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A simultaneous triplex *Taq*Man real-time PCR approach for authentication of caprine and bovine meat, milk and cheese

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ABSTRACT

Goat foodstuffs are considered as healthy foods with high nutritional value. This study demonstrated the development and validation of a triplex real-time PCR on the basis of species-specific and species-conservative *Taq*Man probes for the simultaneous identification of caprine and bovine DNA in meats, milk and cheeses with a prerequisite designed endogenous control. In this research, caprine and bovine meat, milk and cheese were specifically identified via developed primers and probes, and the limits of detection of this methodology were 0.005 and 0.01 ng DNA of milk and cheese from goat, and 0.01 and 0.05 ng DNA of milk and cheese from cow. Taken together, this approach was elaborated to address dairy adulteration issues to eliminate the fraud of economically motivated goat milk and cheese adulteration by adding cow milk.

1. Introduction

Today, more than ever, the safety, quality and composition of food have caused comprehensive attention with increasing public concern. Furthermore, authentic and accurate labelling is fundamental to underpin fair-trade practices in markets. To strengthen mandatory labelling with actual information, suppress and punish the behaviour of food adulteration, and reconstruct the confidence of consumers, a great many of regulations are enshrined in the international and national laws. Nevertheless, robust analytical techniques are requisite for implementation of regulations and consequences of non-compliance in the food industry.

To date, a large number of analytical methodologies have been developed, optimised and validated for authentication of foods (e.g., meat and milk products) by means of identification of species origin (Clark & Mora Garcia, 2017; Di Pinto et al., 2017; Kumar et al., 2015; Rahmati, Julkapli, Yehye, & Basirun, 2016). A variety of methods based on DNA showed high specific and sensitive qualities for identification of animal species from raw and processed meats and dairy products (Clark & Mora Garcia, 2017; Di Pinto et al., 2017; Rahmati et al., 2016). The authenticity of poultry meat, beef, milk and cheese was identified by conventional PCR-based methods (Golinelli et al., 2014; Mane, Mendiratta, & Tiwari, 2012; Soares, Amaral, Mafra, & Oliveira, 2010). Some real-time PCR methods using EvaGreen or SYBR Green have been developed for authentication of meats and dairy products (Agrimonti, Pirondini, Marmiroli, & Marmiroli, 2015; Sakalar, Ergun, & Akar, 2015). Last, but not least, *Taq*Man real-time PCR has become increasingly relevant in the analysis of food products including meat samples, and was shown to be more specific and effective than real-time PCR using a fluorescent dye (Kesmen, Gulluce, Sahin, & Yetim, 2009; Kim, Yoo, Lee, Hong, & Kim, 2016; López-Calleja et al., 2007; Zhang, Fowler, Scott, Lawson, & Slater, 2007).

Goat milk exhibits some prominent nutritional characteristics regarding the richness in proteins, vitamins, minerals, and small fat molecules, which endow it with highly digestible features compared with milk from other species (Di Pinto et al., 2017; Golinelli et al., 2014). More importantly, some people who suffer from cow milk allergy can assimilate nutritional components from goat milk (Ribeiro & Ribeiro, 2010; Silanikove, Leitner, Merin, & Prosser, 2010). Due to the increasing price and decreasing availability of goat milk, some dairy producers and dealers could be tempted to commit adulteration of goats' milk and dairy products with cows' milk. Such fraudulent behaviour would violate consumer trust and confidence. Additionally, health reasons (e.g., milk

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protein allergy) require authentic declaration of milk and dairy products. Therefore, authentication of milk and dairy products is significant to ensure food safety as well as to gain consumers' trust. In this context, it is extremely essential and urgent to develop a robust technique for identification of species origins in milk and dairy products.

This research developed a novel triplex real-time PCR method to authenticate goat and cow milk and cheese by means of identifying caprine and bovine DNA with an endogenous control amplification, which was simultaneously used to confirm the process of the PCR reaction and to get rid of possible false negatives. The innovation of this study is related to the design of the species-conservative and species-specific probes, which were simultaneously utilised in the triplex real-time PCR. Considering that the three-channel approach based on different *Taq*Man probes was specific and sensitive, this triplex real-time PCR assay is believed to be an economical and high-throughput approach for authentication of goat and cow milk and cheese.

2. Materials and methods

2.1. Preparation of meats, milk and cheeses

Fresh meat samples [e.g., chevon (goat), beef, sheep, buffalo, yak, pork, horse, camel, donkey, chicken, duck, turkey, rabbit, dog, pigeon, and quail] were purchased from a farmers' market and the DKL shopping mall in Xilinhot, China. Goat, cow and mare milk were obtained from Plain Mountain Pasture in Xilin Gol of Inner Mongolia, and four samples per species were obtained from different individual of same species. Goat cheese (Queserias Entrepinares, Spain) was purchased from Shanghai Rongyue company (Shanghai, China), and cow cheese was obtained from Xilingol Ximulike dairy company (Xilinhot, China).

2.2. Genomic DNA extraction of meat, milk and cheese

The genomic DNA from meat was extracted by the standard CTAB method (Murray & Thompson, 1980). DNA concentration was calculated by Nanodrop2000 (Thermo scientific, USA) at a wavelength of 260 nm. DNA from milk and cheese was extracted by the modified CTAB method. Firstly, 50 mL milk or 50 g cheese was homogenised and defatted with PBS by centrifugation at 13,000× *g* for 10 min at 4 °C, and the supernatant was discarded by wiping the upper cream with a cotton swab. Then, 500 μL PBS was added to the precipitate followed by completely mixing and centrifugation at 13,000× *g* for 10 min at 4 °C, and discarding the supernatant. Next, 540 μL PBS and 60 μL emulsifier were added to the precipitate followed by completely mixing and centrifugation at 13,000× *g* for 10 min at 4 °C, and the supernatant was discarded. Emulsifier consisted of 20 mL Triton-X100 (90%), 125 mL ethanol (95%) and 855 mL NaCl (0.9 g L^{-1}). Lastly, 500 μL PBS was added to the sediment and incubated at 40 °C for 10 min, and the cell pellet was harvested by centrifugation at 13,000× *g* for 10 min at room temperature. Further, the DNA from the above cell pellet was extracted according to the standard CTAB method (Murray & Thompson, 1980).

2.3. The development of primers and probes for the triplex real-time PCR

After alignment of mitochondrial DNA from chevon, beef, sheep, buffalo, yak, pork, horse, camel, donkey, chicken, duck, turkey, rabbit, dog, pigeon, and quail the primers were designed by targeting the species-conservative sequences in the 12S ribosomal gene of mitochondrion DNA to decrease the mismatch of primers in the triplex real-time PCR, the probe of endogenous control was developed by targeting the species-conservative sequences for indicating the process of PCR reaction, and the goat and cow probes were designed by targeting the species-specific sequences for specially detecting DNA from goat and cow meats and dairy products.

Due to too many sequences, the representative sequences were chosen to align to exhibit the target positions for the design of primers and probes in Fig. 1, and the primers and probes are shown in Table 1. In the *Taq*Man real-time PCR system, the caprine-specific probe was labelled with HEX (fluorophore) and TAMRA (quencher), the bovine-specific probe was labelled with FAM (fluorophore) and TAMRA (quencher), and the endogenous control probe was labelled with ROX (fluorophore) and BHQ-2 (quencher).

The innovation of the development was to guarantee the simultaneous triplex real-time PCR with a conservative and effective endogenous control in one PCR reaction. The triplex real-time PCR reaction shared one forward primer and two reverse primers with one different base, and Control-probe could anneal same targeted amplified sequence with Goat-probe and Cow-probe in the real-time PCR reaction based on *Taq*Man probes. Under this technical context, the simultaneous detection of caprine, bovine and endogenous control was executed by the three channels of fluorophores (FAM, HEX and ROX). Goat-HEX, Cow-FAM and Control-ROX represented the caprine, bovine and endogenous control probe, respectively. The designed primers and probes were synthesised and purified using HPLC by Ruibiotech company (Beijing, China).

A similar approach for designing primers and probes to simultaneously identify bovine and equine DNA was previously described in the application of the developed methodology for authenticating mare milk and koumiss adulterated by cow milk and yoghurt (Guo et al., 2018).

2.4. Real-time PCR system and sensitivity assay

Triplex real-time PCR reaction mixtures (20 μL) were: 10 μL Probe qPCR SuperMix (Tansgen, Beijing, China), 0.5 μL each of Goat-LP1, Cow-LP1, Goat-RP1, and Cow-RP1 (all at 10 μmol L^{-1}), 1 μL each of Goat-probe, Cow-probe, and Control-probe (all at 10 μmol L^{-1}), 1 μL template (100 ng μL^{-1}), and 4 μL ddH_2O. Sensitivity assay mixtures (20 μL) based on the simplex real-time PCR reaction were: 10 μL Probe qPCR SuperMix (Tansgen, Beijing, China), 1 μL Goat-LP1 or 1 μL Cow-LP1, 1 μL Goat-RP1 or 1 μL Cow-RP1, 1 μL Goat-probe or 1 μL Cow-probe (all at 10 μmol L^{-1}), 1 μL template, and 6 μL ddH_2O. The triplex and simplex real-time PCR reactions were performed under the following program: 30 s at 94 °C, 40 cycles of 5 s at 94 °C and 31 s at 60 °C (ABI 7300plus).

The sensitivity assay of the triplex real-time PCR assay was evaluated by the limit of detection (LOD). The LOD was determined using 10- and 2-fold DNA serial dilutions (100, 10, 1, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, 0.0001, and 0.00001 ng μL^{-1}). Twenty replicates (in 3 different runs) for each dilution were used for the triplex real-time PCR, and results were analysed as inferred from Probit analysis (Finney, 1971).

To validate the simultaneous triplex real-time PCR assay for authentication of milk, the ability to detect different levels of cow milk in goat milk was determined to simulate adulteration. Artificial binary milk mixtures were blended, then the DNA that was extracted from the binary mixtures was utilised as the template for the real-time PCR. The percentages of cow milk in the mixtures were 1, 5, 10, 30, 70, 90, 95 and 99 (w/w), and the corresponding percentages of goat milk in the mixtures were 99, 95, 90, 70, 30, 10,

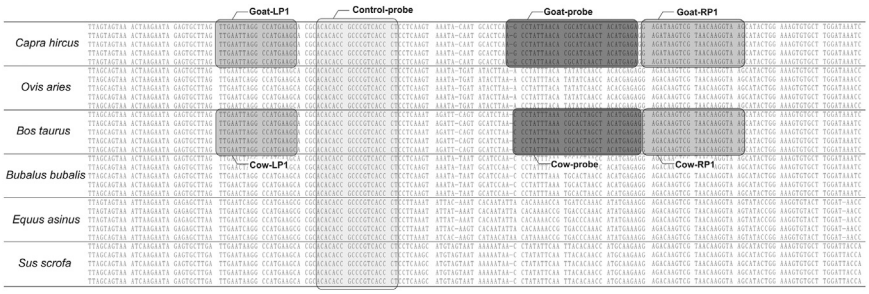

Fig. 1. The target positions for the design of primers and probes in the alignment.

5 and 1 (w/w). Twenty replicates for each mixture were used for the triplex real-time PCR.

3. Results and discussion

3.1. Authentication of meats, milk and cheeses by the triplex real-time PCR based on the developed primers and probes

A specificity assay in the triplex real-time PCR amplification for meats was performed for evaluating the effectiveness of this novel technique. Different kinds of nucleic acid extracted from fresh meats of chevon, beef, sheep, buffalo, yak, pork, horse, camel, donkey, chicken, duck, turkey, rabbit, dog, pigeon, and quail were used as the templates of the *Taq*Man real-time PCR to confirm the developed primers and probes to be caprine and bovine specific in the authentication of meats.

As shown in Fig. 2A,C, the amplification plots of Goat-HEX consistently and specifically appeared in chevon inferred from the triplex real-time PCR, while the Control-ROX was amplified to indicate the reaction of real-time PCR for eliminating false negative results. Meanwhile, the amplification plots of Cow-FAM consistently and specifically appeared in beef, while the Control-ROX was amplified to be an endogenous control (Fig. 2B,C). The Ct values of chevon (3 independent samples per assay, 3 replicates per sample), beef (3 independent samples, 3 replicates), and other species meats (14 different species, 3 replicates per taxon) inferred from the triplex real-time PCR are shown in Table 2. No amplification values were obtained with the DNA from non-target meats; Ct values of chevon and beef identified by Goat-HEX and Cow-FAM were consistently obtained (Table 2). The simultaneous Control-ROX amplification validated the PCR reaction and eliminated false negative results. The above results suggested that the triplex real-time PCR with an endogenous control was effective tool for authentication of caprine and bovine meats.

In addition to meat, milk and cheese are regarded as high-nutritional foodstuffs. The developed triplex real-time PCR was applied to identify authentic milk from goat and cow. As shown in Fig. 3A, the amplification of Goat-HEX were specifically shown in goat milk, while the Control-ROX was amplified to indicate the reaction of real-time PCR for eliminating false negative results. Similarly, the amplification of Cow-FAM consistently appeared in cow milk, while the Control-ROX was amplified as an endogenous control (Fig. 3B).

Mare milk was used as a negative control to validate the specificity of caprine- and bovine-specific primers and probes. As shown in Fig. 3C, the amplification of Goat-HEX and Cow-FAM were not detected in mare milk, while the Control-ROX was amplified successfully. As shown in Fig. 3D, the amplification of Goat-HEX was specifically shown in goat cheese, while the Control-ROX was

Table 1
Triplex *Taq*Man real-time PCR primers and probes.

Primer and Probe	Sequence (5′ to 3′)
Goat-LP1	TTGAATTAGGCCATGAAGC
Cow-LP1	TTGAATTAGGCCATGAAGC
Goat-RP1	CTTACCTTGTTACGACTTATCTC
Cow-RP1	CTTACCTTGTTACGACTTGTCTC
Goat-probe	HEX-TCTCATGTAGTTGATGCGTGTTAATAGGCT-TAMRA
Cow-probe	FAM-CTCTCATGTAGCTAGTGCGTTTAAATAGGG-TAMRA
Control-probe	ROX-ACACACCGCCCGTCACCCT-BHQ-2

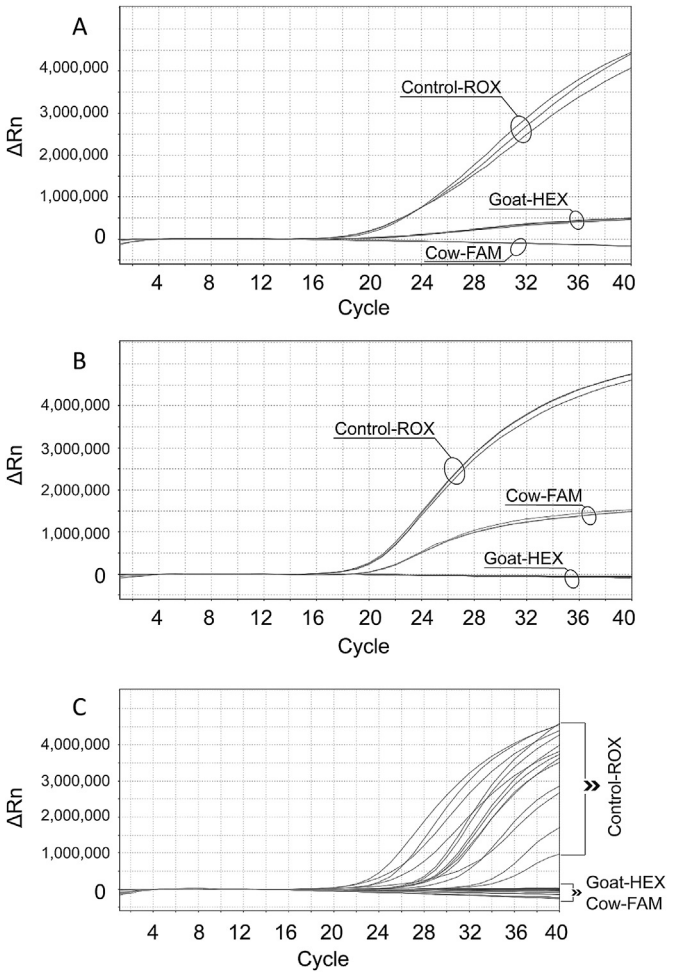

Fig. 2. Triplex real-time PCR amplification plots for authentication of caprine and bovine meats. Caprine and bovine-specific probes labelled with HEX (Goat-HEX) and FAM (Cow-FAM) were utilised to verify the authenticity of chevon (A), beef (B) and other meats (sheep, buffalo, yak, pork, horse, camel, donkey, chicken, duck, turkey, rabbit, dog, pigeon, and quail) (C). Control-ROX was used as an endogenous control. The amplification results were determined with three replicates.

Table 2
The Ct values in the triplex real-time PCR assay for authentication of caprine and bovine meats from 16 species.[a]

Samples	Ct value		
	Goat-HEX	Cow-FAM	Control-ROX
Chevon 1	16.55 ± 0.34	0	15.95 ± 0.01
Chevon 2	19.25 ± 0.14	0	18.25 ± 0.21
Chevon 3	18.17 ± 0.53	0	17.59 ± 0.09
Beef 1	0	18.62 ± 0.11	15.91 ± 0.03
Beef 2	0	20.41 ± 0.50	17.32 ± 0.03
Beef 3	0	18.14 ± 0.47	16.00 ± 0.43
Sheep	0	0	21.03 ± 1.83
Buffalo	0	0	16.82 ± 0.50
Yak	0	0	13.71 ± 0.24
Pork	0	0	16.48 ± 0.07
Horse	0	0	21.85 ± 1.66
Camel	0	0	15.26 ± 2.19
Donkey	0	0	22.63 ± 0.25
Chicken	0	0	20.28 ± 0.52
Duck	0	0	21.17 ± 0.32
Turkey	0	0	23.94 ± 0.94
Rabbit	0	0	16.21 ± 0.65
Dog	0	0	16.14 ± 0.11
Pigeon	0	0	22.61 ± 0.35
Quail	0	0	26.24 ± 0.45

[a] Data (average ± SD) represent three replicates.

amplified to indicate the reaction of real-time PCR, eliminating false negative results. The amplification of Cow-FAM appeared in cow cheese, while the Control-ROX was amplified as an endogenous control (Fig. 3E).

The Ct values of goat milk (4 independent samples per assay, 6 replicates per sample), cow milk (4 independent samples, 6 replicates), mare milk (4 independent samples, 6 replicates), goat cheese (3 independent samples per assay, 6 replicates per sample), and cow cheese (3 independent samples per assay, 6 replicates per sample) inferred from the triplex real-time PCR are illustrated in Table 3. The values of simultaneous Control-ROX were sufficient to verify the process of real-time PCR, and the values of Goat-HEX and Cow-FAM in the three milk samples and two cheeses demonstrated that this triplex real-time PCR was validated as a robust technique for authentication of goat and cow milk and cheeses. In addition, the endogenous control that is spatiotemporally simultaneously amplified in the real-time PCR could be significant for indicating the reaction of PCR and eliminating false-negative results.

3.2. Sensitivity of authentication of goat and cow milk and cheese

After validating of the triplex real-time PCR for authentication of goat and cow milk and cheese, the LOD of the developed primers and probes was determined using the serially diluted DNA from these samples. As shown in Fig. 4 and Table 4, the LOD of the caprine identification in goat milk and cheese were 0.005 and 0.01 ng (confidence limit: 95%). The LOD of the bovine identification in cow milk and cheese were 0.01 and 0.05 ng (confidence limit: 95%). The Ct values of the sensitivity of the authentication of goat and cow milk and cheese are shown in Table 4. The Ct values that were determined by 20 replicates corresponded to the above amplification plots. As shown in Table 4, the Ct values of Goat-HEX increased on dilution of DNA of goat milk and cheese, and the Ct values of Cow-FAM increased on dilution of DNA of cow milk and cheese. It was demonstrated that the real-time PCR assay based on the designed primers and probes was sensitive to authenticate goat and cow milk and cheese.

As shown in Fig. 5, the calibration curves were constructed by plotting the Ct values versus the logarithm of DNA concentration of goat and cow milk and cheese. The calibration curve was determined from 20 replicates. The slopes of the calibration curve were −4.626 for goat milk (Fig. 5A), −4.2763 for goat cheese (Fig. 5B), −4.0976 for cow milk (Fig. 5C), and −4.3176 for cow cheese (Fig. 5D), and the correlation coefficients were 0.9914 for goat milk (Fig. 5A), 0.9984 for goat cheese (Fig. 5B), 0.9966 for cow milk (Fig. 5C), and 0.9979 for cow cheese (Fig. 5D). The amplification efficiencies were 64.5% and 71.3% for goat milk and cheese, 75.4% and 70.5% for cow milk and cheese. We speculate that the integrity of DNA isolated form milk and cheese may harness the

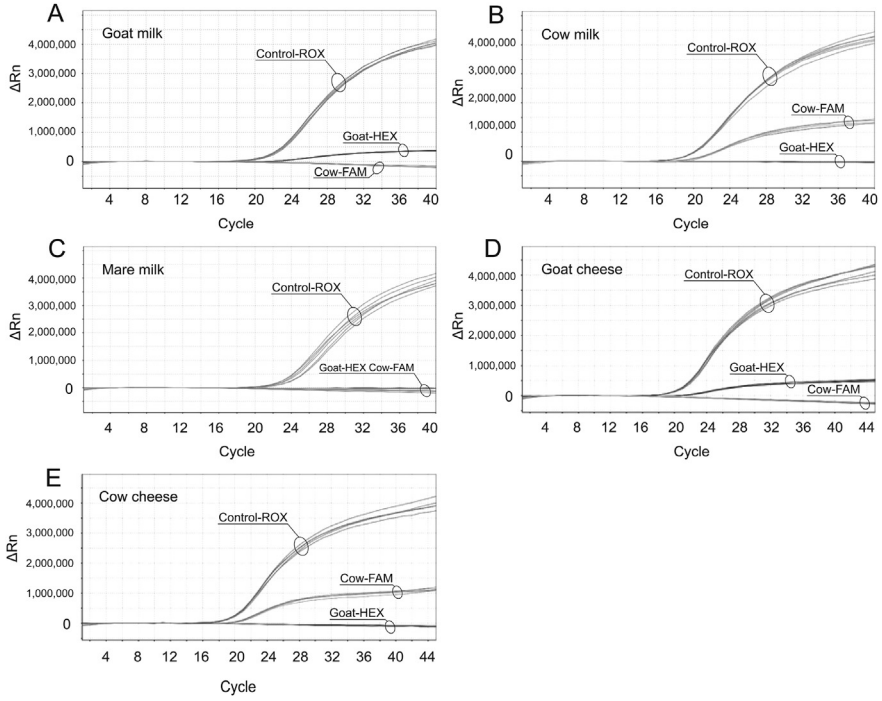

Fig. 3. Triplex real-time PCR amplification plots for authentication of milk and cheeses. Goat-HEX, Cow-FAM, and Control-ROX were utilised to verify the authenticity of goat milk (A), cow milk (B), mare milk (C), goat cheese (D), and cow cheese (E). Control-ROX was used as an endogenous control. The amplification results were determined with six replicates.

Table 3
The Ct values in the triplex real-time PCR assay for authentication of goat and cow milk and cheese.[a]

Samples		Ct value[1]		
		Goat-HEX	Cow-FAM	Control-ROX
Goat milk	1	20.22 ± 0.74	0	13.92 ± 0.66
	2	20.39 ± 0.42	0	14.61 ± 1.75
	3	20.57 ± 0.49	0	14.80 ± 2.52
	4	18.55 ± 0.40	0	17.68 ± 0.54
Cow milk	1	0	17.48 ± 0.85	13.87 ± 0.09
	2	0	17.34 ± 0.46	13.92 ± 0.14
	3	0	17.29 ± 0.65	13.79 ± 0.13
	4	0	20.79 ± 1.09	17.63 ± 3.36
Mare milk	1	0	0	20.16 ± 0.47
	2	0	0	23.02 ± 1.06
	3	0	0	13.79 ± 0.05
	4	0	0	17.07 ± 1.41
Goat cheese	1	19.68 ± 0.31	0	14.35 ± 0.15
	2	20.25 ± 0.24	0	14.6 ± 0.13
	3	20.72 ± 0.24	0	14.84 ± 0.33
Cow cheese	1	0	18.74 ± 0.22	17.71 ± 0.2
	2	0	19.63 ± 0.19	14.34 ± 0.18
	3	0	15.68 ± 0.65	13.83 ± 0.06

[a] Data (average ± SD) represent six replicates. The quality of the DNA in the reactions was 100 ng.

amplification efficiencies of reactions. The real-time PCR system developed in this study demonstrated good calibration linearity, and was thus believed to be sufficient to quantify caprine and bovine DNA in milk and cheese.

To develop primers and probes for authentication of bovine meat, milk and cheese, the *Taq*Man real-time PCR technique was established to quantitatively detect as little as 35 pg bovine DNA (Zhang et al., 2007). Recently, *Taq*Man real-time PCR assays have been developed to identify 25 pg DNA for bovine, 19 pg for buffalo, 2.5 pg for sheep, and 0.35 pg for goat dairy products (Di Domenico, Di Giuseppe, Wicochea Rodriguez, & Camma, 2017). After comparing LOD in this study with published data, the detection of 5 and 10 pg goat DNA from milk and cheese, and 10 and 50 pg cow DNA were appropriate for authentication of caprine and bovine dairy products.

3.3. Validation of the triplex real-time PCR for detection of goat milk adulteration with cow milk

To validate the simultaneous triplex real-time PCR assay for authentication of milk, detection of goat milk adulteration with cow milk was performed by using of Goat-HEX, Cow-FAM and Control-ROX in the simulation of adulteration. As shown in Fig. 6 and Table 5, with increasing levels of cow milk in the mixtures, the Ct values decreased, and the Ct values for 1% and 5% cow percentage were 0. The amplification plots of Cow-FAM consistently appeared in ≥10% cow percentages (confidence limit: 95%).

As shown in Fig. 6 and Table 5, with decreasing levels of goat milk in the mixtures, the Ct values increased, and the Ct values for 1%, 5% and 10% goat percentages were 0. The amplification plots of Goat-HEX consistently appeared in ≥30% goat percentages (confidence limit: 95%). More importantly, the endogenous control (Control-ROX) was consistently amplified in the all eight mixtures (Fig. 6 and Table 5). In short, the simultaneous triplex real-time PCR assay for authentication of milk was validated in the milk mixtures of cow and goat. The above results showed that the triplex real-time PCR with an endogenous control was effective to simultaneously and sensitively detect cow and goat origins in milk mixtures.

Because above 10% adulteration with cow milk would be illegally profitable, we believe this technique is sufficient to

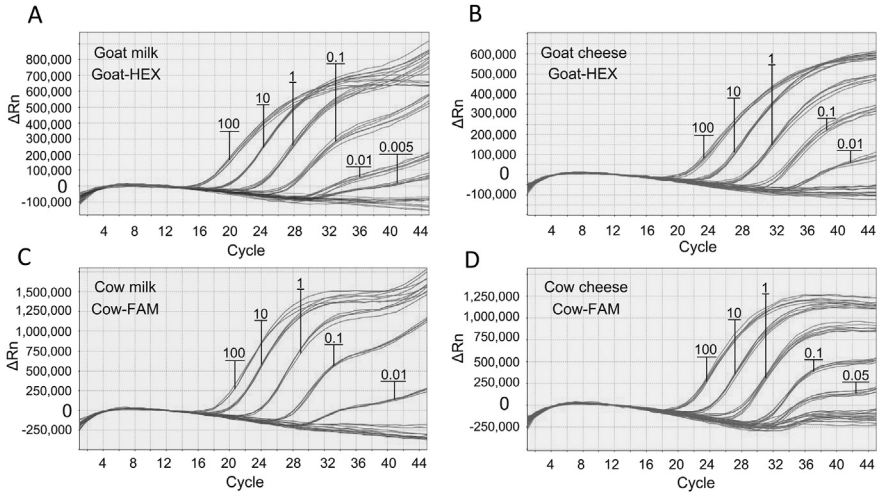

Fig. 4. Simplex real-time PCR amplification plots for the sensitivity of authentication of goat milk (A), goat cheese (B), cow milk (C), and cow cheese (D). The gradient dilutions of DNA extracted from goat and cow milk and cheese were utilised as the templates for the simplex real-time PCR. The concentrations of the diluted DNA from goat milk (A), goat cheese (B), cow milk (C), and cow cheese (D) were 100, 10, 1, 0.1, 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001, 0.0001, and 0.00001 ng μL^{-1}. The amplification results were determined with 20 replicates.

Table 4
The Ct values in the real-time PCR assay for the sensitivity of authentication of goat and cow milk and cheese.[a]

Samples	Input DNA amount (ng)	Ct value	
		Goat-HEX	Cow-FAM
Goat milk	100	15.13 ± 0.28	N/A
	10	19.18 ± 0.46	N/A
	1	23.25 ± 0.46	N/A
	0.1	27.56 ± 0.74	N/A
	0.01	32.8 ± 1.1	N/A
	0.005	35.55 ± 2.05	N/A
	0.0025	0	N/A
	0.001	0	N/A
	0.0001	0	N/A
	0.00001	0	N/A
Goat cheese	100	20.75 ± 0.36	N/A
	10	24.69 ± 0.49	N/A
	1	28.69 ± 0.46	N/A
	0.1	33.19 ± 1.28	N/A
	0.01	37.88 ± 1.24	N/A
	0.005	0	N/A
	0.0025	0	N/A
	0.001	0	N/A
	0.0001	0	N/A
	0.00001		N/A
Cow milk	100	N/A	17.29 ± 0.92
	10	N/A	20.63 ± 1.25
	1	N/A	24.84 ± 0.94
	0.1	N/A	28.89 ± 0.63
	0.01	N/A	33.65 ± 0.7
	0.005	N/A	0
	0.0025	N/A	0
	0.001	N/A	0
	0.0001	N/A	0
	0.00001	N/A	0
Cow cheese	100	N/A	20.19 ± 0.57
	10	N/A	24.28 ± 0.44
	1	N/A	28.55 ± 0.43
	0.1	N/A	32.69 ± 0.38
	0.05	N/A	34.7 ± 0.78
	0.025	N/A	0
	0.01	N/A	0
	0.001	N/A	0
	0.0001	N/A	0
	0.00001	N/A	0

[a] Data (average ± SD) represent 20 replicates. N/A, not applicable.

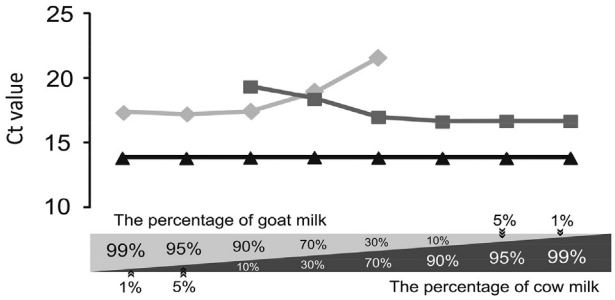

Fig. 6. Triplex real-time PCR assay for identification of goat and cow in the milk mixtures. The artificial binary cow and goat milk mixtures containing 1%, 5%, 10%, 30%, 70%, 90%, 95% and 99% (w/w) of cow in goat, and were blended for the simultaneous triplex real-time PCR: ◆, Goat-HEX; ■, Cow-FAM; ▲, Control-ROX. The quantity of the DNA in the reactions was 100 ng. The results were confirmed with 20 replicates.

Table 5
The Ct values in the triplex real-time PCR for detection of goat and cow milk in the milk mixtures.[a]

Samples	Volume (%)		Ct value		
	Goat milk	Cow milk	Goat-HEX	Cow-FAM	Control-ROX
Mix 1	99	1	17.4 ± 0.25	0	13.85 ± 0.07
Mix 2	95	5	17.2 ± 0.46	0	13.83 ± 0.04
Mix 3	90	10	17.37 ± 0.77	19.37 ± 0.31	13.87 ± 0.04
Mix 4	70	30	18.89 ± 0.67	18.42 ± 0.57	13.9 ± 0.06
Mix 5	30	70	21.56 ± 1.67	17.01 ± 0.3	13.86 ± 0.05
Mix 6	10	90	0	16.66 ± 0.65	13.84 ± 0.04
Mix 7	5	95	0	16.7 ± 0.33	13.83 ± 0.04
Mix 8	1	99	0	16.7 ± 0.37	13.85 ± 0.05

[a] Data (average ± SD) represent 20 replicates.

authenticate goat milk and identify cow milk equal or greater than 10%. In this study, caprine-specific probe cannot detect goat milk less than or equal to 10% in the mixtures, and cattle-specific probe cannot identify cow milk less than or equal to 5% in the mixture inferred from the simultaneous triplex real-time PCR assay (Fig. 6 and Table 5). The concentration of template for the triplex real-time PCR reaction was adjusted to 100 ng. 1% goat or cow milk percentage was equal to 1 ng of caprine or bovine DNA. The LOD

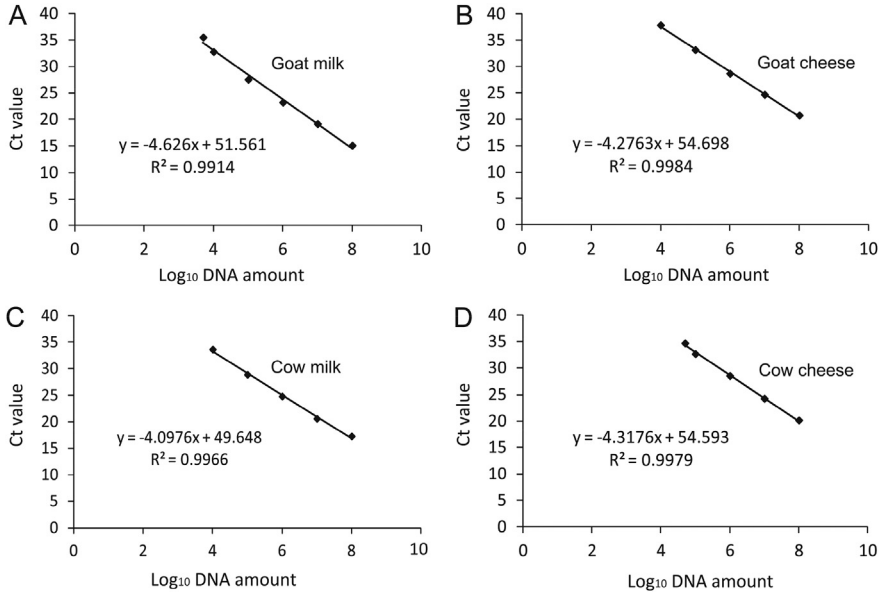

Fig. 5. The calibration curves for quantification of goat (A and B) and cow (C and D) DNA in milk (A and C) and cheeses (B and D) inferred from the simplex real-time PCR.

results suggested that the caprine-specific and bovine-specific probe steadily can detect ≥0.005 ng goat and ≥0.01 ng cow DNA from milk. Nevertheless, in the simultaneous triplex real-time PCR assay the caprine-specific and bovine-specific probe failed to detect 1 ng of goat and cow milk DNA (1% goat and cow milk percentage).

The extreme variation of detection limit between the LOD and the triplex real-time PCR suggested that the simplex real-time PCR based on the designed primers and probe was more sensitive than the triplex PCR for authentication of goat and cow milk. We suspect that the three different probes could compete for the PCR resource in the triplex real-time PCR. The limitation of triplex real-time PCR resource imposed restrictions on the sensitivity of caprine and bovine-specific probe.

4. Conclusions

The objective of this research was to develop and validate a novel triplex *Taq*Man real-time PCR methodology that was applied to authenticate caprine and bovine meat, milk and cheese, and to detect the fraudulent presence of cow milk among goat milk to investigate the prevalence of mislabelling and adulteration.

The simultaneous triplex real-time PCR was verified as being able to identify goat and cow DNA in milk and cheeses with an endogenous control, which was simultaneously used to demonstrate PCR reaction on the rails for eliminating possible false negatives.

This method was based on the design of the species-conservative primers and species-specific probes targeting mitochondrion DNA. With this assay for the detection of animal derived products, goat and cow DNA were specifically identified by employing the primers and probes developed, and the limits of detection were 0.005 and 0.01 ng DNA of milk and cheese from goat, and 0.01 and 0.05 ng DNA of milk and cheese from cow.

Spatiotemporal simultaneous amplification of caprine, bovine and endogenous control in one PCR reaction contributed to lowering the cost of reagents, consumables, and time by half by adding one more probe. In short, the triplex PCR assay for the multi-channel identification of species origins of meat, milk and cheese was verified to be a specific, sensitive and effective technique for authenticating goat and cattle/cow in meats and dairy products, and distinguishing cow milk from goat in the market. In future, the triplex real-time PCR with the designed primers and probes will be applied for the qualitative and quantitative identification in the authentication of foodstuffs from goat and cow.

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