (sec) |
| Extension             | 72 | 15 (sec) |
| Number of Cycles      | 35 |          |
| Final extension       | 72 | 5 (min)  |

**Table 3.** Thermal cycling conditions used in the PCR reaction for species identification of buffalo milk

| PCR program Temp (°C) |    |            |
|-----------------------|----|------------|
|                       |    | Time (min) |
| Initial denaturation  | 95 | 3 (min)    |
| Denaturation          | 95 | 15 (sec)   |
| Annealing             | 64 | 15 (sec)   |
| Extension             | 72 | 15 (sec)   |
| Number of Cycles      | 35 |            |
| Final extension       | 72 | 5 (min)    |

**Specificity and sensitivity assessment:** To evaluate the specificity of the PCR assay, DNA extracted from different animal species, including cow, buffalo, goat, and sheep, was subjected to PCR under identical conditions. Specificity was confirmed by the presence of amplifica-

tion products exclusively in the target species. Sensitivity was determined using serial dilutions of template DNA to establish the lowest detectable DNA concentration. The limit of detection (LOD) was defined as the minimum DNA amount that produced a clearly visible amplification band on the agarose gel.

**Method validation:** Validation of the PCR method was performed by assessing key performance parameters, including specificity, sensitivity, repeatability, and robustness. Repeatability was evaluated by performing the PCR assays in triplicate on different days under the same experimental conditions. To evaluate the robustness of the developed PCR method, small but deliberate variations were introduced in the experimental parameters. Specifically, the annealing temperature was varied by  $\pm 1$  °C and  $\pm 2$  °C (57 °C and 61 °C, relative to the optimized 59 °C), and the primer concentration was tested at  $\pm 10\%$  of the standard protocol. Each variation was performed in triplicate to ensure consistency.

**Data analysis:** PCR results were evaluated qualitatively based on the presence or absence of species specific amplification bands. Gel images were analyzed using ImageJ software, and the results were reported descriptively.

## Results

### Assessment of the Quality of Extracted DNA:

Genomic DNA of appropriate quality was successfully extracted from all cow and buffalo milk samples. Spectrophotometric analysis showed that the A260/A280 absorbance ratios ranged between 1.8 and 2.0, indicating good purity of the extracted DNA. In addition, electrophoretic analysis on 1% agarose gel confirmed the presence of clear and intact DNA bands without noticeable degradation.

To evaluate the quality and amplifiability of the extracted DNA, PCR amplification was performed using 18S primers. The results of the PCR amplification showed that the expected fragment was successfully amplified in all samples. The presence of clear and distinct bands indicated that the extracted DNA was of sufficient quality and suitable for subsequent species specific PCR analyses.

### PCR Amplification Results for Identification of Milk Species Origin:

The PCR amplification results demonstrated that the use of species specific primers successfully generated fragments of the expected sizes for each species. In samples corresponding to cow milk, a clear amplification band of 118 bp was observed, whereas buffalo milk samples produced a distinct band of 142 bp. The amplified bands

showed high clarity and adequate intensity, indicating the efficiency and optimal performance of the PCR conditions. Moreover, the absence of non specific bands or unintended amplification products confirms the high specificity of the primers and the proper optimization of the PCR reaction conditions used in this study.

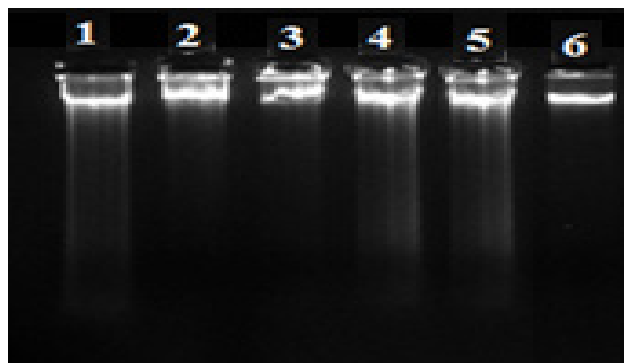


**Fig 1.** Electrophoresis of DNA extracted from cow and buffalo milk samples on 1% agarose gel. Samples loaded in lanes 1–3 correspond to DNA extracted from cow milk, while samples in lanes 4–6 correspond to DNA extracted from buffalo milk.

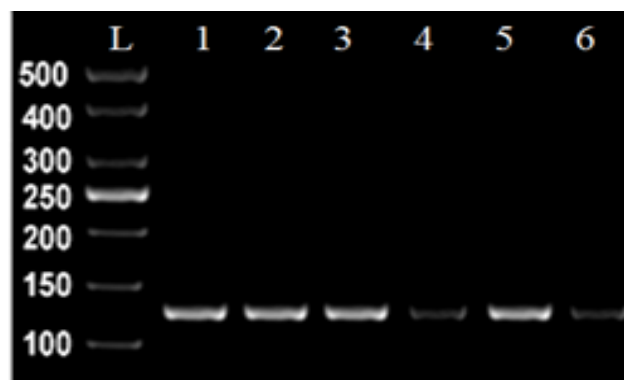


**Fig 1.** PCR amplification results of DNA extracted from milk samples using 18S primers. PCR products were visualized on agarose gel, indicating successful amplification of the target fragment. Lanes 1–3 correspond to DNA extracted from cow milk, while lanes 4–6 correspond to DNA extracted from buffalo milk.

### Specificity of the Designed Primers:

The specificity of the designed primers was evaluated using DNA extracted from milk of different animal species, including cow, buffalo, goat, and sheep. PCR amplification results demonstrated that each primer pair produced a DNA fragment of the expected size only in the target species, while no amplification bands were observed in the non target species.

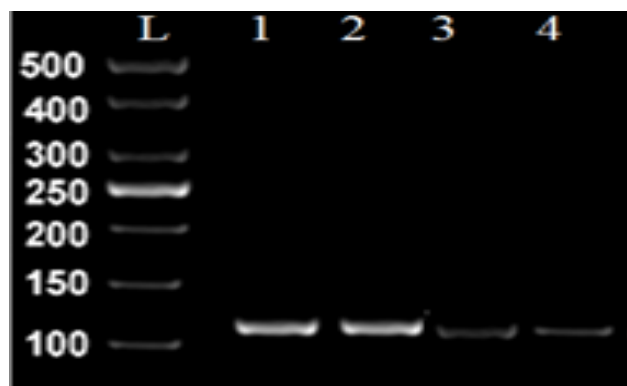


**Fig 3.** Agarose gel electrophoresis (1.5%) of PCR amplification products obtained from cow milk samples.

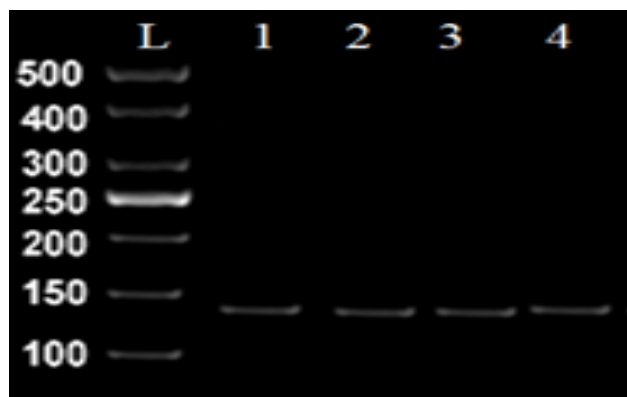


**Fig 4.** Agarose gel electrophoresis (1.5%) of PCR amplification products obtained from buffalo milk samples.

The absence of amplification products in non target species indicates the high specificity of the designed primers and confirms their suitability for accurate identification of the species origin of cow and buffalo milk under laboratory conditions.

#### Evaluation of Method Sensitivity and Limit of Detection:

To determine the sensitivity of the developed method, serial dilutions of DNA extracted from cow and buffalo milk samples were analyzed. The PCR results demonstrated that the method was capable of detecting the target species DNA at low template concentrations. The limit of detection (LOD) was determined to be 0.1 ng of template DNA, which was defined as the lowest DNA concentration that produced a clearly visible amplification band on agarose gel electrophoresis. These findings indicate that the developed PCR method exhibits adequate sensitivity for application in milk authentication and species identification studies.

#### Repeatability of the PCR Method:

To evaluate the repeatability of the method, PCR assays were performed in three independent replicates on different days. The results obtained from all replicates were consistent in terms of the presence of species specific bands and the relative intensity of the amplified products, with no noticeable variation among the experiments. These findings demonstrate the good repeatability and reliability of the developed PCR method under laboratory conditions.

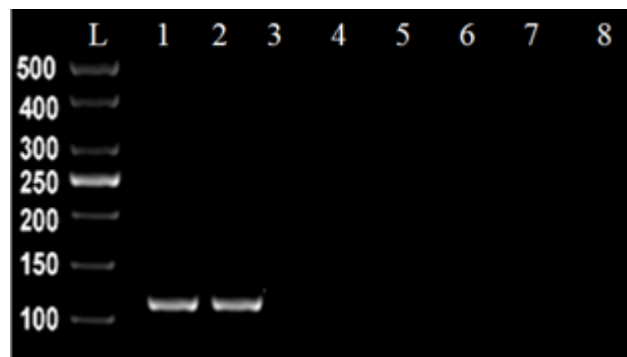


**Fig 5.** PCR specificity analysis of cow specific primers using DNA extracted from different livestock species, including buffalo, goat, and sheep. Lanes 1 and 2 correspond to cow milk samples, lanes 3 and 4 to buffalo milk samples, lanes 5 and 6 to goat milk samples, and lanes 7 and 8 to sheep milk samples.

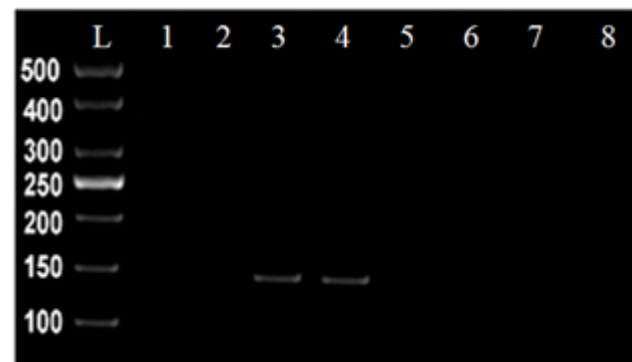


**Fig 6.** PCR specificity analysis of buffalo specific primers using DNA extracted from different livestock species, including cow, goat, and sheep. Lanes 1 and 2 correspond to cow milk samples, lanes 3 and 4 to buffalo milk samples, lanes 5 and 6 to goat milk samples, and lanes 7 and 8 to sheep milk samples.

#### Robustness of the PCR Method:

To evaluate the robustness of the developed method, minor variations were introduced into selected PCR parameters, including the primer annealing temperature and the concentration of template DNA. The results indicated that

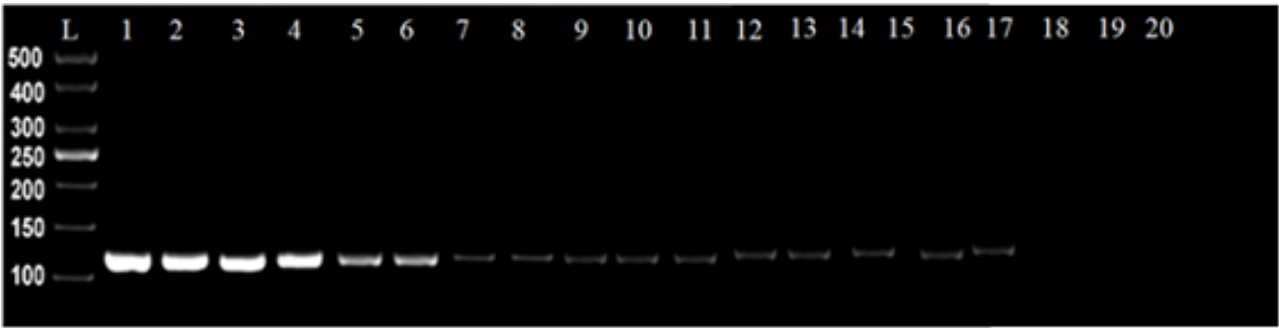


**Fig 7.** PCR results of serial dilutions of cow DNA used to determine the limit of detection of the method.

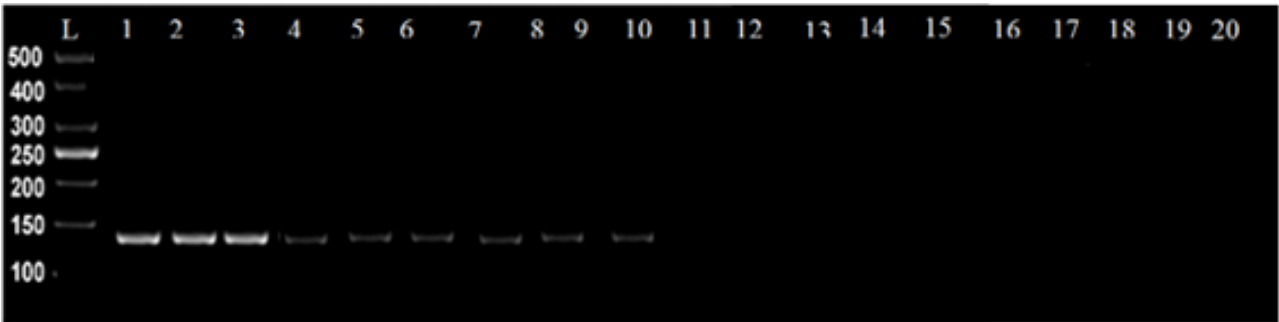


**Fig 8.** PCR results of serial dilutions of buffalo DNA used to determine the limit of detection of the method.

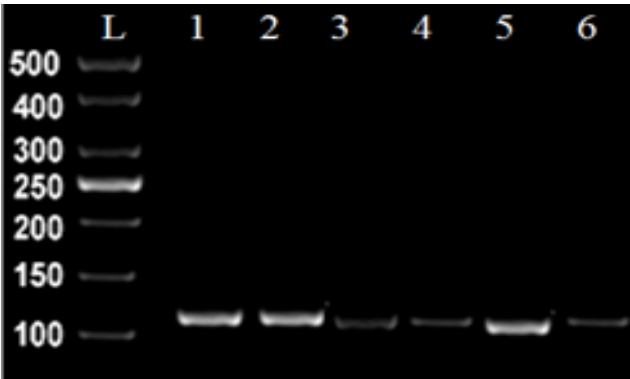


**Fig 9.** Repeatability of the PCR assay for cow DNA in three independent replicates.

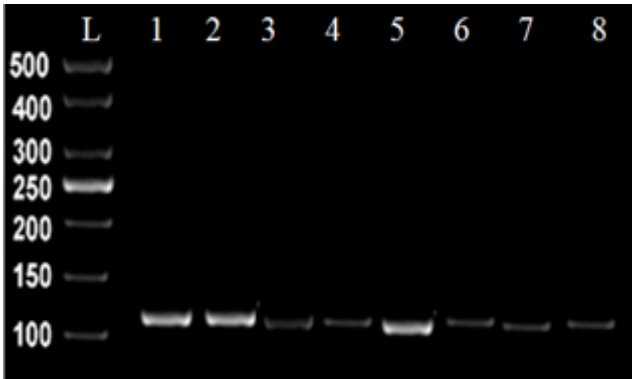


**Fig 11.** Evaluation of the effect of minor variations in PCR conditions on amplification efficiency and specificity of the method.

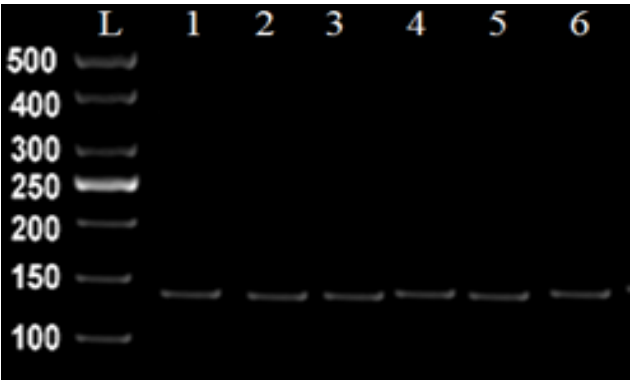


**Fig 10.** Repeatability of the PCR assay for buffalo DNA in three independent replicates.

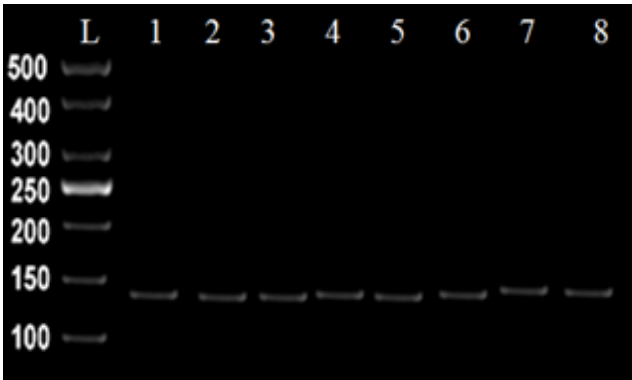


**Fig 12.** Evaluation of the effect of minor variations in PCR conditions on amplification efficiency and specificity of the method.

**Table 4.** Robustness testing of the PCR assay under varying experimental conditions.

| Parameter Variation                                                                                                      | Detection Success  | Rate (%)   | P-value* |
|--------------------------------------------------------------------------------------------------------------------------|--------------------|------------|----------|
| Annealing Temp.                                                                                                          | 58 °C (-1 °C)      | 100% (3/3) | > 0.05   |
|                                                                                                                          | 59 °C (Optimal)    | 100% (3/3) | -        |
|                                                                                                                          | 60 °C (+1 °C)      | 100% (3/3) | > 0.05   |
| Primer Conc.                                                                                                             | 0.45 µM (-10%)     | 100% (3/3) | > 0.05   |
|                                                                                                                          | 0.50 µM (Standard) | 100% (3/3) | -        |
|                                                                                                                          | 0.55 µM (+10%)     | 100% (3/3) | > 0.05   |
| *P-values were calculated using One-way ANOVA to compare band intensities; P > 0.05 indicates no significant difference. |                    |            |          |

these variations did not significantly affect the specificity or efficiency of the amplification, and the expected species specific bands remained clearly detectable on agarose gels. These findings demonstrate that the developed PCR method exhibits good robustness and maintains reliable performance under minor variations in experimental conditions.

Statistical analysis using One-way ANOVA (or Student's t-test) demonstrated that slight variations in annealing temperature and primer concentration had no significant impact on the PCR performance or the clarity of the target bands ( $P > 0.05$ ). These results confirm the high robustness of the method for species identification in different laboratory settings.

## Discussion

The present study developed and validated a conventional PCR-based method for species identification in cow and buffalo milk, with emphasis on analytical specificity, DNA suitability, and practical applicability for dairy authenticity control. The findings demonstrate that the proposed assay is capable of reliably discriminating between bovine and buffalo milk through species-targeted amplification, while also confirming DNA extract suitability through complementary quality-control steps, including spectrophotometric assessment, agarose gel evaluation, and amplification with universal 18S markers. Taken together, these results support the suitability of PCR-based species authentication as a robust molecular tool for routine milk origin verification, especially in contexts where economic adulteration, mislabeling, and species substitution can compromise both consumer trust and regulatory compliance.

Milk from different animal species differs significantly in nutritional composition, technological properties, and economic value. Buffalo milk, for example, typically possesses higher fat, protein, total solids, and certain bio-

active fractions than cow milk, which directly influences its industrial value and consumer perception. These differences also create incentives for substitution or undeclared blending, particularly in premium dairy products and regional specialty foods [1,2]. In this context, analytical methods that can distinguish the species origin of raw milk with high confidence are essential not only for fraud detection but also for traceability systems, quality assurance programs, and label verification. Over the last decade, food authenticity research has increasingly emphasized molecular methods because DNA targets offer greater taxonomic specificity and often remain detectable even when compositional parameters are variable or processing history complicates physicochemical assessment [3,4].

The rationale for selecting PCR as the analytical platform in the present study is strongly supported by the broader food authenticity literature. Compared with protein-based techniques such as electrophoretic or immunological assays, DNA-based methods generally provide better specificity at the species level and are less affected by physiological, seasonal, dietary, and lactational variation [5,6]. In dairy matrices in particular, the performance of protein-based markers can be influenced by protein denaturation, proteolysis, heat treatment, and matrix complexity, whereas appropriately designed PCR assays can still detect species-related DNA targets at low concentration if DNA extraction and amplification conditions are optimized [7]. Conventional PCR also remains highly attractive for laboratories that require a cost-effective, accessible, and transferable method without dependence on expensive instrumentation such as real-time PCR systems or sequencing platforms. Thus, while more advanced molecular technologies continue to expand, a well-validated conventional PCR assay remains highly relevant for routine authenticity screening in many regulatory and academic settings [8,9].

One important aspect of the present work is the successful extraction of amplifiable DNA from milk samples. Milk is widely recognized as a challenging matrix for nucleic acid analysis because it contains relatively low numbers of somatic cells and can include inhibitory substances such as fat, calcium, proteins, and residual enzymes that interfere with PCR performance [10]. Moreover, DNA yield in milk is frequently lower than in solid tissues, and extraction efficiency may vary depending on species, fat content, storage conditions, and centrifugation strategy [11]. In the current study, the extraction protocol generated DNA of adequate purity and integrity, as indicated by spectrophotometric analysis and agarose gel electrophoresis. Importantly, amplification with universal 18S primers further demonstrated that the extracted DNA was not merely present, but also suitable for downstream PCR. This additional verification step is scientifically important because NanoDrop values alone do not fully predict amplifiability; DNA may appear quantitatively acceptable while still being degraded or co-extracted with inhibitors [12]. Therefore, the integration of spectrophotometric, electrophoretic, and PCR-based quality assessment increases confidence that negative results in species-specific PCR are true negatives rather than artifacts of poor template quality.

The species-specific amplification patterns obtained in this study indicate that the selected primers effectively distinguished cow and buffalo DNA. Such discrimination is especially important in bovine–buffalo systems because these milks are commercially related, are sometimes co-distributed within the same supply chains, and may be difficult to differentiate by gross physicochemical properties alone, particularly in raw liquid matrices. The success of the assay is likely attributable to careful primer design, with attention to species specificity, melting temperature, and avoidance of secondary structures. Previous studies have similarly shown that mitochondrial targets are particularly advantageous for species authentication because mitochondrial DNA is present in multiple copies per cell and often yields better sensitivity than nuclear targets in low-template foods [13,14]. Although the exact analytical target selection may differ among studies, the broader conclusion is consistent across the literature: well-designed PCR primers can reliably discriminate closely related food species when sequence regions with sufficient interspecies divergence are selected [15].

The observed analytical specificity of the assay is one of its most significant strengths. In milk authentication, specificity is critical because false-positive amplification from non-target species can undermine legal defensibility and practical utility. The inclusion of non-target species, such as goat and sheep, in specificity assessment provides

a meaningful challenge to the assay and strengthens the argument that the observed amplification is species-restricted. This is especially relevant in mixed dairy production regions where cross-species handling can occur and where analytical methods must distinguish not just one target from one non-target, but one target from multiple plausible alternative species [16]. Recent methodological reviews in food authentication have repeatedly emphasized that specificity testing should include phylogenetically related and commercially relevant non-target species, rather than relying only on target-template amplification [17]. The present findings align with that principle and suggest that the assay has practical potential for routine use in surveillance or quality control programs. Sensitivity is another central criterion for evaluating authenticity assays, particularly when the analytical goal includes detection of low-level adulteration. Although the present study was designed primarily around species identification rather than quantitative adulteration measurement, the successful amplification across diluted DNA templates indicates that the method is capable of detecting target DNA at relatively low concentrations. This feature is important because economically motivated adulteration may involve partial substitution rather than complete replacement [18]. In dairy fraud investigations, methods with insufficient sensitivity may fail to detect low-percentage addition of a cheaper milk source, particularly when the target matrix is diluted, stored, or subjected to handling conditions that reduce DNA quality. Numerous studies published over the last decade have confirmed that PCR-based assays, especially those targeting mitochondrial loci, can provide analytically useful sensitivity for identifying species admixture in milk and dairy products [19,20]. Although real-time PCR and digital PCR can outperform conventional PCR in quantification and trace-level detection, conventional endpoint PCR still offers valuable sensitivity for screening purposes when assay conditions are properly optimized [21]. The use of universal 18S PCR as a quality-control measure deserves further discussion because it addresses a recurrent but sometimes underappreciated issue in food DNA analysis: the distinction between extraction success and amplification suitability. Food matrices can yield DNA that appears measurable yet remains partially degraded or inhibitor-rich. In such cases, failure of species-specific PCR may be misinterpreted as absence of the target species. By showing that DNA from milk samples was amplifiable with a conserved eukaryotic target, the study adds an internal layer of analytical confidence. Similar strategies have been recommended in recent authenticity and traceability studies, where endogenous controls or universal primers are used to minimize false-negative

interpretation and strengthen method validation [22,23]. In practical laboratory workflows, such controls are particularly valuable when analyzing raw biological fluids, processed dairy products, or archived samples in which DNA quality may be heterogeneous.

Another important implication of the present study is its relevance to routine food authenticity laboratories that may not have access to advanced molecular infrastructure. During the last decade, there has been major progress in qPCR, high-resolution melting analysis, next-generation sequencing, LAMP, biosensors, and digital PCR for food species identification [24–27]. These technologies offer notable advantages, including higher analytical sensitivity, multiplexing capacity, rapidity, or quantitative resolution. However, they also require more specialized equipment, method standardization, or cost investment. In contrast, conventional PCR remains a practical option for many laboratories, particularly in resource-constrained settings or in contexts where the primary objective is binary species authentication rather than absolute quantification. The present assay therefore occupies an important methodological niche: it is sufficiently robust for species discrimination, yet simple enough to support broader implementation in routine food control.

The findings also have implications for the regulatory and commercial management of milk labeling. Accurate identification of species origin is important for at least four reasons. First, it protects consumers against economic fraud. Second, it supports fair trade for producers of higher-value milk types. Third, it contributes to traceability and certification systems, particularly for geographically distinctive or premium dairy products. Fourth, it may be relevant for specific nutritional or allergenic considerations, since different milk species vary in protein composition and digestibility profiles [28,29]. Although the current study focused on raw milk, the assay framework could potentially be extended to fresh dairy derivatives, provided that DNA extraction is adapted to matrix complexity. This point is particularly relevant given that adulteration often occurs not only at the raw milk level but also in value-added products where species identity contributes strongly to market price [30].

From a methodological perspective, the repeatability and robustness considerations in this study enhance confidence in assay reproducibility. In analytical authentication, reproducibility across runs, operators, and small variations in reaction conditions is essential if a method is to move beyond proof-of-concept and toward routine application. The study's validation approach—evaluating specificity, sensitivity, repeatability, and robustness—is in line with current expectations for applied molecular methods in food control [31]. Robustness testing is espe-

cially useful because PCR performance can be affected by seemingly minor changes in annealing temperature, DNA template amount, reagent quality, or thermal cycler performance. A method that maintains correct species assignment under modest variation is more likely to be transferable across laboratories.

Despite its strengths, the present study also has limitations that should be acknowledged. First, the work focused on raw milk samples, and therefore the findings should not be generalized without caution to highly processed dairy matrices such as cheese powders, fermented products, sterilized milk, or products subjected to severe thermal treatment. Heat processing, fermentation, drying, and prolonged storage can fragment DNA and reduce amplifiable target length, thereby affecting assay performance [32,33]. Second, the use of conventional PCR provides qualitative identification but not accurate quantification of species proportions in mixtures. For regulatory situations requiring threshold determination or precise measurement of adulteration levels, real-time PCR or digital PCR may be more appropriate [34,35]. Third, although the specificity panel included relevant non-target species, future studies could expand the testing set to include additional dairy or non-dairy species encountered in broader food supply chains. Finally, interlaboratory validation would be an important next step before formal adoption in standard testing frameworks.

Future research can build on the present findings in several directions. One logical extension is the development of a multiplex PCR format that simultaneously amplifies cow, buffalo, and an internal control within a single reaction, thereby improving throughput and reducing reagent consumption. Another valuable direction would be validation in commercial dairy products, including mixed milks, cheeses, yogurts, and heat-treated products, to determine the real-world analytical window of the method [36]. Comparative evaluation against qPCR, HRM, or digital PCR could also help clarify the trade-offs between cost, sensitivity, quantifiability, and operational simplicity. In addition, the integration of standardized reference materials and blind-coded sample trials would strengthen evidence for regulatory readiness. Given the increasing global emphasis on food authenticity, fraud prevention, and traceability, such developments would be timely and highly relevant.

Overall, the present study demonstrates that a PCR-based approach can provide a scientifically sound and operationally feasible method for distinguishing cow and buffalo milk. The combination of optimized DNA extraction, careful species-specific primer design, complementary DNA quality assessment with universal 18S amplification, and analytical validation through specificity and sen-

sitivity testing resulted in a coherent framework for milk species authentication. In the broader context of dairy fraud control, these findings reinforce the continuing value of molecular diagnostics as reliable tools for verifying species origin. While advanced molecular platforms are increasingly available, conventional PCR remains a powerful and accessible approach when developed and validated rigorously. The method described here therefore represents a practical contribution to authenticity testing and offers a useful basis for future expansion into processed products, mixture analysis, and routine regulatory screening.

## Conclusion

The present study demonstrated that the PCR-based method developed for the species-specific identification of cow and buffalo milk provides a reliable, sensitive, and specific approach for dairy authenticity assessment. Successful extraction of amplifiable DNA from milk samples, confirmed by spectrophotometric analysis, agarose gel electrophoresis, and 18S rRNA PCR, indicated that the applied extraction protocol was suitable for downstream molecular analysis. The designed species-specific primers showed high discriminatory power, generating clear amplification patterns for the target species without cross-reactivity with non-target species.

The method also exhibited satisfactory analytical performance in terms of specificity, sensitivity, and repeatability, suggesting its suitability for routine laboratory application in milk authentication and quality control. Given its relative simplicity, cost-effectiveness, and reproducibility, the proposed assay can serve as a practical tool for the detection of species origin in raw milk and may contribute to the prevention of species-related adulteration and mislabeling in dairy products.

Nevertheless, further studies are recommended to evaluate the applicability of this method in processed dairy matrices and mixed-species samples, as well as to compare its performance with more advanced quantitative molecular approaches such as real-time PCR and digital PCR. Overall, the findings of this study support the potential of conventional PCR as an effective platform for species authentication of cow and buffalo milk in food control laboratories.

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## Declarations

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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