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REAL-TIME PCR DETECTION OF FRAUDULENT ADDITION OF BOVINE MILK TO CAPRINE AND OVINE MILK

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Abstract

Adulteration of caprine (*Capra hircus*) and ovine (*Ovis aries*) milk and dairy products by cheaper bovine (*Bos taurus*) milk is a common fraud attempted with the desire of profit. The intention to shorten and simplify the control of such deceits has encouraged the development of various analytical methods. Detection of bovine DNA from somatic cells using real-time PCR is a promising approach for qualitative and quantitative determination of bovine milk presence in milk and other dairy products.

In our study, we evaluated specific real-time PCR with BOS/MAN system to detect bovine and mammalian DNA. First, the most efficient method of DNA extraction was selected followed by optimization of real-time PCR protocol with SybrGreen I and TaqMan chemistry. Then, the standard curves were designed and the area of linearity, efficiency, sensitivity and specificity of the BOS/MAN systems were determined. Sensitivity determined theoretically with standard plasmid DNA was 0.6 pg/µL and practically with standard milk mixtures was 0.5 % of bovine milk in the tested samples.

We confirmed that the BOS/MAN system is adequate for qualitative detection of bovine milk in caprine and ovine milk. Although some researchers claim to perform successful quantification using real-time PCR for bovine milk, we could not repeat their results. The main issue in absolute quantification of bovine milk in samples consisted of mixed milk is the variable number of somatic cells in the milk. Information about this is surprisingly limited, what will be the focus discussion.

***Key words**: Real-time PCR, Milk adulteration, Quantification.*

1. Introduction

Food adulteration is a problem in the food industry. The adulteration of milk is achieved in two basic ways. The first is by dilution of the milk with water, and the second is by dilution of the more expensive kinds of milk (e.g., caprine milk, ovine milk) with a less expensive milk (e.g., bovine milk) [1 to 4]. The adulteration of milk with water can be detected by determination of the freezing point. The adulteration of caprine or ovine milk with bovine milk can be detected by analysis of the milk proteins or DNA. The current official method for the detection of bovine milk in milk or milk products from other dairy animals is isoelectric focusing of the caseins [5]. This is based on extraction of the caseins from the milk or milk products, isoelectric focusing of these caseins, and comparison of the protein bands obtained with standard milk containing 0% and 1% bovine milk. This analysis is laborious, time-consuming, and relatively expensive, so other alternative approaches are being investigated. Alternative methods must have a detection limit of 0.5% adulteration or lower, exclude the possibility of false-positive results, and be suitable also for the detection of bovine milk in long ripened cheeses.

Alternative methods for the detection of adulteration of caprine and/or ovine milk and/or milk products include PCR and real-time PCR (qPCR) [6 - 12]. The main issue in the quantification of bovine milk in samples of mixed milks is the variable number of somatic cells that might be present in the milk. If the starting concentrations of somatic cells in the bovine milk used to prepare adulterated caprine or ovine milk are not known, this limits the accurate quantification of the level of adulteration. To our knowledge, only Dąbrowska *et al*. [6] and Mayer *et al*. [12] have considered this problem of the somatic-cell concentrations and the quantification of the DNA.

The aim of our study was the application of a qPCR-based method for the detection of bovine milk in mixtures of bovine and caprine or bovine and ovine milk, and consideration of the possible causes of errors in the qPCR quantitative analysis of this adulteration with bovine milk.

2. Materials and Methods

2.1 Milk samples and DNA extraction

Samples of fresh whole bovine (*Bos taurus*), caprine (*Capra hircus*) and ovine (*Ovis aries*) milk were obtained from different Slovenian producers. The samples were divided into smaller aliquots and stored at -80 ^{0}C. The total DNA was extracted from 1 mL samples of the milk and milk mixtures using NucleoSpin Food kits (Macherey-Nagel, Düren, Germany), following the protocol described previously [13]. The concentration and purity of the extracted DNA were determined from the absorbance at 260 nm (A260) and the A260/A280 ratio, respectively, using a LAMBDA Bio PLUS spectrophotometer (Perkin-Elmer, Norwalk). Standard bovine DNA was obtained from DNA extracted from bovine milk, amplified with the mammalian system (see section 2.2), and ligated into the plasmid of *Escherichia coli* JM109 high-efficiency-competent cells (Promega, Madison, USA); this was used for optimisation of the qPCR. The DNA extracted from 10 samples of bovine milk, six samples of caprine milk, and six samples of ovine milk were used for the determination of the qPCR specificity. The standard milk mixtures were prepared with the bovine and caprine milk (caprine milk A) and the bovine and ovine milk (ovine milk A), such that they contained 5%, 10% and 20% bovine milk. Other milk mixtures were prepared on the same way as the standard milk mixtures, with the same bovine milk, but with different caprine milk (caprine milk B) and ovine milk (ovine milk B).

2.2 qPCR

All of the qPCR reactions were carried out in an ABI PRISM 7500 instrument (Life Technologies). Two systems were used for the qPCR, as reported by López-Calleja [7]: a bovine (BOS) system with BOS primers amplified a 252-bp amplicon specific for *B. taurus*; and a mammalian (MAN) system with MAN primers amplified a 426-bp amplicon specific for mammalian systems. Universal thermal cycling conditions were used for all of the reactions (10 min at 95° C, 40 cycles of 15 s at 95° C, and 1 min at 60° C). The detailed protocols of these qPCR reactions were as described previously [13].

2.3 Simulation of possible combinations of somatic cell concentrations

Simulations were performed using data from routine somatic cell concentrations measured in Slovenian bovine [14], caprine and ovine milk in 2012 [15]. As the distribution of somatic cells in the milk is not based on a normal Gaussian distribution; the log-normal statistics was used. From the data provided, we calculated the 95% probability range and central value (median) of the somatic-cell concentrations for bovine, caprine and ovine milk.

3. Results and Discussion

According to the aim of the study, we introduced and optimized the qPCR, constructed standard curves, and practically and theoretically showed the influence of the somatic-cell concentrations in the milk mixtures on the bovine milk quantification obtained by qPCR.

3.1 Optimisation of qPCR

Comparisons of the efficiencies and correlation coefficients (R^2) of the standard curves obtained by amplification of sequentially diluted standard bovine DNA showed the best performance of qPCR for the BOS system when using 300 nM BOS forward primer and 600 nM BOS reverse primer, and for the MAN system when using 300 nM MAN forward primer and 900 nM MAN reverse primer. The optimal probe concentration was 50 nM for both systems (results not shown). The specificity of qPCR with SybrGreen I was 88.8% and 100% for the BOS and MAN systems, respectively, and 100% for both systems when used with TaqMan chemistry (Table 1). The theoretical sensitivity obtained with standard bovine DNA was 0.0015 ng per reaction mixture. The practical sensitivity was determined with amplification of the caprine and ovine milk mixtures containing the different percentages of bovine milk. The results show that the practical sensitivity was 0.5% bovine milk in caprine or ovine milk. The R^2 for the BOS and MAN systems were good (0.98/0.99), although the efficiencies for the BOS and MAN systems were low (62.0% to 85.3%; Table 2). As the BOS and MAN systems had better efficiencies with SybrGreen I than with TaqMan chemistry, we used the SybrGreen I chemistry for the further experiments.

Table 1. qPCR parameters for the BOS and MAN systems, as optimised for the SybrGreen I and TaqMan chemistries

qPCR parameter	SybrGreen I		TaqMan	
	BOS system	MAN system	BOS system	MAN system
Specificity (%)	88.8	100.0	100.0	100.0
Theoretical sensitivity (ng per reaction)	0.0015	0.0015	0.0015	0.0015
Practical sensitivity (%)	0.5	/	0.5	/
Correlation coefficient (R^2)	0.98	0.99	0.99	0.98
Efficiency (%)	85.3	69.9	82.9	62.0

3.2 Quantification of the bovine milk adulteration

The standard curves for quantification of the bovine DNA with qPCR were generated from qPCR data obtained with standard milk mixtures that consisted of bovine and caprine milk, and bovine and ovine milk (data not shown) [9]. These standard curves defined the logarithm of the percentage of bovine milk in the mixtures (abscissa) according to the corresponding ΔCt (ordinate), which was calculated as the difference between the Ct obtained with the BOS system (Ct_{BOS}) and that obtained with the MAN system (Ct_{MAN}) for each milk mixture (i.e., $\Delta Ct = Ct_{BOS} - Ct_{MAN}$). The linear trends were analysed, and the equations for the linear regression and R^2 were calculated: bovine plus caprine milk mixture, $y = -4.87x + 7.06$, $R^2 = 0.99$; bovine plus ovine milk mixture, $y = -2.22x + 3.05$, $R^2 = 0.95$.

The data for the quantification of the bovine milk in mixtures #1 to #3 (Table 2: caprine milk A) showed small deviations from the true values, as these samples were prepared from the same milk as the calibration curve. However, the deviations from the true values were much higher in mixtures #4 to #6 (Table 2: caprine milk B), as these mixtures were prepared with the second caprine milk sample. Similar results were obtained with the mixtures of bovine and ovine milk (Table 2: mixtures #7 to #9, ovine milk A; #10 to #12, ovine milk B). The reason for these deviations might be different concentrations of somatic cells in caprine milk A and caprine milk B, and also in ovine milk A and ovine milk B. However, there might also be other reasons, such as the efficiencies of amplification, although these would be expected to be of less importance as the same BOS and MAN systems were used for all of the milk mixtures.

Table 2. qPCR results for the bovine DNA in the mixtures of bovine and caprine, and bovine and ovine, milk

Caprine or ovine milk	Mixture number (#)	Bovine milk in mixture (%)	qPCR result for bovine DNA (%)
Caprine milk A	1	20	15.1
	2	10	11.3
	3	5	5.7
Caprine milk B	4	20	10.2
	5	10	13.8
	6	5	7.9
Ovine milk A	7	20	34.0
	8	10	6.0
	9	5	2.1
Ovine milk B	10	20	5.6
	11	10	1.7
	12	5	2.4

3.3 Simulation of milk mixtures with different concentrations of somatic cells and their effects on quantification of bovine milk using qPCR

Somatic cell counts are known to vary to widely between individual milk samples [16]. To better understand the role of the somatic cells in these qPCR analyses of bovine milk, we also analysed the available data for the concentrations of somatic cells in bovine, caprine and ovine milk in Slovenia. Figure 1 shows an example of the distribution of the concentrations of somatic cells in ovine milk samples in Slovenia for 2012 [14]. We also collected these data for the concentrations of somatic cells in bovine and caprine milk samples, as analysed in Slovenia in 2012 (Table 3) [15].

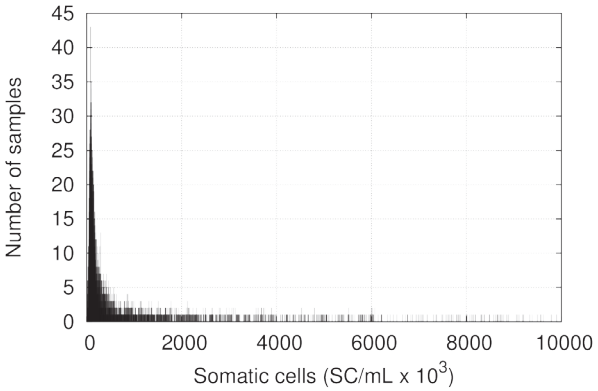

Figure 1. Distribution of the concentrations of somatic cells in ovine milk samples in Slovenia in 2012 [14]

The range of the concentrations of somatic cells that includes 95% of the milk samples is very wide for all three types of milk, thus demonstrating the difficulty for the quantification of bovine milk from somatic cells (Table 3).

Table 3. Somatic cell concentration ranges in bovine, caprine and ovine milk samples analysed in Slovenia in 2012 [14, 15]

Parameter	Bovine milk	Caprine milk	Ovine milk
95% range of somatic cell concentration (SC/mL)	10×10^3 – $2\,655 \times 10^3$	33×10^3 – $5\,605 \times 10^3$	60×10^3 – $5\,725 \times 10^3$
Median somatic cell concentration (SC/mL)	162×10^3	432×10^3	587×10^3

- SC, somatic cells

To illustrate the quantification problem caused by these uneven concentrations of somatic cells, we simulated examples of the caprine milk adulterated with 10% bovine milk using three different normally

occurring somatic-cell concentrations, and calculated the resulting percentages of bovine somatic cells in these mixtures of bovine and caprine milk (Table 4). As the quantification relies on the ratio of the bovine DNA and mammalian DNA, and as the DNA source is somatic cells in the milk, the ratio of the somatic cells is highly correlated to the amount of bovine milk determined by qPCR. According to the results of these simulations, the quantity of bovine milk determined changes together with the somatic cell count. Thus the quantification using standard curves will only be accurate when the concentrations of somatic cells in the standard samples and the tested adulterated samples are similar. These data thus indicate the possible effects of changing somatic-cell concentrations on the qPCR quantification. Indeed, Dąbrowska at el. [6] also indicated that 5% bovine milk with 8 $\times 10^5$ somatic cells can produce the same relative quantity of bovine DNA as 20% bovine milk with 5 $\times 10^5$ somatic cells. Thus, as indicated in Table 4 for the present quantification, only where the somatic-cell concentrations of the standard samples and the tested adulterated samples are the same (2 $\times 10^5$ somatic cells/mL) is the amount of added bovine milk confirmed as 10% using qPCR. This means that the results from these analyses can only be correct when the milk used for the preparation of the standard curves has a similar somatic-cell concentration as the bovine, caprine and ovine milk samples. Thus we can confirm that the somatic-cell concentrations in these milks influence the accuracy of the qPCR quantification [12].

Table 4. Simulation of the effects on the qPCR of variable somatic-cell concentrations in mixtures of 10% bovine milk and 90% caprine milk

Somatic cell concentration (cells/mL $\times 10^3$)		qPCR bovine milk analysis
Bovine milk	**Caprine milk**	**(%)**
50	200	3.0
200	200	**10.0**
400	200	18.2
50	700	0.8
200	700	3.1
400	700	6.0
50	1500	0.4
200	1500	0.5
400	1500	2.9

4. Conclusions

- Our data indicate that qPCR can be successfully used for the detection of bovine milk in samples of caprine and ovine milk that are adulterated with bovine milk.

- However, for the quantification of bovine milk in mixtures of bovine and caprine or ovine milk, the accuracy of the qPCR is affected by the concentrations of the somatic cells in these different types of milk, with further research needed to minimize these deviations.

- In practice, the extreme values of somatic-cell concentrations indicated for these milks in the present analysis would, however, probably not occur in samples from an actual market. This might allow the quantification of bovine milk in adulterated caprine and ovine milk with acceptable accuracy.

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5. References

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