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A Novel Approach to the Quantification of Bovine Milk in Ovine Cheeses Using a Duplex Polymerase Chain Reaction Method

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A duplex Polymerase Chain Reaction (PCR) method able to detect bovine milk in ovine cheeses was developed. This method is based on the mitochondrial 12S and 16S rRNA genes to generate fragments of different lengths. The proposed methodology presents an alternative DNA extraction procedure faster and more economical than the kits commercially available. A linear normalized calibration curve was obtained between the log of the ratio of the bovine band intensity and the sum of bovine and ovine band intensities versus the log of cow's milk percentage. The method was applied successfully to the detection and quantification of raw, pasteurized, and powdered bovine milk in different cheeses. The proposed duplex PCR provides a simple, sensitive, and accurate approach to detect as low as 0.1% bovine milk in cheeses and to quantify bovine milk in ovine cheeses in the range of 1−50%.

KEYWORDS: Cheese; Polymerase Chain Reaction; species identification; quantification; food authenticity

INTRODUCTION

Milk species identification in cheeses has received great attention in recent years. In particular, the identification of bovine and ovine milk in cheeses has a remarkable importance because of the possibility of detecting fraudulent procedures such as the substitution of ovine milk for bovine milk. The seasonal oscillations and the much lower ovine milk yield, together with the much lower price of bovine milk, are the main reasons for this adulteration. In addition, some cheeses are manufactured from defined amounts of each type of milk. Thus, authenticity issues in cheese analyses are challenged not only by milk species identification (e.g., the qualitative detection of bovine milk) but also by the need for quantitative determination of bovine milk in ovine cheeses manufactured from raw or processed milk.

Different analytical approaches have been applied for identification purposes; among these, immunological (*1*−*3*), electrophoretical (*4*−*5*), and chromatographic (*1*, *6*−*9*) are worth mentioning. The present European Community reference method for bovine milk detection is based on isoelectric focusing of β-casein (*10*). Nevertheless, few methods describe milk species quantification. A method based on isoelectric focusing and cation-exchange HPLC of *p*-κ-casein (*9*) has been proposed for quantitative analyses. However, as the estimated percentage of bovine milk in mixed cheese is strongly affected by the casein content of milks used for cheese manufacture, the results were approximate. On the other hand, methods for milk species quantification based on the whey protein fraction suffer from a shortcoming, as that fraction is more sensitive to heating than the casein fraction. Thus, such methods can cause false negatives when sterilized or powdered milk has been used in the cheese manufacture (*5*−*11*). Excessive proteolysis during cheese ripening can also be disadvantageous for quantification.

More recently, biomolecular techniques such as Polymerase Chain Reaction (PCR) have received particular attention. It is possible to use milk as a source of DNA and as a substrate for PCR. Plath et al. (*12*) applied PCR amplification and enzyme restriction of nuclear DNA to differentiate cow, goat, and ewe β-casein genes. Alternatively, methods based on mitochondrial DNA (mtDNA) have been successfully applied to detect the target species in cheeses using a simplex PCR (*13*) or a multiplex PCR (*14*, *15*). However, attempts to use the PCR technique as a quantitative tool in food analysis are mainly restricted to genetically modified organisms and contaminating microorganism screening. A simple approach to estimate the proportion of cow's milk in cheeses is described by Maudet and Taberlet (*13*), where these authors use the sigmoid augmentation of DNA amount versus cow's milk percentage. Another approach for quantification, but to estimate pork in ground beef and pâté, uses a linear relation of pork percentage versus the ratio of band intensity value to the band intensity of a 100% pork sample (used as an external standard) (*16*). However, it should be pointed out that accurate quantification is influenced by a number of variations that can occur during sample preparation or in the course of the PCR, and minor variations in reaction conditions are greatly magnified during the amplification process. These variations may partly be

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overcome by normalizing the amount of PCR products of the specific template with respect to an internal reference template as described for quantitative competitive PCR (*17*). Therefore, a DNA competitor has to be constructed, as described in an approach to quantify bovine species in meat and bone meal samples (*18*). Alternatively, real time PCR should be used to obtain reliable estimates of bovine species (*19, 20*).

In the present paper we describe a simple duplex PCR method able to identify bovine and ovine species in cheeses and to quantify bovine milk in ovine cheeses using a normalized calculation.

MATERIALS AND METHODS

Reference Cheese Samples. Reference cheeses were prepared in the laboratory using mixtures of bovine (Frísia breed) and ovine (Churra and Bordaleira breeds) raw milks obtained from local farmers. Cheeses were prepared according to the classical ovine cheese procedure from raw milk, to determine the amount of cow's milk. One pure ovine cheese, one pure bovine cheese, and eight mixture cheeses containing the following percentages of bovine milk, 50%, 20%, 10%, 5%, 2.5%, 1%, 0.5%, and 0.1%, were prepared. Aliquots of fresh and ripened cheese samples were collected and analyzed to study the influence of ripening on DNA integrity.

Cheese Samples for Validation of the Method. To validate the estimation approach, several blind tests were carried out using ovine cheeses with undisclosed proportions of cow's milk. Eight mixture cheeses containing the following percentages of bovine raw milk were prepared: 0.5%, 1%, 2.5%, 5%, 17% (two samples), and 33% (two samples). To verify the detection of powdered milk, two sets of cheeses containing 1% and 30% reconstituted bovine powdered milk in ovine raw milk were prepared.

Ten samples of soft and ripened Portuguese cheeses containing raw and pasteurized milks purchased from supermarkets (six labeled with ovine milk, three labeled with bovine/ovine milk, and one labeled with ovine/bovine milk) were analyzed to evaluate the applicability of the method to dairy products from the retail trade.

DNA Extraction. Samples of 200 mg of cheese were dissolved in 1.4 mL of 4 M guanidium isothiocyanate buffer using vortex mixing and heating in a water bath at 55 °C until homogenization. β-Mercaptoethanol (100 μL) and 500 μL of cooled ethanol (−20 °C) were added with brief mixing. The pellets resulting from centrifugation (16000*g* for 5 min) were dissolved in 50 μL of 6 M guanidium hydrochloride buffer with the addition of 10 μL of silica suspension in water (50%, v/v). After incubation at room temperature for 15 min, mixing every 2 min to allow DNA adsorption to the silica particles, the mixture was centrifuged (16000*g* for 5 min). The resultant pellet was resuspended in 80 μL of 6 M guanidium hydrochloride buffer and centrifuged (16000*g* for 2 min). The pellet was washed with 3 × 1 mL of washing solution (70% ethanol/50 mM Tris, pH 7.2/1 mM EDTA). After the last centrifugation (16000*g* for 5 min) and rejection of the washing solution, the pellet was centrifuged again briefly and the remaining solvent removed by pipeting. To ensure complete removal of solvent, which might inhibit PCR, the pellet was air-dried for 20 min at 50 °C. The DNA was eluted from the silica particles by the addition of 100 μL of Tris−EDTA buffer (10 mM Tris/1 mM EDTA, pH 7.5) and incubation at 50 °C for 20 min. Silica particles were separated from the DNA extract by centrifugation (16000*g* for 5 min), and the supernatant was transferred to a clean tube and stored at 4 °C.

PCR Amplification. Duplex PCR amplification was performed in a 25 μL total reaction volume containing 4 μL of pure DNA extract (ca. 5 ng), 15 mM Tris−HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, a 0.2 mM concentration of each dNTP (Invitrogene, Carlsbad, CA), 5 pmol of each bovine primer, 3 pmol of each ovine primer, and 1 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Branchburg, NJ). The primers used were the same as those proposed by Bottero et al. (*15*) and synthesized by MWG-BIOTECH AG (Ebersberg, Germany). The mitochondrial 12S rRNA and 16S rRNA genes generated fragments of 256 and 172 bp's, respectively, for bovine and ovine species.

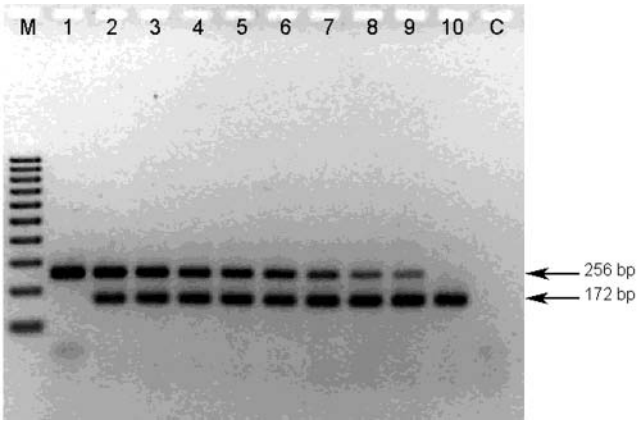

Figure 1. Agarose gel electrophoresis of PCR products amplified with 35 cycles from DNA of reference cheese samples. Key: M, 100 bp ladder; lane 1, bovine cheese; lanes 2–9, mixture ovine/bovine cheeses containing 50%, 20%, 10% 5%, 2.5%, 1%, 0.5%, and 0.1% bovine milk, respectively; lane 10, ovine cheese; lane C, negative control.

PCR amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc., Watertown, MA) using the following conditions: initial denaturation step at 94 °C for 5 min; 25−35 cycles at 94 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; a final extension at 72 °C for 5 min.

Amplified fragments were resolved on a 2% agarose gel electrophoresis carried out in Tris acetate/EDTA buffer for 75 min at 120 V, stained with ethidium bromide (0.4 μg mL^{-1} for 5 min), and destained in distilled water for 30 min. The agarose gel was visualized under UV light. A digital image was obtained using a Kodak Digital Science DC120, and the fluorescence intensity was measured using Kodak Digital Science 1D image analysis software.

Statistical Analysis. The intensities of the bands were used to obtain normalized calibration by regression analysis with 35 and 25 PCR cycles.

Student's *t* test was used to measure the significance of differences between percentages of bovine milk in known cheese samples and the mean results obtained with the reference curve of PCR of 25 cycles. A regression analysis between the actual and estimated amounts of bovine milk with 95% confidence intervals was performed. SPSS for Windows version 11.5 (SPSS Inc., Chicago, IL) was used for statistical treatment of the results.

RESULTS AND DISCUSSION

Method Optimization. The extracts of the cheese samples studied originated the PCR fragments of 172 and 256 bp's for ovine and bovine milks, respectively. The results showed the applicability of the proposed primers to Portuguese bovine and ovine breeds, as observed by Bottero et al. (*15*) for Italian breeds. Moreover, the amplification of the DNA extracts obtained with the procedure described above was compared successfully to the one obtained with the DNA extracts following the Dneasy tissue protocol (Qiagen GmbH, Germany), as this is the DNA extraction method most frequently applied to cheese (*13*−*15*, *21*) and milk (*22*, *23*). The results showed similar amplicons using both extraction procedures (data not shown), suggesting the use of the proposed method as a faster and more economical alternative to the commercial kits.

Figure 1 shows the amplification results of standard cheeses using 35 cycles, where it is possible to observe that 0.1% bovine milk can be detected using a duplex PCR. The same detection limit of 0.1% was obtained using the amplification of cow's mtDNA in goat's cheese (*13*), being lower than that obtained using a duplex PCR method with the same primers for cow (*15*) and also lower than the official isoelectric focusing method (*10*). Both methods allowed the detection of 0.5% cow's milk.

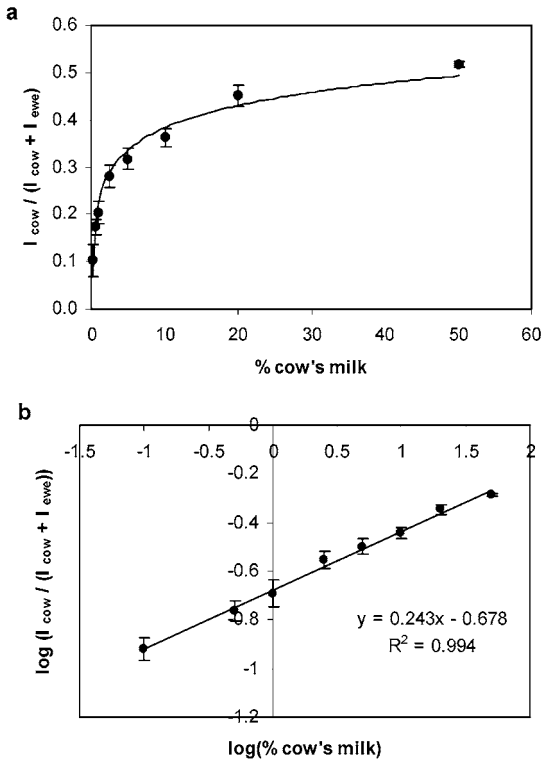

Figure 2. Normalized calibration curves for quantification of bovine milk in ovine cheeses obtained with a 35-cycle PCR: (**a**) relative band intensities versus cow's milk percentage; (**b**) logarithm of relative band intensities versus logarithm of cow's milk percentage. Band intensities for cow (I_{cow}) and for ewe (I_{ewe}) were obtained with image analysis software, Kodak Digital Science. Values are means of replicate assays ($n = 4$).

The intensity of the bands of the PCR products concerning reference cheeses was used to obtain a relation with cow's milk percentage. To overcome variations that might occur during sample preparation and thus affect the PCR, it is necessary to normalize the intensity of the target band. Using a duplex PCR, two products of each reaction are obtained; thus, if we relate the target band intensity (bovine) to a second band intensity (ovine), we can normalize the intensity because both products are equally affected by the presence of PCR inhibitors. As the sum of both bands should be constant, we relate the target band intensity to the sum of both bands. The normalized intensities for cow were calculated by the ratio of the band intensities for cow (I_{cow}) and the total band intensities for cow and ewe ($I_{cow} + I_{ewe}$). **Figure 2a** shows the curve of the relative intensity of the bands [$I_{cow}/(I_{cow} + I_{ewe})$] versus the percentage of cow's milk in cheeses, and **Figure 2b** depicts the same relationship but using a logarithmic scale in both axes. For quantitative purposes, linear relations allow more reliable determinations, so using logarithmic scales, it was possible to obtain a linear normalized calibration curve with a good correlation coefficient in the range of 0.1−50% (**Figure 2b**). However, the response factor should be the closest possible to unity to enable accurate determinations. For that purpose, the number of PCR cycles was optimized to decrease band saturation and thus increase the response factor (slope), without compromising excessively the sensitivity of the method. A PCR of 25 cycles (**Figure 3**) was considered a good compromise among linearity (**Figure 4**), response factor of the method (0.512), and sensitivity (1%).

The proposed method describes a normalized procedure without needing to construct a DNA competitor as internal standard, which makes it even simpler than the quantitative competitive PCR. Several drawbacks could be pointed out for the gel-based systems along with problems related to image processing (*24*). As mentioned above, the real time PCR can be used for quantitative PCR purposes and considered more accurate as it does not need post-PCR analysis. However, the simplicity and the relatively low-cost equipment needed to implement the proposed method compared with those of the real time PCR instrumentation are advantageous for routine analyses.

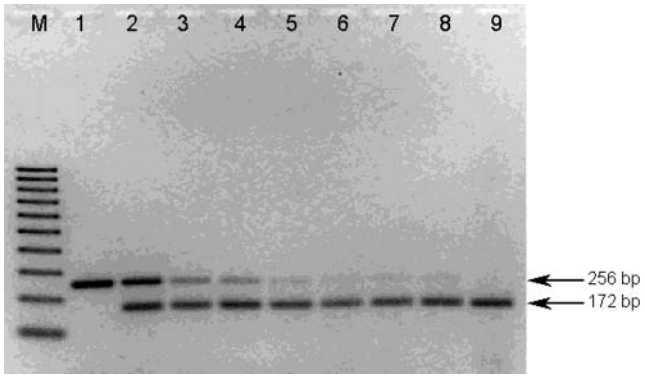

Figure 3. Agarose gel electrophoresis of PCR products amplified with 25 cycles from DNA of reference cheese samples. Key: M, 100 bp ladder; lane 1, bovine cheese; lanes 2–8, mixture ovine/bovine cheeses containing 50%, 20%, 10% 5%, 2.5%, 1%, and 0.5% bovine milk, respectively; lane 9, ovine cheese.

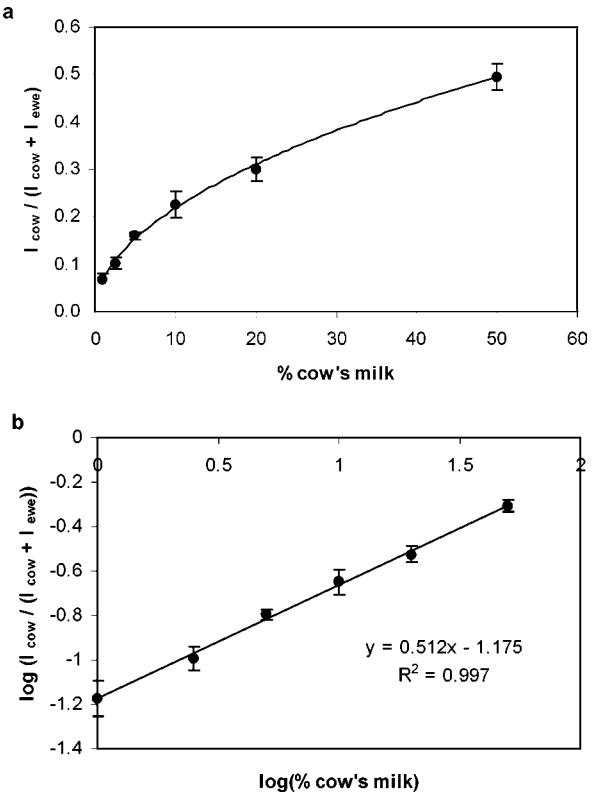

Figure 4. Normalized calibration curves for quantification of bovine milk in ovine cheeses obtained with a 25-cycle PCR: (**a**) relative band intensities versus cow's milk percentage; (**b**) logarithm of relative band intensities versus logarithm of cow's milk percentage. Band intensities for cow (I_{cow}) and for ewe (I_{ewe}) were obtained with image analysis software, Kodak Digital Science. Values are means of replicate assays ($n = 6$) obtained with three different PCR.

Prediction of Bovine Milk in Ovine Cheeses. The bovine milk content in ovine cheeses of initially unknown samples was

Table 1. Determination of Bovine Milk Percentage in Ovine Cheeses by a Duplex PCR Using the Calibration Curve with 25 Cycles (**Figure 4b**)

sample	% cow's milk (actual)	% cow's milk[a] (mean predicted)	SD[b]	CV[b] (%)
1	0.5	nd		
2	1	0.939	0.28	29.4
3[c]	1	3.04	0.48	15.1
4	2.5	2.85	0.17	6.13
5	5	5.11	1.0	19.5
6	17	13.4	1.8	13.6
7	17	12.5	2.1	16.7
8[c]	30	37.0	1.5	5.64
9	33	35.8	1.2	4.08
10	33	37.3	3.9	3.46

[a] Values are means of replicate assays ($n = 4$). nd = not detected. [b] SD = standard deviation. CV = coefficient of variation. [c] Percentage of reconstituted bovine powdered milk.

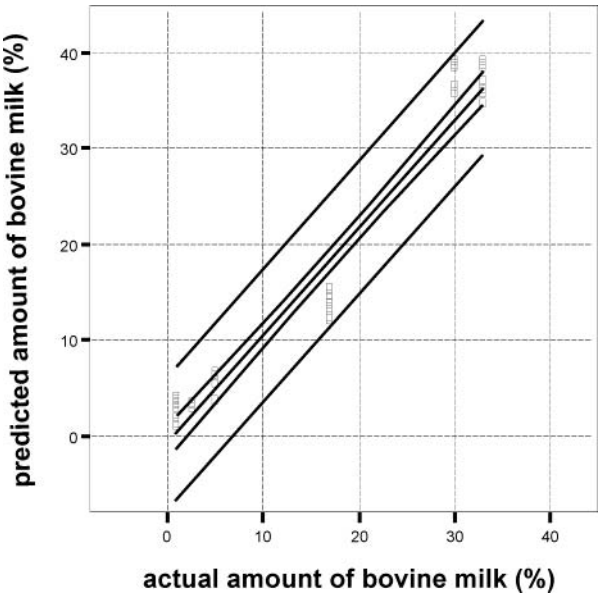

Figure 5. Regression analysis between the predicted bovine milk percentage in ovine cheeses and the actual value. $Y = -0.93 + 1.13X$, $R = 0.97$, $n = 37$.

easily quantified using the standard curve in the range of 1−50% (**Figure 4b**). **Table 1** shows predicted values of bovine milk percentages of cheese samples using the calibration curve of 25 PCR cycles. The correlation between estimated and actual values was 0.97 (**Figure 5**). The results show that the duplex PCR with 25 cycles provides a good estimate of the bovine milk proportion in ovine cheeses, including cheeses prepared with bovine powdered milk. The mean t value was 2.404, indicating that the predicted mean bovine milk content in known samples was not significantly different ($P > 0.05$).

The duplex PCR was applied to 10 commercial cheese samples to verify the label statements (**Table 2**). Eight samples confirmed the information given by labeling, while for two cheeses labeled with bovine/ovine milks only bovine milk was detected. In one sample also labeled with bovine/ovine milks it was possible to detect both species; however, because the bovine milk proportion was higher than 50%, only the extrapolation of the linear model makes it possible to obtain an estimate (91%). These findings are indicative of possible fraudulent procedures due to the substantial reduction or elimination of ovine milk. This fact suggests the development of a new model

Table 2. Results of Duplex PCR Performed on Portuguese Cheeses from the Retail Market

label	PCR results	estimated cow's milk[a] (%)	label	PCR results	estimated cow's milk[a] (%)
bovine/ovine	bovine	100	ovine/bovine	ovine/bovine	23.3 ± 3.3
ovine	ovine	nd	ovine	ovine	nd
ovine	ovine	nd	bovine/ovine	bovine	100
ovine	ovine	nd	ovine	ovine	nd
ovine	ovine	nd	bovine/ovine	bovine/ovine	>50

[a] Values are means of replicate assays ($n = 4$). nd = not detected.

for the quantification of the ovine milk amount in mixture cheeses with bovine/ovine milks.

Final Remarks. In this work a new method of DNA extraction of cheese samples is proposed as a faster and more economical procedure than the commercial kits available.

The duplex PCR described in this paper proved to be a fast and sensitive method that can be applied to the detection of raw or processed cow's milk in cheeses. Thus, it can also be applied to different types of cheeses, including those prepared with reconstituted powdered milk, contrarily to the protein-based methods available. The method provides a simple quantitative prediction of bovine milk adulterations in ovine cheeses by means of a linear normalized calibration curve in the range of 1−50%.

As the results obtained with the samples purchased from the market indicate possible fraudulent practices by drastic reduction of ovine milk, a similar method is being implemented to estimate the proportion of ovine milk in mixture cheeses (with less than 50% ovine milk).

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