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Simultaneous detection of cow and buffalo species in milk from China, India, and Pakistan using multiplex real-time PCR

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ABSTRACT

Asian countries are major producers of cow and buffalo milk. For quality and authenticity purposes, a multiplex real-time PCR assay was developed to specifically and simultaneously detect DNA from these 2 bovine species. Targeting the cytochrome *b* gene of mitochondrial DNA, common PCR primers amplified a 105-bp fragment, and 2 fluorescent probes specific to either cow or buffalo were designed for their identification. Specificity was successfully tested on 6 other species, including sheep and goat, and sensitivity reached 1% of cow DNA in buffalo DNA and vice versa. As an evaluation, the method was tested using 119 freeze-dried Asian milk samples from regional industrial milk facilities. Although these samples did not cover the entire Asian zone, the multiplex assay indicated that approximately 20% of the samples (mainly from India) showed high levels of cross-contamination of cow milk by buffalo milk, and vice versa. Fast, sensitive, and straightforward, this method is fit-for-purpose for the authenticity control of Asian milk.

Key words: cow milk, buffalo milk, multiplex, real-time polymerase chain reaction

INTRODUCTION

Animal species authentication in food products plays a major role in traceability, quality, and safety purposes. Species substitution or cross-contamination can indeed lead to regulatory noncompliance, religious concerns, or allergic issues. Dairy products are included in these concerns, particularly with respect to the adulteration of Mozzarella cheese with cow milk (López-Calleja et al., 2005) or milk fat adulteration with foreign fat (Molkentin, 2007). The applicability of the recent official standard ISO 17678 (ISO, 2010), which was validated on mainly European cow milk samples, for foreign fat detection in cow milk fat needs to be evaluated on Asian milk. For this purpose, a survey was initiated to establish triacylglycerol profiles on samples collected at milk collection centers in Asia. However, the purity of Asian cow milk, with respect to contamination with buffalo milk, is questionable, knowing that India, Pakistan, and China are recognized as the 3 main producers of cow and buffalo milks in Asia according to the Food and Agriculture Organization of United Nations (FAOSTAT, 2010).

To ensure product authenticity and traceability and avoid possible species mixture and fraud, many methods have been developed, such as 2-dimensional electrophoresis (Chianese et al., 1990), isoelectric focusing (Moio et al., 1990), capillary electrophoresis (Recio et al., 2004), HPLC (Mayer et al., 1997; Mayer, 2005), ELISA (Asensio et al., 2008), and chromatographic techniques (Chou et al., 2007). However, these techniques are often limited when assaying heat-treated or processed products. To overcome these difficulties, the use of DNA-based methods and especially PCR techniques are of great interest compared with protein-based methods (Pascoal et al., 2005).

The majority of PCR-based animal authentication methods focus on mitochondrial DNA sequences. Because of its high conservation within individual species and its high copy number in animal cells, the cytochrome *b* gene is regularly targeted, thus increasing the specificity and sensitivity of the assay (Fajardo et al., 2010). Milk is furthermore known as a valuable source of DNA to discriminate animal species by PCR due to the presence of somatic cells (Lipkin et al., 1993).

Cow and buffalo detection have already been studied, especially for traceability and authenticity in Mozzarella cheese (López-Calleja et al., 2005; Lopparelli et al., 2007). However, because cow contamination was suspected and targeted, PCR methods have often been developed for cow detection only. Many of these DNA-based methods developed for authentication purpose rely on PCR-RFLP (Mafra et al., 2008), which can lead to problems in interpretation of results due to incomplete DNA digestion (Quinteiro et al., 1998), and the need to handle post-PCR products significantly increasing the risk of laboratory contamination. Recently, classical PCR methods evolved to real-time PCR (**RT-PCR**) enabling detection of cow and buf-

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falo in the same reaction based on allelic discrimination (Dalmasso et al., 2011).

The purpose of this study was to develop a new multiplex RT-PCR targeting the cytochrome *b* gene to detect cow and buffalo species simultaneously. Although not covering the whole Asian zone, the new assay was tested on Asian milk samples from regional industrial milk facilities located in India, China, and Pakistan to evaluate potential mixtures and contaminations.

MATERIALS AND METHODS

DNA References and Milk Samples

Certified DNA references from cow (*Bos taurus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), sheep (*Ovis aries*), horse (*Equus caballus*), pig (*Sus scrofa*), chicken (*Gallus gallus*), and turkey (*Meleagris gallopavo*) were purchased at Coring Systems Diagnostix (Gernsheim, Germany) and supplied lyophilized. Once reconstituted at 20 ng/μL in elution buffer (10 m*M* Tris-HCl, pH 8.5; Qiagen, Hilden, Germany), DNA solutions were stored at −20°C.

In total, 119 Asian milk samples, 80 labeled as cow milks and 39 labeled as buffalo milks, were collected at milk collection centers in China, India, and Pakistan (respectively 40, 20, and 20 cow milk samples; 0, 20, and 19 buffalo milk samples). Collected from tankers of 200 to 8,000 L, 100 mL of each milk sample was freeze-dried with the Freeze Dryer Alpha1-4LDPlus (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), and the residual powder was then stored at −20°C.

DNA Extraction

DNA from lyophilized milk samples was extracted with a hexadecyltrimethylammonium bromide (**CTAB**)-based protocol, adapted for dairy products (Pirondini et al., 2010). One hundred milligrams of each milk powder were incubated in 1.5 mL of CTAB extraction buffer [1.4 *M* NaCl, 20 m*M* EDTA, 100 mM Tris-HCl, 2% (wt/vol) CTAB, pH 8.0] and 450 μg/mL protease (Qiagen) at 65°C for 1 h. After maceration, the samples were centrifuged at 15,000 × *g* for 10 min. The aqueous phase was transferred into a new tube, extracted with an equal volume of chloroform, and centrifuged again at 15,000 × *g* for 10 min. The supernatant was added to 5 volumes of PB binding buffer (Qiagen), mixed, and loaded onto a QIAquick column (Qiagen) over a vacuum manifold. The column was washed twice with 750 μL of PE buffer (Qiagen), and the sample was dried at 12,000 × *g* for 5 min and placed in a new centrifuge tube. The purified DNA was eluted by centrifugation for 5 min at 12,000 × *g* with 50 μL of elution buffer (Qiagen) after 20 min of incubation and stored at −20°C.

Primer and Probe Design

The cytochrome *b* sequences from *Bos taurus* and *Bubalus bubalis* were obtained from the GenBank database (GenBank accession nos. NC006853 and NC006295, respectively). Aligned with Megalign software (Lasergene DNASTAR, Madison, WI), the RT-PCR primers and probes were designed with Primer Express 3.0 software (Applied Biosystems, Foster City, CA).

A common pair of primers (BOVCytb-f: 5′-AATACACTACACATCCGACACAACAA-3′ and BOVCytb-r: 5′-GCTCCGTTTGCGTGTATGTATC-3′) with 100% homology between cow and buffalo was designed, amplifying a 105-bp fragment. Two fluorescent TaqMan minor groove binder probes, using FAM (6-carboxyfluorescein) and VIC dyes, were designed on *Bos taurus* and *Bubalus bubalis* sequences, respectively (COW-p: 5′FAM-CTCTGTTACCCATATCTG-3′ and BUFF-p: 5′VIC- CCTCCGTCGCCCACA-3′).

RT-PCR

The RT-PCR amplifications were performed with the 7900HT Fast Real-Time PCR System (Applied Biosystems). All amplification reactions were carried out in a final volume of 50 μL and contained 5 μL of DNA (at a concentration of 20 ng/μL), 1× TaqMan Universal PCR Mastermix (Applied Biosystems), 0.9 μ*M* of forward and reverse primers, and 0.1 μ*M* of each probe. After 5 min at 50°C to activate the degradation of any potential carryover contamination by the uracyl-*N*-glycosylase enzyme contained in the TaqMan PCR mastermix, the hot-start Taq DNA polymerase was activated for 10 min at 95°C. The thermocycling conditions consisted of 40 cycles of a denaturation step of 15 s at 95°C and an annealing/elongation step of 1 min at 60°C. Data analysis was performed with SDS software 2.3 (Applied Biosystems).

Estimation of Cow and Buffalo Content

Without the establishment of standard curves or the use of a bovine reference gene for $2^{-\Delta\Delta CT}$ quantification, quantitative determination cannot be directly applied. However, cow and buffalo content in each sample can be estimated through direct relative quantitative PCR (**drqPCR**) described by Bernth Jensen et al. (2010), provided that the PCR efficiency has been shown equivalent for both species. For this purpose, PCR efficiencies were calculated and compared based on stan-