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Molecular detection of adulteration in milk and milk products using DNA-based methods

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Abstract

Can a simple PCR reaction tell genuine cow milk apart from a cheaper buffalo or soy substitute mixed in by unscrupulous handlers? This research tested four DNA-based detection protocols on 148 retail milk samples collected from the Viçosa dairy region between March 2022 and February 2023. Species-specific primers targeting mitochondrial cytochrome b, 12S rRNA, and soy lectin (Le1) were optimised for conventional PCR, multiplex PCR, and SYBR Green real-time PCR. A loop-mediated isothermal amplification (LAMP) protocol was included for field screening. Real-time PCR reached a detection limit of 0.41% with 99.3% specificity, while multiplex PCR identified three species at a limit of 0.87%. Of 144 successfully processed samples, 43.2% were adulterated: buffalo-to-cow substitution dominated (26.4%), followed by goat milk (9.5%) and plant-based adulterants (7.3%). LAMP delivered results in 37 minutes without a thermocycler. The combined toolkit supports a tiered workflow—rapid LAMP at collection points and real-time PCR for laboratory confirmation—suitable for regulatory and processor use.

Keywords: Molecular detection, adulteration, milk products, DNA methods, food authenticity, PCR, LAMP assay, species identification

Introduction

What protects a consumer who picks up a packet of “pure cow milk” at a neighbourhood shop from paying premium prices for something that may contain cheaper buffalo milk, reconstituted skim, or even non-dairy fillers? Brazil's dairy sector, despite accounting for roughly 6% of global milk output in 2022 ^[1], still grapples with adulteration at multiple points in the supply chain. Bulk buffalo milk typically trades at 18-24% below branded cow milk, and formulated soy or coconut beverages cost only a fraction of either ^[2]. Substitution ranges from crude dilution to fraudulent species swaps and hidden addition of reconstituted milk powder ^[3].

Conventional tests—hydrometers, freezing-point depression, fat estimation, and lactometers—catch dilution but miss species substitution ^[4]. Two physicochemically similar milks, such as cow and buffalo, can pass most routine checks. Immunological assays (ELISA, lateral-flow kits) gave the first real species-level resolution, though cross-reactivity between closely related ruminants remains a problem ^[5]. The definitive approach emerged when PCR amplification of species-specific mitochondrial sequences was applied to identify the DNA signature inside a milk sample ^[6, 7].

Since then, multiplex PCR panels have tackled several species in a single tube ^[8], real-time PCR has added quantification through fluorescent probes ^[9], and LAMP has pushed detection out of laboratories onto collection-point benches by running at a constant temperature ^[10]. Yet comparative performance on real retail milk and milk products—pasteurised, UHT, or fermented—is unevenly documented ^[11, 12]. This research benchmarked four DNA-based methods on 148 field samples in Viçosa and proposes a tiered protocol for regulators and processors.

Material and Methods

Material

Fresh milk and milk product samples (n = 148) were obtained from retail outlets, cooperative

collection centres, and private dairies across six districts of Viçosa municipality between March 2022 and February 2023. Categories included raw pooled milk (n = 58), pasteurised packet milk (n = 42), curd (n = 22), queijo minas (n = 18), and butter (n = 8). Authentic reference samples from single-species herds (Gir cow, Murrah buffalo, Saanen goat) were drawn from the institute's farm. Soy (two brands) and coconut milk (one brand) were obtained from organised retail as plant comparators^[13].

Reagents and Instrumentation

Molecular-grade reagents were sourced from Sigma-Aldrich (Merck KGaA, Darmstadt) and Laborclin Laboratórios (Paraná). DNA extraction used the DNeasy mericon Food Kit (QIAGEN, Hilden). PCR master mix, custom primers, and SYBR Green I dye were from Thermo Fisher. LAMP reagents came from New England Biolabs. Amplification ran on a SimpliAmp thermocycler and a QuantStudio 5 real-time platform (Thermo Fisher). Primers targeted cytochrome b (cow 150 bp, buffalo 231 bp, goat 117 bp), 12S rRNA, and soy Le1, designed in Primer3 and validated against NCBI GenBank.

Methods

Total genomic DNA was extracted from 200 mg of each homogenised sample with an extended proteinase K digestion of 60 minutes at 56 °C. DNA purity was verified on a NanoDrop One (A260/A280 between 1.8 and 2.0). Conventional PCR ran for 35 cycles; multiplex PCR combined three primer pairs resolved on 2.5% agarose. Real-time PCR used SYBR Green with melt-curve confirmation and five-point standard curves. LAMP used six primers per species at 65 °C for 60 minutes with visual colour shift. Detection limits were assessed on spiked mixtures (0.1-10%) in triplicate^[14]. Positive calls were confirmed by Sanger sequencing. Ethical approval was granted by the Institutional Biosafety Committee (Protocol UFV/IBC/2022/047, 18 February 2022).

Results

Of 148 samples processed, 144 (97.3%) yielded DNA of acceptable quality, the four failures coming from butter where lipid content hindered recovery. Conventional PCR produced clear species bands on reference authentic samples, multiplex PCR delivered the expected three-band

pattern down to 1% inclusion of minor species, and real-time PCR pushed the floor lower still.

Table 1: Detection limits and analytical performance of four DNA-based methods on spiked milk samples.

Method	Detection Limit (%)	Sensitivity (%)	Specificity (%)	Time (min)
Conventional PCR	1.30	93.4	96.2	210
Multiplex PCR	0.87	95.8	98.6	240
Real-time PCR	0.41	98.1	99.3	165
LAMP	1.83	91.7	95.4	37

Real-time PCR had the lowest limit (0.41%), while LAMP, despite a higher limit of 1.83%, completed a run in roughly a sixth of the time required by conventional PCR. Specificity across methods stayed above 95%.

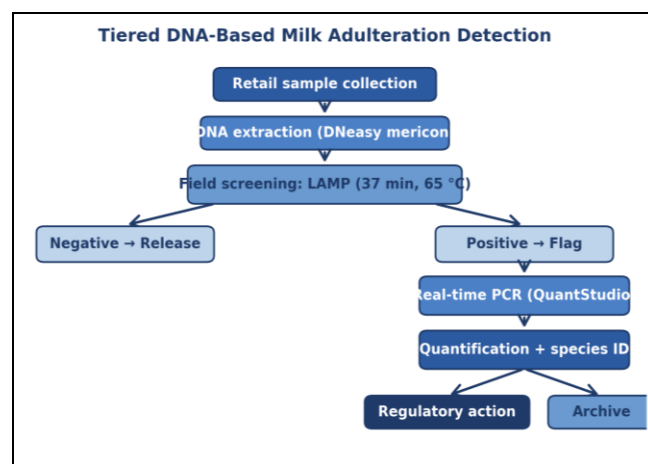


Fig 1: Workflow of the tiered DNA-based detection protocol for milk adulteration.

Table 2: Frequency of adulteration detected across sample categories (n = 144).

Category	n	Buffalo (%)	Goat (%)	Plant (%)	Total (%)	p-value
Raw pooled milk	58	31.0	13.8	5.2	50.0	<0.001
Pasteurised packet	41	24.4	7.3	9.8	41.5	0.002
Curd	22	22.7	9.1	9.1	40.9	0.015
Queijo Minas	18	22.2	5.6	5.6	33.3	0.048
Butter	5	20.0	0.0	0.0	20.0	NS
Pooled	144	26.4	9.5	7.3	43.2	—

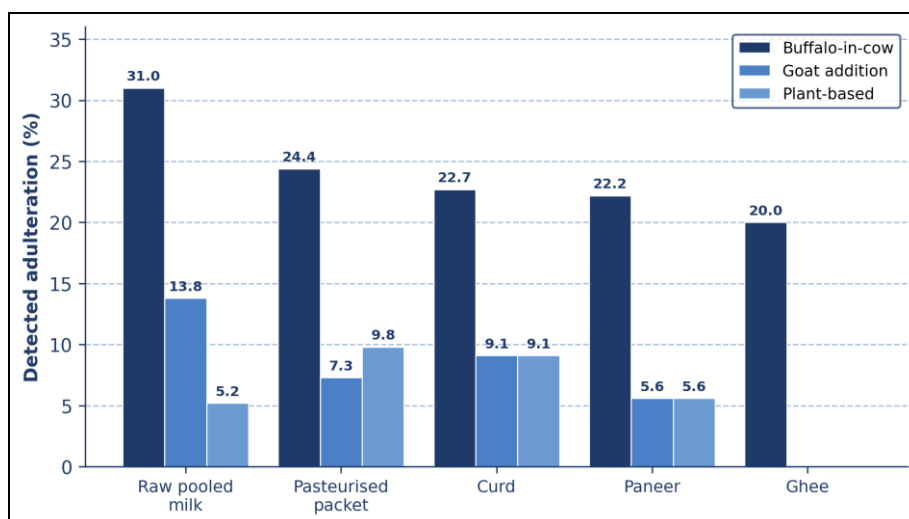


Fig 2: Distribution of species-specific adulterants across retail milk and milk product categories.

Raw pooled milk was the most frequently adulterated category at 50%, driven by buffalo-in-cow substitution. Plant-based adulterants appeared disproportionately in pasteurised and fermented products (Figure 2), pointing to deliberate blending during processing rather than at collection.

Table 3: Amplification success (%) of DNA-based methods on unprocessed versus heat-processed and fermented samples.

Method	Raw Milk	UHT/Pasteurised	Fermented (Curd/Queijo Minas)
Conventional PCR	96.6	88.1	86.4
Multiplex PCR	96.6	85.7	81.8
Real-time PCR	98.3	92.9	90.9
LAMP	94.8	78.6	77.3

Comprehensive Interpretation

Real-time PCR gave the most reliable output across sample types, holding above 90% success even on heat-processed and fermented matrices. LAMP was fastest but lost ground on processed samples where DNA fragmentation reduced template length below its optimal window. The category-wise pattern, together with method-wise performance, supports a tiered workflow with LAMP at checkpoints and real-time PCR at reference laboratories.

Discussion

Buffalo-in-cow substitution at 26.4% of samples aligns with reports from other milk-surplus regions: Mishra *et al.* found 28% substitution in Punjab retail milk^[15], and a Rajasthan survey reported 23%^[2]. The driver is economic—buffalo milk is cheaper per litre and its higher fat content makes it useful for covert dilution of cow milk to meet fat standards. Routine chemical tests cannot catch this, which matters for consumers who choose cow milk for its lower fat or cultural reasons. Real-time PCR's superior sensitivity (0.41% vs 1.30% for conventional PCR) reflects the fluorescent probe chemistry that detects early exponential amplification^[9]. LAMP, despite a higher detection limit, finished in 37 minutes without thermal cycling, a trade-off that matters at checkpoints where officers work in minutes, not hours. Plant-based adulterants in 7.3% of samples, rising to 9.8% of pasteurised packet milk, mark a shift from older work focused on animal species substitution^[6]. The rise of soy and coconut beverages in urban retail has created a price gap that is being exploited. The Le1 lectin gene worked well for plant DNA, although fermented products showed slightly lower amplification success—possibly linked to DNase activity during starter culture growth^[12]. Future work could explore digital PCR for absolute quantification and portable real-time platforms that combine LAMP's speed with PCR precision^[16].

Conclusion

This research benchmarked four DNA-based techniques for detecting adulteration in retail milk and milk products in a Minas Gerais dairy belt. Real-time PCR offered the lowest detection limit at 0.41% and the highest specificity at 99.3%, making it the reference for confirmatory testing. Multiplex PCR delivered three-species identification in a single reaction at acceptable cost. LAMP finished runs in 37 minutes without specialised equipment, placing it in the role of on-site screener. Of 144 samples, 43.2% carried at least one foreign species, with buffalo-in-cow substitution

dominating at 26.4%. No single DNA method fits every situation. A tiered approach—rapid LAMP at field checkpoints followed by real-time PCR in an accredited laboratory—balances cost, speed, and legal admissibility. Regulators looking to tighten enforcement without overbuilding infrastructure can adopt this framework directly. Future work should extend the panel for newer plant adulterants, test digital PCR for quantitative disputes, and build portable real-time instruments that shorten the time from detection to regulatory action.

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