



AUTHENTICATION OF CAPRINE MILK AND CHEESE BY COMMERCIAL QPCR ASSAY

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ABSTRACT

The objective of the study was to investigate potential adulteration of commercial caprine milks and cheeses with bovine milk using commercial qPCR assay. The assay comprised of bovine-, ovine- and caprine-specific primers and TaqMan probe and mammalian internal control. Specificity, sensitivity, linearity, reproducibility and efficiency of the bovine assay were tested as well. Specificity was verified by running reaction on the DNA of other milk-producing species (caprine and ovine) and made-up bovine-caprine (v/v) milk mixes. In both experiments, a bovine DNA fragment was amplified whereas no amplification was obtained from the other species. Sensitivity, linearity, reproducibility and efficiency were tested on 10-fold dilution series of 10 ng bovine DNA. The assay has shown good linearity ($R^2 = 0.983$) within whole range, with efficiency of 86% and excellent reproducibility (SD around the C_T for the technical replicates <0.5). The sensitivity was adequate, as calculated LOD and LOQ were 1.44 pg and 2.94 pg of bovine DNA, respectively. Finally, the assay was used to authenticate 5 caprine milk samples and 5 caprine cheese samples, purchased from local supermarkets. Totally, 1 milk sample has shown the fluorescence signal, which exceeded baseline in cycle 39.01 ± 0.69 . However, the signal was above LOD and LOQ suggesting that there could not be unambiguously declared any adulteration with bovine milk. Amplification of bovine-specific DNA was not observed in the other samples indicating products were not adulterated. The commercial qPCR assay has proved that real-time PCR assays, as well as DNA-based techniques in a general, are the excellent and reliable tools for fighting with frauds in the food industry and protecting the public health.

Keywords: bovine; caprine; adulteration; qPCR

INTRODUCTION

Milk in its natural form has a high nutritive value as it is a one of the best sources of quality proteins, fats, carbohydrates, vitamins and minerals. It is easily digestible and hence is readily absorbed and thus is especially important for infants, nursing women, children and elderly people (Poonia et al., 2016; Azad and Ahmed, 2016; Barłowska et al., 2011).

Accounting for more than 80% of world milk production, bovine milk is the most universal raw material for processing, which is reflected in the broadest spectrum of manufactured products (Barłowska et al., 2011; Haug, Høstmark and Harstad, 2007).

Caprine milk is similar to bovine milk with around 87% water, 67% energy, 3.3% protein, 4.0% fat and 4.6% carbohydrates. Caprine milk differs from bovine and human milk in several ways, among them higher digestibility and lower lactose content. (Osman, Aradaib and Musa, 2013). The high dispersion state facilitates the digestion process of this milk and its products. Even though the nutrient contents in caprine milk are slightly

lower than those in bovine milk, its composition allows for a wide range of uses, such as consumption milk, and even to some extent as a therapeutical product (low content or lack of α_{s1} -casein) and most of all, as the raw material for dairy processing (Barłowska et al., 2011; Jung et al., 2011; Haenlein, 2004).

Milk and dairy product adulteration came into global concern after breakthrough of melamine contamination in Chinese infant milk products in 2008 (Azad and Ahmed, 2016). Identification of animal species origin in dairy products has become more and more important, with regard not only to accurate consumer information and legal aspects (e.g. labelling and guarantee requirements), but also to public health (bovine milk proteins are potential allergens, even if present in very low quantities) (Minimi et al., 2009; Zeleňáková et al., 2016). Moreover, bovine milk was reported as the main dairy product responsible for human adverse reaction (Osman, Aradaib and Musa, 2013).

In the production chain or during processing, there can be distinguished intentional or unintentional contamination

(Zhang et al., 2007). Regarding the former, undeclared bovine milk is frequently admixed with caprine and ovine milk during the manufacture of caprine and ovine cheeses, mainly due to the lower yield from goats and ewes, together with the much lower price of bovine milk (Klančnik et al., 2015). However, milk origin in the products can not be identified by the consumer. Moreover, they are sold at different prices under various product names (Kemal Seçkin, Yilmaz and Tosun, 2017).

Proving conclusively that adulteration or contamination has occurred requires the detection and quantification of food constituents. This can be difficult because the materials replaced are often biochemically very similar and food matrices are extremely complex and variable (Zhang et al., 2007). Poonia et al. (2016) and Azad and Ahmed (2016) reviewed numerous methods, based on electroimmigration, immunological reactions and chromatography, which have been used for of milk origin authentication in dairy products. The methods usually use lipids and proteins as a target analytes.

More recently, DNA molecules have received much attention and been the target compounds for species identification based on PCR because they are thermally more stable than lipids and proteins (Caldwell, 2017). Therefore, PCR-based methods have been designed and applied to dairy products for authenticating caprine milk. These methods can detect very small amounts of bovine milk in caprine milk (Kotowicz et al., 2007; Hutu et al., 2013). However, quantifying inability, inaccuracy and cross contamination have been suggested as the main disadvantages of end-point PCR. To overcome the limitations, several real-time PCR assays have been proposed to detect and quantify bovine milk in caprine cheese (Mininni et al., 2009) and in caprine milk (Jung et al., 2011). These studies showed that PCR-based methods have a potential in addressing food adulteration.

The study was aimed to test performance of bovine-specific TaqMan real-time PCR assay and to reveal potential adulteration of purchased caprine milks and cheeses.

MATERIAL AND METHODOLOGY

Sample preparation

Ultra-high-temperature processed commercial bovine (*Bos taurus*), caprine (*Capra hircus*) and ovine (*Ovis aries*) milks were purchased from several national food retailers in Nitra, Slovakia. Moreover, 5 UHT treated caprine milk samples (CM-1 – 5) and 5 caprine cheese samples (CCH-1 – 5) were randomly selected in market for authentication. Samples were transported to the laboratory and stored at 4 °C. Sensitivity, linearity, reproducibility and efficiency tests of the bovine qPCR assay were carried out with DNA extracted from bovine milk. Milk mixtures of bovine milk in caprine milk were prepared for further DNA extraction and assay specificity test. Five different mixtures, containing 50, 10, 5, 1, and 0.5% (v/v) bovine and caprine milks, were prepared in a final volume of 1 mL.

DNA extraction

DNA was extracted using the InnuPREP DNA Mini Kit (Analytik Jena, Jena, Germany) rendering an elution

volume of 250 µL DNA according to the manufacturer's instruction. DNA samples were quantified using the QuantiFluor dsDNA system (Promega) with Quantus™ Fluorometer (Promega).

Real-time PCR reaction

With the InnuDETECT Cheese Assay, bovine and caprine DNA were identified by using specific primers complementary to respective species. An internal positive control was incorporated in the InnuDETECT Cheese Assay kit. The internal control coamplified with the primers used for the qPCR reaction. PCR amplification was performed according to the manufacturer's recommendations by adding 10 µL 2x MasterMix, 3 µL Primer/Probe Mix bovine, 1 µL Internal control, 5 µL of sample and the mixture was filled up to 20 µL. Real-time qPCR assay was performed with a LightCycler (Roche, Germany) based on the TaqMan principle. Bovine and caprine DNA have been detected in separated tubes (FAM channel) in order to reach the maximum sensitivity. Internal Control was used as an amplification control (HEX channel). Real-time PCR cycling parameters were optimised based on manufacturer's manual: Initial denaturation 95 °C, 120 s, followed by 40 cycles of 95 °C, 10 s of denaturation, 62 °C 45s of annealing/elongation and finally, absolute quantification analysis. All reactions were run in triplicate. In qPCR, 10 ng of bovine DNA was considered as 100% bovine milk. Amount of amplified DNA, isolated from bovine-caprine milk mixtures, commercial caprine milks and cheeses, corresponded to ~10 ng DNA as well.

Data analysis

Primary real-time PCR data were analysed by the LightCycler Software 4.1.1.21 (Roche, Germany) and the threshold cycle (C_T) was calculated. C_T values of standard curve replicates (Y) and \log_{10} (DNA amount) (X) were analysed using XLSTAT (Addinsoft, 2016) software and a linear regression equation of the C_T value plotted against the \log_{10} (DNA amount) was calculated. The total DNA in prepared milk mixtures was estimated using the model. Limit of detection (LOD) and limit of quantification (LOQ) was calculated.

RESULTS AND DISCUSSION

DNA extracted from a sample of 100% bovine milk was used for the sensitivity and efficiency determination of the TaqMan real-time PCR assay. Linear range of positive amplification for the bovine milk assay was achieved over five log units, which extended from 10 ng to 0.001 ng bovine DNA (Figure 1, Table1).

Sensitivity

Parameters of the model for linear detection and quantification range are shown in Table 2. The assay showed good linearity, with correlation coefficient of $R^2 = 0.983$ and efficiency of 86%. The LOD and LOQ were 1.44 pg and 2.94 pg, respectively. This corresponded to cut-off in C_T of 37.80 and 36.64, respectively.

Table 2 Parameters of the model.

Source	Value	95% CI Value	Standard error	t	Pr > t
Intercept	27.214	26.708, 27.720	0.234	116.272	<0.01
Slope	-3.724	-4.016, -3.432	0.135	-27.558	<0.01

Specificity

Detection system was tested for its selectivity and cross-reactions to other milk-producing species. The bovine-specific system amplified fragment from bovine DNA whereas no amplification was obtained from ovine and caprine DNA. The internal control amplified fragment from different mammalian species (cow, sheep and goat), with similar C_T values.

Quantification of bovine DNA in milk mixtures and cheeses

Table 3 summarises mean $C_T \pm SD$ values and mean bovine DNA in target matrixes as predicted by linear regression model. Detection of bovine DNA in milk mixtures was achieved even in samples containing 0.5% of bovine milk. Totally, in 1 caprine milk sample (CM-3), a bovine-specific signal was observed after 40th cycle. Since LOD and LOQ were defined, C_T values above these limits were considered as either non-specific amplification or possible amplification of bovine trace DNA due to the accidental contamination during manufacturing. Amplification of bovine-specific DNA was not observed in the other samples suggesting products were not adulterated.

Molecular techniques using DNA technology to combat fraud, improve traceability and distinguish between closely related species are being increasingly utilised in food

forensic analysis (Caldwell, 2017).

The PCR assays rely on the amplification of known DNA sequences. Conventional end-point PCR utilise agarose gels to identify targets via fragment length. In PCR-RFLP technique, DNA is amplified and then cut into smaller fragments using restriction enzymes (Caldwell, 2017).

During last decade, end-point PCR and PCR-RFLP was widely used for milk species identification in dairy products. Branciari et al. (2000) used PCR-RFLP of cytochrome b (cyt-b) gene to investigate the adulteration rate of feta cheeses, made from mixture of ovine and caprine milk, with less expensive bovine milk. The restriction enzymes *HaeIII* and *Sau3AI* differentiated DNA of bovine, ovine, and caprine milk. The LOD of undeclared milk admixture was about 1%.

Bottero et al. (2003) developed end-point PCR-RFLP, based on mt12S and mt16S rRNA, for simultaneous detection of bovine, ovine and caprine milk in dairy products. In total, 19 cheeses from the retail trade were analysed, of which fifteen samples confirmed the information given by labelling, while four did not. The LOD of caprine DNA was 0.125 ng in mixture of all three species DNA. Regarding the bovine milk addition to caprine milk, LOD was 0.5% (v/v).

Lanzilao et al. (2005) developed PCR-RFLP method, targeting t cyt-b gene, for the identification of the 4 animal species of main interest in the dairy industry (cow, sheep,

Table 1 Sensitivity of bovine-specific assay.

Dilution (%)	DNA amount (ng)	$\log_{10}(\text{DNA amount})$	Mean $C_T \pm SD$
100	10	1.00	23.36 \pm 0.23
10	1	0.00	26.54 \pm 0.08
1	0.1	-1.00	32.09 \pm 0.37
0.1	0.01	-2.00	34.90 \pm 0.16
0.01	0.001	-3.00	37.80 \pm 0.21

Regression of C_T by $\log_{10}(\text{DNA})$ ($R^2 = 0.983$)

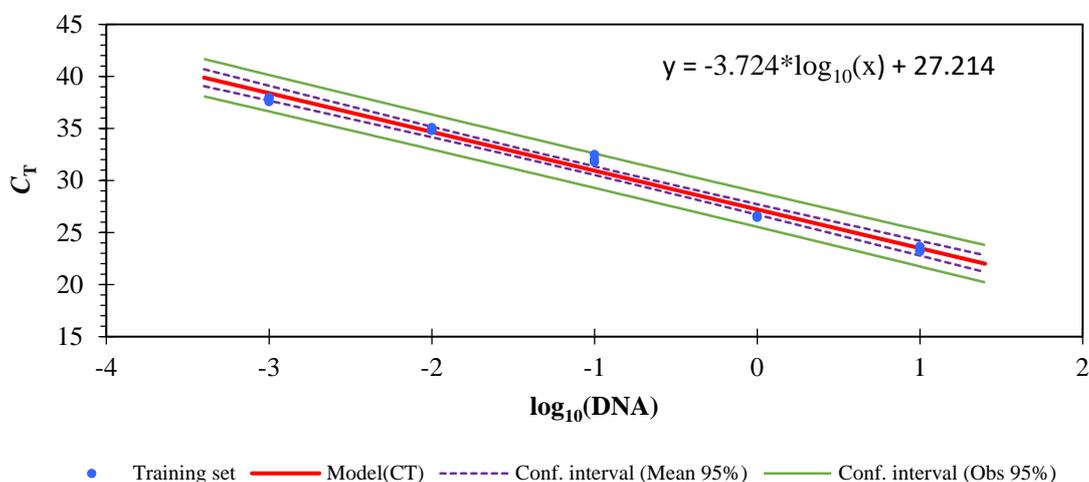


Figure 1 Linear range of detection and quantification of the bovine qPCR assay.

goat and buffalo). The comparative analysis of the 92 cyt-b sequences belonging to the 4 species allowed identification of 2 highly conserved regions, which were used to design 2 universal primers for the PCR amplification of cyt-b gene. The *in silico* analysis allowed identification of a set of species-specific restriction endonucleases (*Hae*III, *Taq*I, and *Mwo*I), which generated easily analysable species-specific restriction profiles of amplified cyt-b gene. The system was developed for both purified DNA and DNA extracted from meat or dairy products and finally tested on mixed samples, indicating its applicability to foodstuffs.

Due to the pattern overlapping, some studies reported ambiguous species identification. For this reason, species-specific end-point PCR has been proposed by several authors. For instance, **Maudet and Taberlet (2001)** suggested singleplex end-point PCR for detecting bovine milk in caprine cheese. Targeting the bovine-specific deletions of D-loop (cyt-b), it was possible to design bovine-specific primers and to identify the presence of less than 0.1% (w/w) of bovine milk in model mixture cheeses. In addition, the analysis of an agarose gel digital image allowed a rough estimation of the percentage of bovine milk used in adulteration.

Maudet and Taberlet (2002) applied end-point PCR detection of Prim'Holstein's milk, a *Bos taurus* breed not allowed for cheese making of some French cheeses (e.g. Reblochon, Abondance and Beaufort). The design of species-specific primers targeting the mt12 S rRNA gene enabled the specific detection of bovine milk in ovine and caprine milk mixtures.

López-Calleja et al. (2004) developed end-point PCR assay for specific identification of bovine milk in ovine and caprine milk by using forward primer targeting conserved region of mt12S rRNA gene along with a reverse primer specific for *Bos taurus*. The technique was applied to raw, pasteurised, and sterilised bovine-ovine and bovine-caprine binary milk mixtures, enabling the

specific detection of bovine milk, with LOD of 0.1% (v/v).

Cheng, Chen and Weng (2006) used primers targeting highly conserved regions in bovine mtDNA to reveal adulteration of caprine milk with bovine milk through end-point PCR. Random sampling of different brands of caprine milk powder and tablets showed that 25% of caprine powders and 50% of caprine milk tablets were adulterated. Using the assay, as low as 0.1% (v/v) of bovine milk in caprine milk could be identified.

Besides, in a more advanced PCR format, duplex PCR using two pairs of primers targeting mtD-loop region, has also been successfully used to identify up to 1% bovine milk in the caprine milk. A total of 54 milk samples were examined. In 33 samples, the bovine DNA was detected, while 21 samples produced the caprine-specific amplicon only (**Kotowich, Adamczyk and Bania, 2007**).

Similarly, a duplex PCR has been applied by **Mafra et al. (2007)** for the detection of both bovine and caprine milk in caprine milk cheeses using primers targeting the mt12S rRNA genes. It was possible to quantify cheese adulteration with bovine milk in the range of 1% – 60% (w/w). The duplex PCR technique allowed the detection of 0.1% (w/w) of bovine milk in caprine milk cheese. The proposed method was successfully applied to cheeses with defined amounts of bovine milk and commercial cheese samples. The results showed the fraudulent addition of bovine milk in three samples labelled as pure caprine milk cheeses and the omission of caprine milk mentioned on the label of two cheeses containing mixed milk.

Gonçalves et al. (2012) presented quadruplex PCR of species-specific mtDNA targets followed by fragment size analysis by capillary electrophoresis enabling detection of at least 1% (v/v) relative proportion of bovine, ovine, caprine and buffalo milk in binary mixtures.

More recently, **Hutu et al. (2013)** used two pairs of primers targeting mt12s rRNA gene to detect and quantify the percentage of bovine milk adulteration in products

Table 3 Mean $C_T \pm SD$ values mean bovine DNA in target matrixes (bovine-caprine milk mixtures, commercial caprine milks and cheeses) as predicted by the model.

Target matrix	Mean bovine $C_T \pm SD$	Mean bovine DNA (95% CI) (ng)
B/C-50% (v/v)	27.96 ±0.30	0.61 [0.46, 0.82]
B/C-10% (v/v)	30.71 ±0.21	0.12 [0.09, 0.15]
B/C-5% (v/v)	32.44 ±0.12	0.04 [0.03, 0.05]
B/C-1% (v/v)	35.55±0.17	6.06 x 10 ⁻³ [4.33 x 10 ⁻³ , 8.47 x 10 ⁻³]
B/C-0.5% (v/v)	37.40 ±0.24	1.97 x 10 ⁻³ [1.32 x 10 ⁻³ , 2.93 x 10 ⁻³]
CM-1	ND	-
CM-2	ND	-
CM-3	39.01 ±0.69	-
CM-4	ND	-
CM-5	ND	-
CCH-1	ND	-
CCH-2	ND	-
CCH-3	ND	-
CCH-4	ND	-
CCH-5	ND	-

Note: ND: not detected.

labelled as caprine milk or bovine-caprine mixture products. The method was validated on 10 standard cheeses: two for each species and eight products with mixed milk, containing different proportions (0.1% – 50% (w/w)) of bovine and caprine milk.

Unlike the end-point PCR, real time PCR utilise fluorescent signal to identify target via threshold cycle (C_T) (Caldwell, 2017).

Zhang et al. (2007) conducted TaqMan real-time PCR assay using a bovine-specific primer pair for the cyt-b gene and mammalian-specific cyt-b probe. LOD of the assay was <35 pg of bovine DNA and showed no cross-reaction with ovine, caprine or porcine DNA. The system has been successfully used to measure bovine DNA in fresh and processed meat, milk and cheese, and would be useful for bovine species identification and quantitative authentication of animal-derived products.

In the study of Mininni et al. (2009), a TaqMan real-time PCR assay was developed to detect and quantify bovine milk in caprine and ovine cheeses, based on two target genes. The cyt-b gene of *Bos taurus* was used to detect and quantify bovine DNA. The nuclear gene Myo, mt18S rRNA and mt16S rRNA were used alternatively as universal reference markers. Caprine (n = 30) and ovine (n = 51) cheese samples were purchased and analysed and most were shown to be contaminated by bovine milk. Regarding the sensitivity, LOD of cyt-b assay for bovine DNA corresponded to 0.2% (w/w) of bovine for standard caprine and ovine cheeses. Next, LOD of 16S assay for bovine DNA corresponded to 0.5% (w/w) for ovine cheese, the 0.5% (w/w) for ovine cheese obtained by the 16S assay, the 1% (w/w) for the 16S assay in caprine cheese. LOQ of Myo and 18S assays were 1% (w/w) for both species.

Rentsch et al. (2012) developed and interlaboratory validated two multiplex TaqMan real-time PCR assays to determine DNA of bovine, ovine and caprine in milk and cheese. For caprine DNA, milk and cheese assays showed amplification efficiency of 85% and 116%, respectively. Linear detection and quantification range was 0.32 – 32 ng of caprine DNA ($R^2 = 0.99$) of the total DNA in both assays.

Agrimonti et al. (2015) described a unique quadruplex SYBR Green real-time PCR platform for the simultaneous detection of milk ingredients (bovine, buffalo, ovine, goat) in dairy products and for quantification of bovine milk in the same products. The methodology enabled the detection of DNA from *Bos taurus* in mixes of milk and cheeses with a LOD = 0.1%. A good correlation ($R^2 > 0.9$) between peaks' area of derivative of melting curves of amplicons and percentages of bovine milk in bovine-caprine milk mixtures and bovine-caprine cheeses, allowed for an estimation of bovine DNA in a dynamic range 0.1 – 10% and 0.1 – 5%, respectively.

Di Domenico et al. (2017) developed and validated 4 TaqMan real-time PCR assays for species identification in dairy products. Totally, 18 commercial samples were analysed. Moreover, the authors were first who confirmed analysis of the samples by IEF, the official European Union reference method. The PCR assays were based on the amplification of a short sequence of mt12S rRNA or cyt-b. The analysis conducted on milk mixtures at the 1% level showed C_T values within the range of linearity

($R^2 \geq 0.99$) of the standard curve for every species tested. Amplification efficiency for all species was $\geq 96\%$. The method revealed a very high level of repeatability. For each assay, DNA from the other species was tested as non-target DNA, using the same amount, and no cross-amplifications were observed. The LOD was 25 pg for bovine, 19 pg for buffalo, 2.5 pg for ovine and 350 fg for caprine, which corresponded to 0.5% (v/v) of bovine, <0.5% (v/v) of buffalo, 0.05% (v/v) of ovine and <0.05% (v/v) of caprine milk admixture.

The milk can be used as a source of DNA because it contains many somatic cells, mostly leucocytes but also epithelial cells from the animal (Sakaridis et al., 2013). Due to the fact that each somatic cell has several copies of mitochondrial DNA and as there are approximately 1000 mitochondria in each somatic cell, there should be expected sensitive detection of milk species (Klančnik et al., 2015). Besides, short amplicons enhance the possibility of amplification in dairy products that have undergone intense treatments such as pasteurization, UHT treatment, rennet or acid coagulation, drying, fermentation, ripening, smoking, high pressure treatment, pH modification, and irradiation (Di Domenico et al., 2017). Nevertheless, accurate quantitative determination of different milk percentages in milk mixtures and cheeses is still problematic. Since DNA is derived from somatic cells that can vary depending on physiological and non-physiological (e.g. mastitis) levels and because several factors in cheese technology may influence the final DNA concentration, DNA-based methods can only provide approximate values (Di Domenico et al., 2017).

CONCLUSION

Adulteration of milk and dairy products is being serious issue not only from economic aspect but also consumer health. To ensure that products meet strict legislation criteria and to avoid adulteration, reliable assays should be used in routine. The commercial assay tested in the study has proved that real-time PCR is accurate and sensitive tool, which has the good potential to reveal adulteration. Taking into account the facts, qPCR-based techniques should be used for semi-quantitative purposes only.

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