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Simultaneous identification of bovine and equine DNA in milks and dairy products inferred from triplex *Taq*Man real-time PCR technique

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ABSTRACT

Koumiss is a popular dairy product in many lands, traditionally prepared from mare milk with spontaneous fermentation. Mare milk and its fermented derivates are more expensive than cow milk and its fermented derivates, and the possibility exists for producers and dealers to adulterate equine products with bovine items. In this work, we described the development of a triplex real-time PCR based on the species-specific *Taq*Man probes (Ruibio Biotec Company, Beijing, China) for identification of bovine and equine DNA in milks and dairy products. In addition, a novel designed endogenous control was simultaneously amplified to eliminate possible false negatives. With this methodology, bovine and equine DNA were specifically identified by employing developed primers and probes. The limits of detection of this method were 0.001 ng for cow milk, yogurt, and mare milk, and 0.005 ng for sour soup and koumiss, respectively. In addition, the triplex real-time PCR assay for authentication of animal-derived products was effectively validated using binary DNA and milk mixtures, exhibiting well in terms of specificity, sensitivity, and reproducibility. In short, the triplex PCR assay was verified to be a time-saving and money-saving technique for the identification of bovine and equine DNA in milks and dairy products.
Key words: triplex, identification, bovine, equine

INTRODUCTION

Mare milk is regarded to be close to breast milk in its nutritional composition. In addition, horses and humans assimilate various components of food with the monogastric digestive system, and their milks are therefore similar to each other (Park, 2009). Xilingol herdsmen yield koumiss by the natural fermentation of mare milk. Koumiss has been appealing to Mongolians in Xilin Gol of Inner Mongolia, where it has been applied as a traditional Mongolian medicine to cure intestinal dyspepsia, hypertension, dyslipidemia, and so on (Rong et al., 2015; Yao et al., 2017). The above nutritional and medical properties have contributed to the prosperity of the market for mare milk and its dairy products, and consequently have attracted interest for herdsmen and producers in various regions and countries. Nevertheless, the lower milk yield of mare and seasonal milk production determine a higher price compared with cow milk. As a result of a shortage of original material and dispersal natural fermentation in the nomad's yurt, koumiss is more expensive than fermented products derived from cow milk. The big difference in price makes it attractive for herdsmen and producers to adulterate mare with cow milk, and koumiss with sour soup, which is the nickname of acidic whey (the byproduct in the production of Mongolian traditional cheese) in Xilingol.

Various analytical methodologies have been developed to identify the animal species from which meat and milk have been obtained (Anguita et al., 1997; Cozzolino et al., 2002; Ferreira and Cacote, 2003; López-Calleja et al., 2007a; Ballin, 2010; Nakyinsige et al., 2012; Rahmati et al., 2016). Many of these analytical methods for milks and dairy products are based on immunology (Anguita et al., 1997), mass spectrometry (Cozzolino et al., 2002), and chromatographic assay (Ferreira and Cacote, 2003). These protein-based techniques are very specific, but are lowly sensitive, especially for the identification of animal-derived products from thermally processed products. Recently, some DNA-based methods exhibited specific and sensitive features for the discrimination of animal species for heat-treated materials (Ballin, 2010; Rahmati et al., 2016). The PCR-based methods were developed to detect poultry meat adulteration (Soares et al., 2010), beef adulteration (Mane et al., 2012), and bovine milk adulteration with goat cheese (Golinelli et al., 2014). Real-time PCR-based methods using SYBR Green were applied to identify the DNA of cow, goat, sheep, and buffalo for dairy adulteration (Agrimonti et al., 2015). In particular, real-time PCR results can be automati-

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Table 1. *Taq*Man real-time PCR primers and probes (Ruibio Biotec Company, Beijing, China)

Primer and probe	Sequence[1] (5′ to 3′)
LP1	TTGAAT(C/T)AGGCCATGAAGC
Bovine-LP1	TTGAATTAGGCCATGAAGC
Equine-LP1	TTGAATCAGGCCATGAAGC
RP1	CTTACCTTGTTACGACTTGTCTC
Bovine-probe	FAM-CTCTCATGTAGCTAGTGCGTTTAAATAGGG-TAMRA
Equine-probe	HEX-TTCATATGTTTGGGTCACGGTTTTATGT-TAMRA
Control-probe	ROX-ACACACCGCCCGTCACCCT-BHQ-2

[1]FAM = fluorescent reporters 6-carboxyfluorescein; HEX = hexachlorofluorescein; ROX = carboxy-X-rhodamine; TAMRA = tetramethylrhodamine; BHQ-2 = black hole quencher 2.

cally achieved without electrophoresis and exhibit the ability of quantitative analysis.

This work aimed at proposing a novel triplex real-time PCR methodology that was introduced to identify bovine and equine DNA in milks and dairy products with an endogenous control amplification, which was simultaneously used to eliminate possible false negatives. The system was based on the design of relative species-conservative primers and species-specific probes targeting 12S ribosomal gene of mitochondrion DNA, which was more specific and sensitive than conventional PCR, and regarded as a less expensive and more throughput assay compared with simplex real-time PCR.

MATERIALS AND METHODS

Preparation of Milks and Dairy Products

Fresh meat samples of beef, horse, mutton, pork, chicken, duck, goose, dog, rabbit, cat, and carp were purchased from 109 supermarkets and DKL shopping mall in Xilinhot. Cow and mare milk were obtained from Plain Mountain Pasture in Xilin Gol of Inner Mongolia, and 5 samples per species were milked from different individuals. The yogurt, koumiss, and sour soup were collected from Mongolian yurts across the Xilingol grassland, and 5 samples per type originated from different individual families.

Genomic DNA Extraction of Milks and Dairy Products

The DNA from milks and dairy products were isolated by the modified cetyltrimethyl ammonium bromide method. First, 50 mL of milks and dairy products were defatted with centrifugation at 13,000 × *g* for 10 min at

Table 2. The cycle threshold (Ct) values in the real-time PCR assay for bovine and equine detection in the meats of 11 species

	Ct value[1]		
Sample	Cow-FAM	Mare-HEX	Control-ROX
Beef	16.18 ± 0.14	0	15.88 ± 0.09
Horse	0	16.35 ± 0.32	15.97 ± 0.17
Mutton	0	0	15.5 ± 0.17
Pork	0	0	15 ± 0.04
Chicken	0	0	N/A[2]
Duck	0	0	N/A
Goose	0	0	N/A
Dog	0	0	N/A
Rabbit	0	0	N/A
Cat	0	0	N/A
Carp	0	0	N/A

[1]Data (average ± SD) represent 3 replicates. FAM = fluorescent reporters 6-carboxyfluorescein; HEX = hexachlorofluorescein; ROX = carboxy-X-rhodamine.

[2]N/A = not applicable.

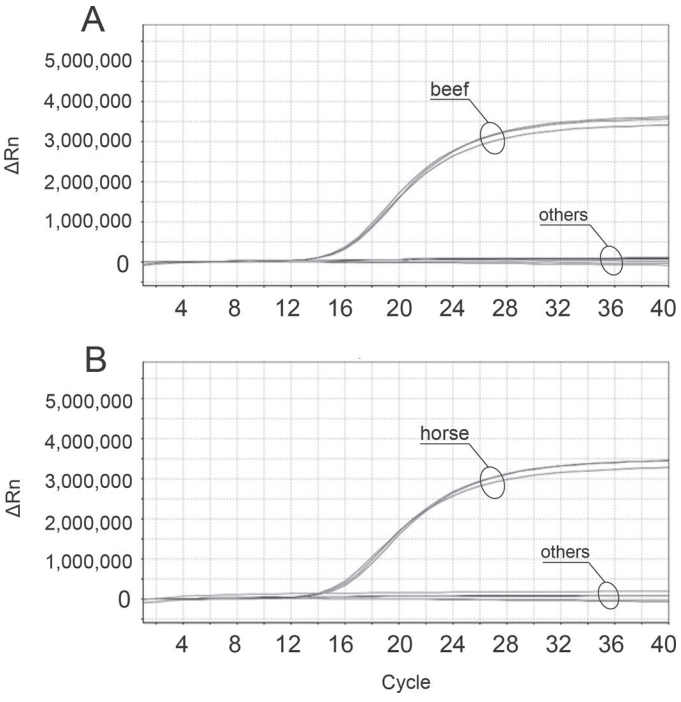

Figure 1. Specificity of bovine and equine primers and probe in the 11 meats. (A) Eleven meats (beef, horse, mutton, pork, chicken, duck, goose, dog, rabbit, cat, and carp) were amplified with bovine-specific primers and probe. (B) Eleven meats were amplified with equine-specific primers and probes. The results were confirmed by 3 replicates. ΔRn = change in normalized reported value.