PLOS One

RESEARCH ARTICLE

TaqMan qPCR quantification of the *Cytb* gene for precise determination of camel milk in dairy products

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Citation: Na Q, Si R, Ming L, Yi L, He J, Jambal T, et al. (2026) TaqMan qPCR quantification of the *Cytb* gene for precise determination of camel milk in dairy products. PLoS One 21(2): e0342999. https://doi.org/10.1371/journal.pone.0342999

Editor: Tzen-Yuh Chiang, National Cheng Kung University, TAIWAN

Received: September 28, 2025

Accepted: January 29, 2026

Published: February 25, 2026

Data availability statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This study was supported by the National Key Research and Development Program (grant number 2020YFE0203300) and the Alxa League Science and Technology

Abstract

Camel milk often faces quality control problems because it is vulnerable to adulteration with inferior dairy sources or plant-derived additives. Many existing DNA-based detection methods rely on suitable endogenous reference genes. This study aimed to develop an absolute quantification strategy based on real-time quantitative polymerase chain reaction using TaqMan probes. A recombinant plasmid standard containing camel-specific *cytochrome b* (*Cytb*) genes was designed. The resulting standard curve revealed high linearity ($R^2 = 0.9982$) across six orders of magnitude, enabling precise quantification of copy numbers. The primers and probes demonstrated high specificity for camel DNA, and the plasmid standards met quality criteria (A260/280 = 1.82; concentration deviation <2%). The method achieved a sensitivity of 6.39×10^2 copies/μL. For samples containing 5%–100% of camel milk, the coefficient of variation ranged from 0.99% to 5.20%, and the recovery rates for spiked products ranged from 97.5% to 107.5%. By providing absolute quantification without requiring a reference gene, this method offers a robust solution for detecting camel milk adulteration in dairy products.

1. Introduction

Camel milk is a unique dairy product valued for its rich profile of bioactive constituents that contribute to various health-promoting effects. It contains high levels of natural antimicrobial components, such as lactoferrin, lactoperoxidase, immunoglobulins, and lysozyme [1]. Fermentation or enzymatic digestion can further release functional bioactive peptides, such as the antidiabetic peptide FFIFTCLLAVVLAK and low-molecular-weight immunomodulatory peptides capable of suppressing pro-inflammatory cytokines such as interleukin-6 and tumor necrosis factor-α [2]. Camel milk exhibits hypoallergenicity

Program (grant number AMTM2022-01). The funders had no role in study design, data collection and analysis, the decision to publish, or the preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

due to the presence of β-casein, elevated immunoglobulin levels (154 mg/100 mL), and the absence of β-lactoglobulin [3]. These immunoglobulins can mitigate allergic responses by binding Fc receptors and reducing immunoglobulin E levels. Moreover, camel milk also contributes to metabolic well-being through beneficial shifts in gut microbiota, suppressing harmful bacteria while promoting beneficial strains such as *Akkermansia muciniphila*, which has relevance to nonalcoholic fatty liver disease [4]. Notably, its glycomacropeptide effectively inhibits *Escherichia coli* adhesion at concentrations as low as 0.24–0.28 g/L, 20 times more potent than cow's milk [5].

The growing consumer demand for camel milk, driven largely by its purported nutritional value, has also heightened concerns regarding its quality and safety. According to statistics provided by the Food and Agriculture Organization, the global camel milk consumption reached 4.1 million tons in 2023 [6]. This expanding market has unfortunately incentivized economically motivated adulteration, with producers sometimes blending camel milk with lower-cost alternatives, such as cow's or goat's milk, to lower production costs and increase profit margins [7]. In contrast to plant-based additives, differentiating between milk from various animal species is considerably challenging due to minimal disparities in their color, odor, and taste [8]. Besides compromises in product quality, such practices may pose health risks to consumers by introducing allergens from other milk types [9].

The present study aimed to briefly summarize the existing qualitative and quantitative analytical approaches for detecting milk adulteration in camel milk [10–12]. Qualitative methods identify if milk from other species is present, while quantitative techniques determine the exact proportion of this milk. Immunoassay-based approaches, which target species-specific proteins, have been extensively adopted for rapid preliminary screening [13]. Furthermore, spectroscopic fingerprinting leverages characteristic compositional profiles to enable high-speed detection of camel milk adulteration [14]. In addition, highly sensitive methods such as liquid chromatography and mass spectrometry have been employed to detect trace levels of adulterant proteins from species [15]. However, these protein-based analytical techniques exhibit substantial limitations. Thermal treatments can alter protein tertiary structures and degrade antigenic epitopes [16]. This, in turn, can result in variability in target availability and reduced detection accuracy. In contrast, DNA-based identification methods offer considerable advantages as they rely on species-specific genetic sequences and benefit from the inherent stability of the DNA molecule [17]. The robust phosphate backbone of DNA, along with its capacity to undergo renaturation following heat-induced denaturation, makes DNA a more dependable target for species discrimination than proteins [18].

Conventional polymerase chain reaction (PCR) has become the predominant DNA-based technique for detecting milk adulteration because of its high sensitivity. By employing specific primers, PCR enables the exponential amplification of targeted fragments from genomic DNA. When paired with agarose gel electrophoresis, it facilitates the qualitative detection of adulterants in camel milk [19]. The advent of real-time quantitative PCR (qPCR) further enabled real-time monitoring of amplification through fluorescence signals, removing the reliance on gel electrophoresis [20–22]. In recent developments,

isothermal amplification techniques, such as loop-mediated isothermal amplification [23] and recombinase polymerase amplification [24], have emerged as a significant advancement in the field. These methods do not require thermal cyclers, while their use of multiple primers enables efficient amplification of target sequences at a constant temperature. Species identification in such methods is typically facilitated by the analysis of either single-copy nuclear genes or multi-copy mitochondrial DNA. A number of studies have used normalized PCR targeting nuclear genes to detect bovine milk adulteration in camel milk [25]. However, the low copy number of nuclear sequences, especially in processed products where DNA is often degraded, can restrict detection sensitivity. In contrast, multi-copy mitochondrial genes maintain relatively high levels even after processing, making them more suitable for sensitive detection [26]. As an example, a study employed a mitochondrial *cytochrome b* (*Cytb*) gene–based assay to detect eight potential adulterants in camel milk, achieving a detection limit as low as 0.1% [27].

In 2024, China updated its national food safety standards to specify that camel milk–based formula must contain at least 80% camel milk solids, with the remaining proportion allowed to consist of approved supplementary ingredients [28]. This policy change marks a shift in regulatory perspective: the presence of non-camel components is no longer automatically interpreted as adulteration. As a result, analytical efforts have shifted from multispecies screening to the precise quantification of camel milk content. The existing detection methods primarily depend on multispecies primer sets that target DNA of foreign species (e.g., bovine or caprine), providing only qualitative or semi-quantitative analysis. Although useful for initial screening, these assays are unsuitable for precise quantification of camel milk content. Relative quantitative PCR approaches have been explored, but their accuracy depends on several critical factors, including the stability of the selected internal reference gene, the assumption of equivalent and consistent amplification efficiencies between the target and reference genes, inter-run variation, and the complexity of constructing a standard curve. These challenges limit their ability to quantify across different samples and batches, ultimately failing to meet the new national standard requirements for precise content determination.

To overcome these limitations, this study developed an absolute quantification method using qPCR targeting a camel-specific mitochondrial *Cytb* gene sequence. The approach involved cloning a target fragment into a plasmid to generate a standard with known copy numbers. A standard curve was constructed by plotting Ct values against serial dilutions of the standard, allowing for the absolute quantification of camel-derived DNA in test samples. This method demonstrated high specificity and sensitivity, thereby supporting compliance with the updated national standard and contributing to reliable quality control of camel milk products.

2. Materials and methods

2.1. Sample collection, preparation, and DNA extraction

Fresh camel milk (*Camelus bactrianus*) and cow's milk (*Bos taurus*) were collected from a farm in the Right Banner of Alxa (China). The samples were stored at −20°C until further use. Laboratory-modeled adulteration was performed by preparing binary mixtures of camel and cow's milk containing known proportions of cow's milk (100%, 90%, 80%, 60%, 40%, 20%, and 0%) (*w*/*w*) in camel milk. All the milk samples were centrifuged at 4000*g* and 4°C to remove the fat present in the milk. The samples were freeze-dried and then stored at −80°C for 24 h to produce usable milk powder. Whole-genome DNA extraction from milk samples was performed using the Sangon Ezup food genomic DNA extraction kit (Shanghai, China), following a slightly modified version of the method described by Wu et al., 2022 [29]. Powdered milk samples (250 mg) were weighed into a 2-mL centrifuge tube. Subsequently, 1000 μL of GMO buffer solution and 20 μL of proteinase K (20 mg/mL) were added to the tube, and the sample was then incubated at 65°C for 30 min in a Yiheng DK-8AX incubator (Shanghai, China). The treated sample was then subjected to a chloroform precipitation process, followed by adsorption onto a silica gel column [29]. Finally, 50 μL of TE buffer was added to elute the silica gel column, yielding the DNA samples. Once isolation was complete, the purity and concentration of the isolated DNA were determined spectrophotometrically using the absorbance ratio A260/280 on a DS-11 spectrophotometer (DeNovix, Wilmington, USA). All isolated DNA samples were stored at −20°C until further use.

2.2. Design of species-specific primers and probes

Complete genome sequences of the camel (*C. bactrianus*: NC_009628.2: 14161–15300) were retrieved from the NCBI database. From these, a conserved region of the *Cytb* gene was selected, and species-specific primers were designed using NCBI Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast). To prevent cross-reactivity with other nontarget species, the species specificity of the selected base pairs was meticulously verified using the NCBI BLASTn suite (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed October 2024). Any sequences exhibiting potential cross-reactivity were subsequently excluded from further experimentation. For detecting cow's milk in camel milk, specific DNA probes were designed for real-time qPCR. Each probe was labeled with a 5-carboxyfluorescein (FAM) (517 nm) reporter fluorescent molecule at the 5' end and a Minor Groove Binder (MGB) at the 3' end to enable target detection. The primers and probes (Table 1) were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). The specificity of the camel *Cytb* primers and probes was assessed against the DNA obtained from bovine and caprine (goat) milk samples. These two species were selected as the primary nontarget models for specificity validation because they represent the most common and economically motivated adulterants in the Chinese dairy market. All primer and probe design, verification, and reporting were performed in strict accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines [30].

2.3. Qualitative PCR, gel resolve, and construction of standard plasmids

PCR amplification was performed in a total volume of 25 μL, comprising 2 μL of DNA extract, 4 μL of 10 × PCR buffer, unless otherwise specified, reagents were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). Then: 100 mM Tris-HCl, pH 8.8; dNTP mix (100 mM; Sangon; Cat. #B500056); TaqPlus DNA polymerase (5 U/μL; Sangon; Cat. #B600090) and each of primer (10μM) (Table 1). The remaining volume was adjusted with ddH_2O. The reactions were carried out in a SimpliAmp thermal cycler (Thermo Fisher Scientific, Waltham, USA) using the following program: an initial denaturation at 95°C for 3 min; 35 cycles at 95°C for 30 s, 57°C for 30 s and 72°C for 30 s; and a final extension at 72°C for 8 min.

The amplified fragments were analyzed by electrophoresis on a 1.5% agarose gel containing 1 × Gelred (Sangon, Cat. #A616697) for staining and prepared with 6 × loading buffer (Bomai, Guangdong, China) for about 25–30 min at 140 V. The agarose gel was visualized using an ultraviolet (UV) light Gel Doc-It 2 (Analytik Jena, Tewksbury, USA), and the digital images were obtained with Vision Works 8.20 (Analytik Jena, Tewksbury, USA).

The target electrophoretic strips were excised efficiently. The SanPrep Column DNA Gel Extraction Kit (Sangon; Cat. #B518191) was used for the recovery of the target strip gel for subsequent plasmid cloning.

The pMD18-T cloning vector (Takara Bio, Dalian, China) was used for plasmid construction. The amplification products of the plasmid and gel recovery were purified using an ultra-thin DNA product purification kit (Sangon, Cat.

Table 1. Sequences of the primer and probe.

Primer and probe	Sequence	Gene	Length (bp)	GC (%)	Tm (°C)	△*G* kcal/mol
F Primer	5' ATTCTTTGCCTTCCACTTCATC 3'	Cytb	22	40.9	62.45	−3.14
R Primer	5' GGGTTATTAGAGCCTGTTTCGT 3'		22	45.5	64.50	−3.17
Probe	5'FAM-ATTATCACGGCCCTAGTA −3'MGB		18	44.4	66.00	−1.47
Species	**Sequence**	**Gene**	**Length (bp)**			
Camel	ATTCTTTGCCTTCCACTTCATCCTGCCATTTATTATCACGGCCCTAG-TAGCCGTACACCTATTATTCCTACACGAAACAGGCTCTAATAACCC	*Cytb*	93			

https://doi.org/10.1371/journal.pone.0342999.t001

B610367) following enzymatic digestion by BamHI and EcoRI at 30°C and 37°C, respectively. The ligation of the *Cytb* gene fragment and the plasmid was then carried out using T4 DNA ligase (1KU, Sangon, Cat. #600511) at 16°C for 1 h. The cells were then transformed into DH5-α receptor cells (stored at −80°C using the glycerol broth freezing method, 20241016), and positive clones were screened using the ampicillin (Amp) plate. The LB (Amp) plate was then incubated at 37°C for 24 h, and the positive clones were picked. The recombinant plasmids were then extracted using the SanPrep Column Plasmid Mini-Preps Kit (Sangon, Cat. #B518191) and samples were sent to Sangon Biotech Co., Ltd (Shanghai, China) for Sanger sequencing. Sequencing data provided by Sangon were analyzed using standard quality-control pipelines.

2.4. Standard curves

The concentration of the recombinant plasmid was determined using a DS-11 UV micro-spectrophotometer (DeNovix, Wilmington, USA). The plasmid copy numbers were calculated using the following formula: recombinant plasmid copies (copies/μL) = 6.02E + 23 (copies/mol) × plasmid concentration (ng/μL) × 10^{-9}/ [plasmid length (bp) × 650 (Da/bp)]. The total plasmid length used for the standard was 2787 bp. After determining the copy number, six samples were prepared by serial dilution in a tenfold gradient. These samples were subjected to the real-time qPCR reaction system as follows: 2 × TaqMan qPCR mix (10 μL), forward primer and reverse primer (10 μM) (0.4 μL each), TaqMan probe (10 μM) (0.4 μL), template (2 μL), and ddH_2O (7.4 μL), in a total volume of 20 μL. The amplification reaction was performed under the following conditions: 94°C for 15 s, 57°C for 15 s, and 72°C for 15 s, for a total of 45 cycles. Three replicate wells per sample were used for qPCR amplification. The primer sequences used are shown in **Table 1**. A standard curve was constructed using the actual amplified Ct values and the corresponding *Cytb* gene copy numbers of plasmid dilutions.

2.5. Quantitative analysis of authentic samples

Total DNA was extracted from simulated camel milk adulterated with cow's milk using the method described in section 2.1. The *Cytb* gene was then amplified using the same RT-qPCR system and amplification conditions outlined in section 2.4. The *Cytb* gene copy numbers in samples with different concentrations of camel milk were calculated based on their Ct value using the standard curve. A calibration curve was established by plotting the calculated *Cytb* copies against the known mass concentrations of camel milk in the adulterated samples, achieving linear fitting. The method was then employed to determine the content of camel milk in the unknown test samples.

2.6. Statistical analysis

All relevant data were exported using LightCycler 96 software (Roche, Diagnostics GmbH, Mannheim, Germany) and subsequently analyzed using MS Excel. Standard curves were generated using GraphPad Prism versions 8.4.2. All statistical calculations [mean and standard deviation (SD)] were carried out using GraphPad Prism 8.4.2. The mean value obtained was indicative of the central tendency of the data sets. SD was calculated to determine the degree of dispersion of the data distribution and the degree of its deviation from the arithmetic mean. All experiments were conducted in triplicate ($n = 3$) for each set of primers. For the final primer and probe set, all qPCR experiments were conducted in technical triplicates ($n = 3$).

3. Results and discussion

3.1. DNA yield, purity, and integrity

In molecular analysis, the quality of DNA extraction directly influences the reliability of subsequent experiments, particularly in the context of complex matrix samples where the effective removal of PCR inhibitors is critical. Camel milk

is distinguished by its elevated protein, fat, and calcium ion concentrations. The presence of impure DNA samples has been shown to impede PCR reactions. Consequently, the commercial kit of CTAB lysis combined with silica gel column purification was used in this study to overcome the problem of nuclease activity caused by elevated somatic cell count (2×10^5–2×10^6 cells/mL) in camel milk, which was efficiently inhibited by CTAB lysate [31]. Second, the silica gel adsorption column technology selectively removed the lactolipid and casein complexes and effectively removed residual calcium ions, thus significantly improving DNA purity [29].

Quality assessment demonstrated that the extracted genomic DNA had a concentration of 42.01 ± 0.086 ng/μL, with the OD260/280 ratio of 1.96–2.03, thereby adhering to the nucleic acid purity standard for molecular experiments (1.8–2.0). Compared with the conventional phenol–chloroform technique, this method achieved a 40% higher DNA yield and reduced operation time to less than 1 h. Analysis by 1.5% agarose gel electrophoresis revealed that the genomic DNA exhibited a high-molecular-weight band with minimal smearing, thereby meeting the criteria for subsequent PCR experiments (**Fig 1**).

3.2. Specificity testing of primers and probes

Cytb gene has emerged as a predominant DNA marker for detecting adulteration in mammalian products due to its advantageous biological characteristics. These mitochondrial genes possess two key advantages: first, their high cellular copy number enhances detection sensitivity, and second, their sequence length permits the design of compact primers capable of amplifying target fragments even in partially degraded DNA samples. Notably, *Cytb* gene sequences exhibit less than

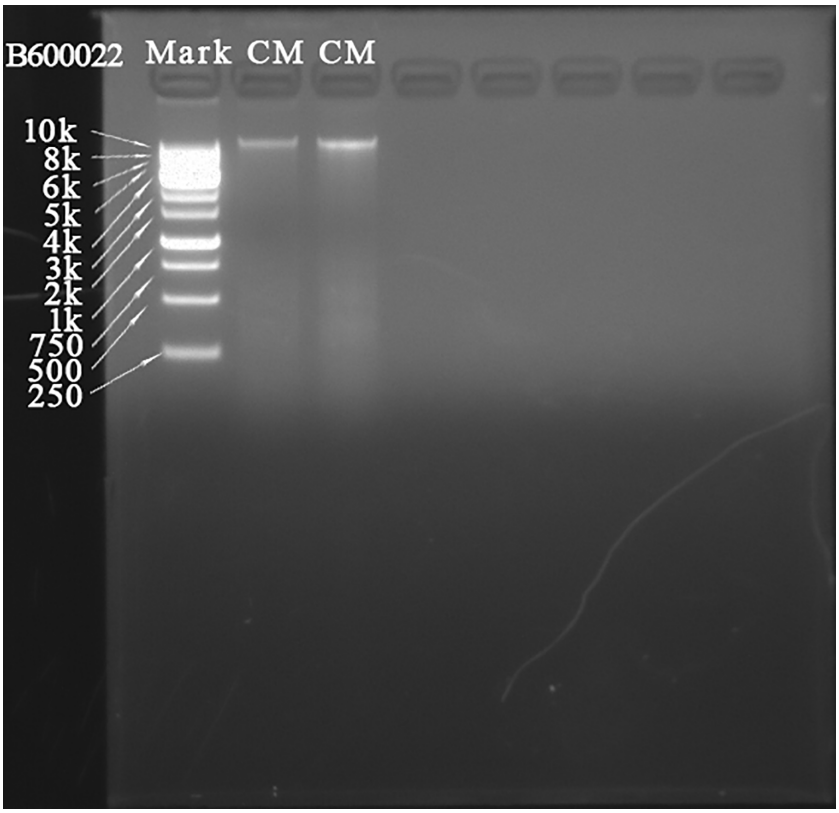

Fig 1. Electropherogram of the 1.5% agarose gel of the whole genomic DNA samples extracted from camel milk. The gel volume was 30 mL, the voltage condition was set to 150 V, the run time was 25 min, and the buffer used was 1 × TAE.

https://doi.org/10.1371/journal.pone.0342999.g001

1% intra-species variation and more than 10% inter-species divergence (Li et al., 2018), making them particularly suitable for species differentiation.

The specificity of primers and probes is a critical factor in achieving accurate PCR amplification [32]. To assess the specificity of camel *Cytb* gene primers designed in this study, cross-amplification tests were conducted using real-time RT-qPCR with DNA extracted from camel, goat, and bovine milk samples. As illustrated in **Fig 2**, distinct patterns in amplification behavior were observed. Camel milk DNA exhibited a characteristic sigmoidal amplification curve with a mean Ct value of 20.043 (±0.15) at the fluorescence threshold of 0.05. In contrast, DNA from goat and bovine milk samples displayed no specific amplification signals (Ct values >40) within 45 PCR cycles. These results demonstrated the primer set's exceptional specificity for camel *Cytb* sequences, with no detectable cross-reactivity to bovine or caprine DNA, the two most relevant potential adulterants in the initial application context of this method. However, the global camel milk trade may involve additional adulterants, including buffalo, sheep, or horse milk. Future studies should aim to validate the assay's specificity against a broader panel of species to further support its international applicability.

3.3. Gel recovery and plasmid reconstruction

As absolute qPCR quantifies a single specific target gene without relying on the reference genes [33], it necessitates the preparation of standardized camel milk–derived DNA samples with predetermined copy numbers. A commonly used approach is to amplify the target DNA fragment from the sample to construct recombinant plasmids, which serves as calibration standards due to their high stability and purity, enabling precise control over both DNA concentration and copies. In this study, the camel *Cytb* gene fragment was first specifically amplified from the DNA of camel milk. After 1.5% agarose gel electrophoresis, the amplification product produced a specific band of 93 bp, which matched with the expected fragment size of the target gene (**Fig 3**). PCR products contain impurities, such as ions, dNTPs, primers, and polymerase, which have the potential to have an adverse effect on subsequent cloning and sequencing. Gel extraction by agarose gel electrophoresis is a well-established and effective method for removing these contaminants and obtaining high-purity DNA samples [34].

Following recovery and purification of the target DNA from agarose gel, the TA cloning strategy was used to ligate the *Cytb* gene fragment into the pMD18-T vector to construct the recombinant plasmid pMD18-T@Cytb. The recombinant plasmid was transformed into *E. coli* DH5α receptor cells via heat shock transformation and blue-white screening on the LB solid medium containing ampicillin. White-positive clones were screened by α-complementary chromogenic reaction,

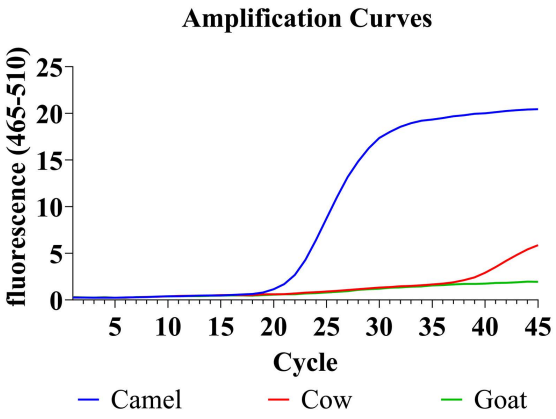

Fig 2. Fluorescence amplification curves of camel-specific primers. The *x*-axis represents the number of cycles and the *y*-axis represents the fluorescence intensity. The curve data represents the average of three assays.

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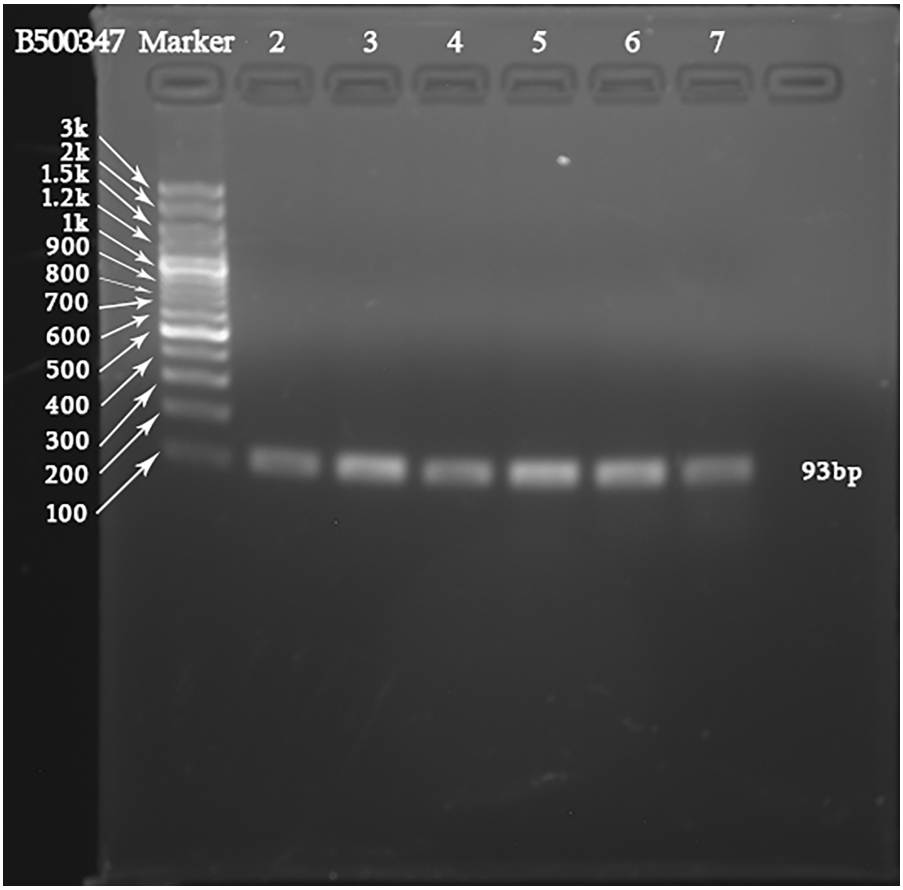

Fig 3. Gel electrophoresis of the *Cytb* gene amplified from camel milk. A 1.5% agarose gel with a 100–3000 bp marker and 1 × TAE buffer. The voltage applied during electrophoresis was 150 V. The second to seventh electrophoretic lanes are filled with *Cytb* amplification products.

https://doi.org/10.1371/journal.pone.0342999.g003

and the recombinant plasmid DNA was prepared in small amounts by alkaline lysis. According to the SnapGene software schematic (Fig 4A), the recombinant plasmid was successfully inserted with 93 bp of *Cytb* gene fragment (red marked area), and the vector backbone retained the intact polyclonal sites (EcoR I and Hind III cleavage sites). To verify the accuracy of the cloning, M13 universal primer sequencing was performed with the recombinant plasmid pMD18-T@*Cytb* and empty vector pMD18-T as positive and negative controls, respectively. The sequencing results confirmed that the total length of the recombinant plasmid was 2785 bp (vector 2692 bp + insert template 93 bp), and the inserted 93 bp sequence was analyzed by comparison and found to be identical to the reference sequence of the camel *Cytb* gene (NCBI accession no. NC_009628.2: 14161–15300) (Fig 4B).

3.4. qPCR detection of camel *Cytb* plasmid

Following sequence verification, the concentration of the recombinant plasmid was quantified using UV spectrophotometry. The purified camel *Cytb* plasmid had a concentration of 192.15 ± 1.78 ng/μL, with an A260/280 ratio of 1.82. The plasmid copy number was calculated to be 6.39×10^{10} copies/μL, using the formula described in section 2.4 (the plasmid length is required in bp for complete calculation). A six-point logarithmic dilution series from 10^0- to 10^5-fold was prepared, generating standard solutions ranging from 6.39×10^2 to 6.39×10^7copies/μL. This concentration range covered the expected detection limits for both pure camel milk and adulterated samples.

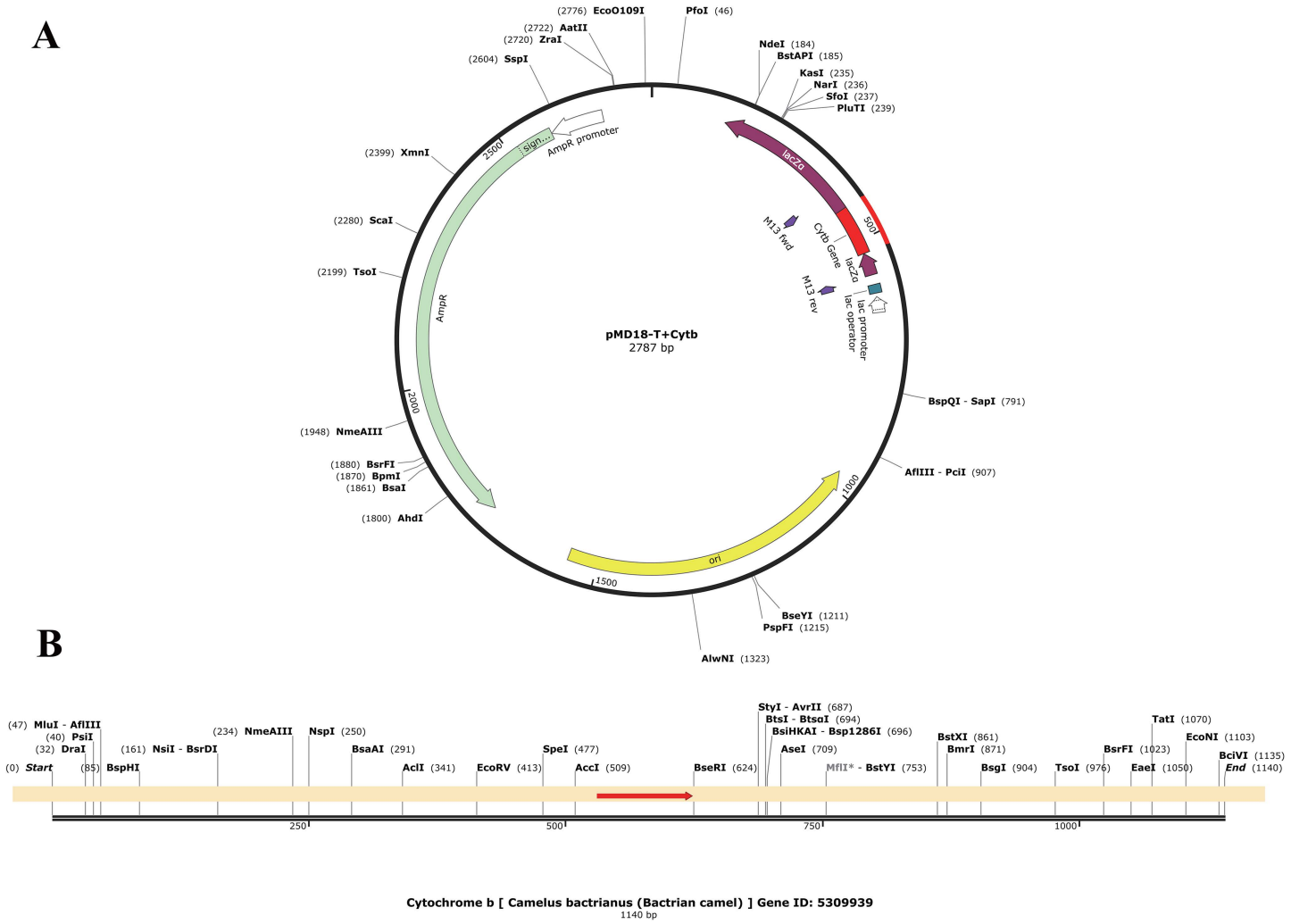

Fig 4. Schematic and verification of the camel Cytb recombinant plasmid. (A) Schematic structure of the pMD-18T+ camel *Cytb* recombinant plasmid. (B) Results of the cloned *Cytb* recombinant plasmid after sequencing compared with the NCBI camel *Cytb* gene. The red region in the circular DNA plasmid is the 93-bp camel *Cytb* gene. In the yellow bands, red arrows are 93-bp amplification products.

https://doi.org/10.1371/journal.pone.0342999.g004

The real-time qPCR amplification of the plasmid standards produced characteristic sigmoidal curves (Fig 5A). A standard curve was established by plotting cycle threshold (Ct) values (*y*-axis) against the logarithm of starting copy numbers ($\log_{10}$; *x*-axis), yielding the regression equation $y = -3.2583x + 39.385$. This model showed excellent linearity, $R^2 = 0.9982$ (Fig 5B), and the amplification efficiency was 103.6%. The presence of primer dimers in the reaction system or the existence of ionic inhibitors can theoretically lead to a significant increase in amplification efficiency [35], although the precise extent of such increases is yet to be determined. In general, real-time PCR assays with amplification efficiencies between 90% and 110% are considered acceptable [36]. Based on the lowest standard that could be quantified with acceptable precision and accuracy [37], the limit of detection and limit of quantification for this qPCR assay were determined to be 146.10 and 434.87 copies, respectively.

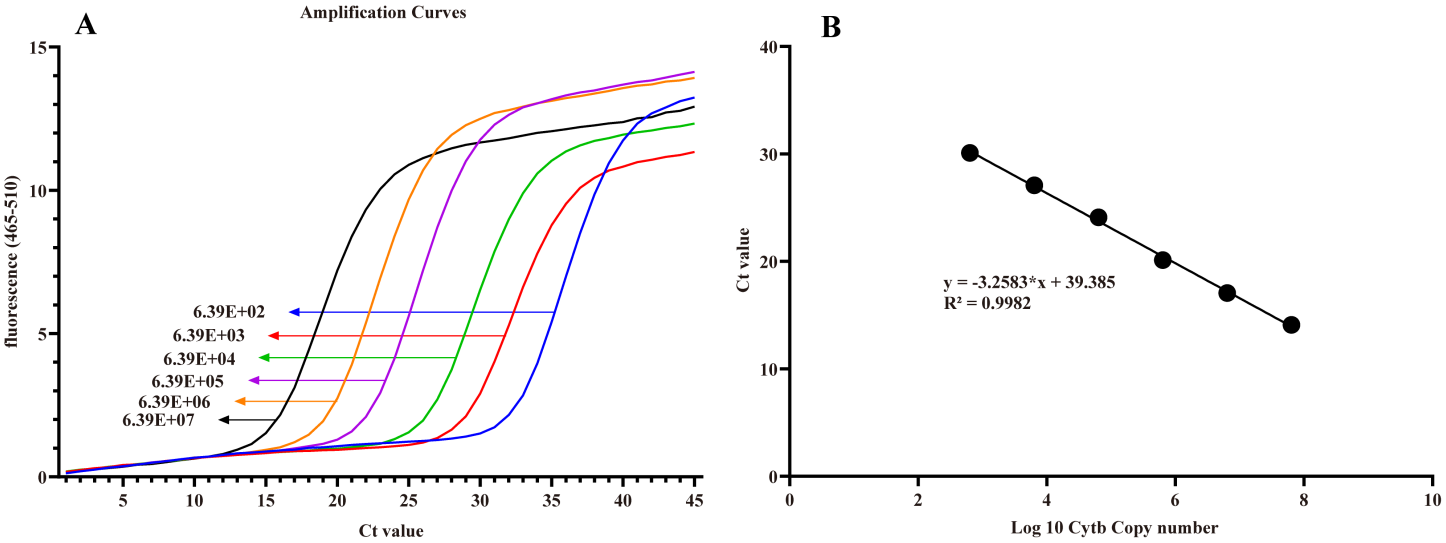

Fig 5. qPCR amplification curves of six standard samples following serial tenfold dilutions (A) and standard curve for *Cytb* recombinant plasmid (B). The *x*-axis represents the $\log_{10}$ copies of six concentrations of standards, and the *y*-axis represents the corresponding Ct values.

https://doi.org/10.1371/journal.pone.0342999.g005

3.5. Method applied to different concentrations of camel milk samples

After PCR amplification of seven artificially prepared samples with varying camel milk concentrations [100%, 80%, 60%, 40%, 20%, 10%, 5% (*w*/*w*)], the Ct values were converted to *Cytb* gene copies using the standard curve equation, as shown in Fig 6A. The 100% camel milk sample exhibited a Ct value of 20.75, corresponding to a *Cytb* gene copy number of 5.31×10^5 copies/μL. In contrast, the 5% camel milk sample exhibited a Ct value of 25.03, which corresponded to 2.53×10^4 copies/μL. The *Cytb* gene copies for the seven concentration samples were calculated using the standard curve equation $y = -3.2583x + 39.385$. A strong linear correlation was observed between the resulting *Cytb* gene copy numbers and the theoretical camel milk proportion (Fig 6B), supporting the establishment of a robust predictive model for estimating the relative content of camel milk in mixed samples. It should be noted that this model quantifies camel-derived DNA, rather than directly measuring milk mass ($R^2 = 0.998$). The observed relationship relies on the assumption that the target DNA quantity is proportional to the camel milk content in the artificially blended samples, a foundational principle underlying DNA-based quantification in food authenticity analysis.

3.6. Method validation and repeatability

The measured and actual values were critically evaluated using a PCR-based amplification technique. DNA was extracted from mixtures of camel milk powder containing varying proportions of camel milk (15%, 50%, and 90%) amalgamated with cow milk powder. The recovery rates of these mixed samples ranged from 97% to 107%, with CV values consistently less than the acceptable range of 5.2% (Table 2). This outcome validates the efficacy of the standardized system and underscores the reliability of the analytical process. To statistically validate the method's performance, one-way ANOVA revealed that the measured DNA contents across the three different concentration groups were significantly different $F(2, 6) = 9351$, $p < 0.001$. This confirms that the assay can effectively discriminate between samples with varying camel milk content. To obtain full regulatory acceptance, more extensive validation, such as comprehensive inter-laboratory robustness testing and analysis of a broader range of commercial matrices, is essential and represents a logical next step. This will be a major focus of our subsequent work.

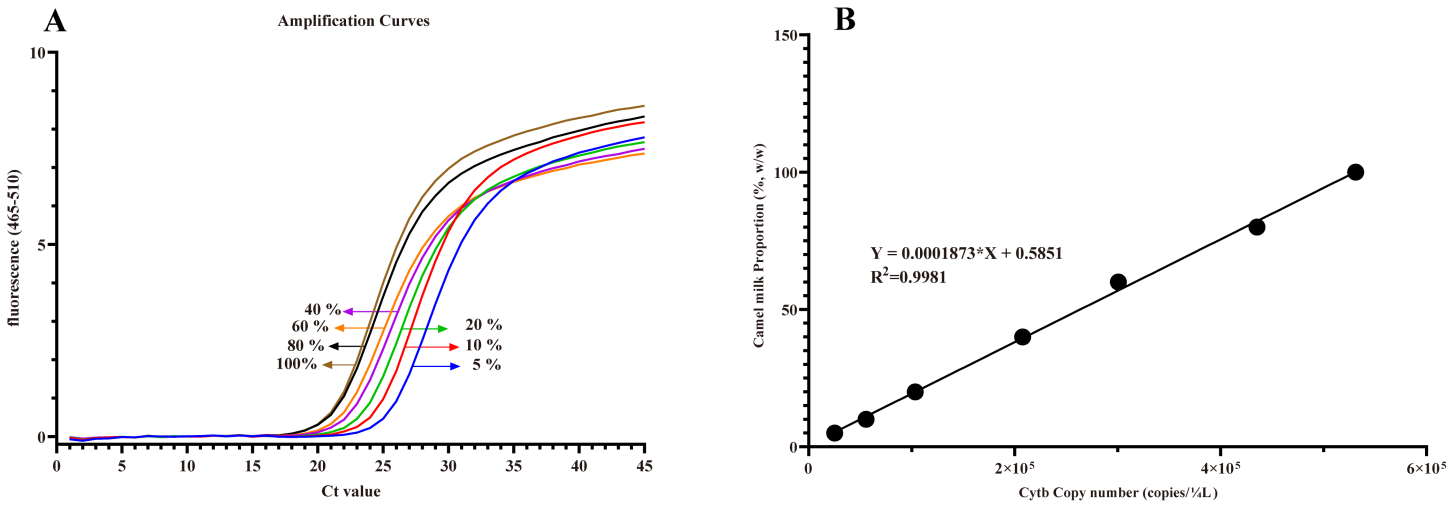

Fig 6. qPCR amplification and standard curve for quantification of camel-derived content. (A) Amplification curves of camel milk samples ranging from 5% to 100%. (B) Standard curves Calibration curve correlating Cytb gene copy numbers with camel milk mass percentage. The *x*-axis represents the $\log_{10}$ copies of six concentrations of standards, and the *y*-axis represents the corresponding Ct values.

https://doi.org/10.1371/journal.pone.0342999.g006

Table 2. Data for the detection of simulated adulteration in camel milk samples according to the actual content of bovine milk.

Actual content (%)	Calculated Content (%)	Mean ± SD (%)	CV (%)	Recovery (%)
15	13.28	13.98 ± 0.73	5.20	107.54
	14.73			
	13.92			
50	48.41	48.75 ± 0.33	0.68	97.5
	49.07			
	48.76			
90	91.24	91.56 ± 0.90	0.99	101.73
	90.86			
	92.58			

https://doi.org/10.1371/journal.pone.0342999.t002

4. Conclusions

In this study, an absolute quantitative qPCR detection system based on the *Cytb* gene was established for the precise detection of camel milk adulteration. Unlike conventional relative quantification methods, the primary innovation of this approach lay in its absolute quantification strategy. This strategy involved the construction of a recombinant plasmid containing the *Cytb* gene as a calibration standard, combined with a specific primer–probe system. This method allowed the direct determination of the absolute content of camel milk in a given sample. It effectively circumvented the limitation of the relative quantification method that relied on reference genes, especially in scenarios where the adulterant substance is unclear or lacks reference genes. As a result, this method could accurately detect the absolute copy number of the target gene.

Regarding technological optimization, this study employed a DNA extraction protocol combining CTAB lysis and chloroform precipitation with silica gel adsorption column purification. This approach effectively mitigated the inhibition of PCR amplification by protease and calcium ions. qPCR is widely used in routine adulteration testing due to its broad dynamic

range and established compatibility with quality-control workflows. This can be primarily attributed to qPCR's broader dynamic range, which accommodates the varying levels of adulteration encountered in real-world samples, its capacity for high-throughput analysis, and its established infrastructure in quality control laboratories, facilitating method adoption and standardization.

From an application perspective, the detection system established in this study laid the groundwork for a standardized method for the detection of camel milk adulteration using a recombinant plasmid as a stable reagent. Despite the current complexity of plasmid preparation process, the demonstrated stability of the standard reagents enables potential scale-up, thereby fostering the wider adoption of absolute quantitative qPCR technology in food adulteration analysis, particularly for nontargeted detection.

Supporting information

S1 File. Supporting information.
(PDF)

S2 File. Raw agarose gel images.
(DOCX)

Acknowledgments

We would like to express our sincere gratitude to Baator Siqin, the owner of a private farm in Alxa City, for providing the camel milk used in the experiments.

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