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Short communication

Detection of undeclared bovine milk in different food matrices by a multi-technique approach

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ABSTRACT

The official method (i.e., isoelectric focusing, IEF) for the detection of bovine milk in cheeses obtained from ovine, caprine or buffalo milk, according to the Commission Implementing Regulation (EU) No 150/2018, was compared with three techniques: lateral flow immunoassay (LFA), ELISA and real-time PCR (RT-PCR). Samples of milk, cheese and vegetable drinks were analysed as control samples and after artificial contamination with bovine milk at different concentrations. All these assays, except for ELISA, showed a good diagnostic performance able to detect milk adulteration at concentrations lower than 1% of bovine milk in caprine, ovine and buffalo milk. Relative sensitivity, specificity and accuracy of both ELISA and RT-PCR compared with IEF were 100%, whereas for LFA they corresponded to 100%, 62.5% and 72.7%, respectively. A good agreement among ELISA and RT-PCR versus IEF was observed but only a moderate agreement between LFA and IEF.

1. Introduction

Milk and dairy products are important components of the human diet due to their high nutritional value, but they are often subjected to fraud that can constitute a risk to human, animal or plant health. One of the most common frauds is the substitution of milk from an animal species with another, for example the addition of bovine milk during the manufacture of buffalo mozzarella (Trimboli et al., 2019) or the use of bovine whey for the production of ricotta declared as deriving from buffalo milk, because it is cheaper and available during the whole year (Cerquaglia, Sottocorno, Pellegrino, & Ingi, 2011).

The official method for the detection of bovine milk and caseinate in cheeses obtained from ovine, caprine or buffalo milk is the isoelectric focusing (IEF) of γ-caseins after plasminolysis (i.e., proteolysis of caseins by plasmin) according to the Commission Implementing Regulation (EU) No 150/2018. This is only a qualitative method, laborious, time-consuming and can show overlapped species-specific bands causing a difficult interpretation of the results (Dal Bosco et al., 2018). Moreover, such method is not able to detect bovine milk in samples of plant origin because weak interfering bands can be observed (Di Domenico, Di Giuseppe, Wicochea Rodríguez, & Cammà, 2017). In Italy, such a method is performed only by few laboratories and also other assays are developed, like molecular techniques, i.e., real-time PCR (RT-PCR). These are sensitive, specific and rapid and can be easily automated, allow quantitative or semiquantitative analysis but require more technical skills (Agrimonti, Pirondini, Marmiroli, & Marmiroli, 2015). Immunological assays, such as ELISA and lateral flow assay (LFA), are among the most widely used techniques for dairy component identification because of their specificity, simplicity and sensitivity. The limitations of such methods can be the requirement for sufficient amounts of antibodies to detect analytes, the extensive purification procedure to eliminate cross-reactivity, and variable affinity (Asensio, González, García, & Martín, 2008).

The need to ensure for consumers that raw milk quality and/or supply dairy products are compliant with what is declared in the label requires the implementation of rapid, simple, effective and

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reliable analytical assays as screening methods. The aim of this study was the comparison of IEF with three alternative techniques, LFA, ELISA and RT-PCR, for analysis of cheese samples. To detect potential fraud and protect consumer interests, these rapid methods were also assessed on milk and vegetable drink samples, which were collected from the market and analysed as control samples and after artificial contamination with bovine milk at different concentrations.

2. Materials and methods

2.1. Samples

Eleven cheese samples (Table 1) and four types of vegetable drinks (soy, rice, almond and oat) were purchased from local retailers. To simulate adulterations, mixtures of bovine/ovine milk, bovine/buffalo milk and bovine milk with vegetable drinks were prepared.

2.2. Real-time PCR

DNA of plant beverages was extracted from 7 mL of each sample using the precipitating cetyltrimethylammonium bromide (CTAB) method (UNI EN ISO 21571:2005). Cow-specific RT-PCR assay was previously designed on *cyt-B* target gene for meat specimens (Cammà, Di Domenico, & Monaco, 2012) and then adapted to milk and cheese samples (Di Domenico et al., 2017).

2.3. ELISA, lateral flow assay and isoelectric focusing

Cheese samples and mixtures of milk were prepared and analysed by commercial ELISA kits using the Ridascreen® Cis (cat. number R4302, R-Biopharm, Darmstadt, Germany) and the Bio-Shield Cow Cheese (cat. number B1748, ProGnosis Biotech, Larissa, Greece) according to manufacturer instructions. Cheese samples and mixtures of milk and bovine milk/vegetable drinks were also tested by the commercial LFA Rapid Test Cow (cat. number R1230/R12120, ProGnosis Biotech), according to manufacturer instructions. Five repetitions or each sample were made. Pure bovine, ovine and buffalo milk samples were used as controls for ELISA and LFA.

IEF for cheese samples was performed according to the EU Commission protocol using ready-to-use polyacrylamide gel (Cerquaglia & Avellini, 2004). Certified reference standards of a mixture of renneted ewes and goats skimmed milk containing 0% and 1% bovine milk were purchased from the Commission Institute for Reference Materials and Measurements (Geel, Belgium). Laboratory interim-standards of 100% renneted bovine milk and renneted buffalo milk containing 0%, 1%, 10%, 20%, 30%, 50% and 70% of bovine milk were produced according to the EU Commission protocol, lyophilised and stored at −20 °C until use.

2.4. Statistical analysis

The results of ELISA, RT-PCR and LFA, applied on cheese samples, were compared with IEF results (gold standard) and Cohen kappa coefficient (κ) was calculated. Sensitivity, specificity and accuracy of each test and their limit of confidence at 95% of probability, were also calculated using Beta distribution.

3. Results

3.1. Analysis of cheese

The IEF results confirmed the presence of the species indicated on the label in all investigated cheese samples. The two commercial ELISA kits identified bovine milk in three samples (one bovine cheese and two *caciotta mista*) at concentrations above the most concentrated standard (10% for the Ridascreen® Cis and 4% for Bio-Shield Cow Cheese). In the other cheese samples, made with ovine, goat and buffalo milk, the concentration of bovine milk was below 1%. The LFA detected the presence of bovine milk in six cheese samples (one bovine cheese, two *caciotta mista*, two *mozzarella di bufala* and *caciocavallo*). Repeatability of LFA was 100% for all tested cheese samples. The results of IEF, ELISA, LFA and RT-PCR are summarised in Table 1. Relative sensitivity, specificity and accuracy of the two commercial ELISA kits and RT-PCR, compared with the gold standard IEF were 100%, as well as the complete agreement ($\kappa = 1$) calculated by the Cohen kappa coefficient (Table 2), while relative sensitivity, specificity and accuracy of LFA were 100%, 62.5% and 72.7%, respectively; the Cohen kappa coefficient was not significant ($\kappa = 0.48$).

3.2. Analysis of milk and vegetable drinks

Pure bovine milk was correctly identified by the two ELISA kits, RT-PCR and LFA. All these methods gave negative results for bovine detection in pure ovine milk samples. Conversely, bovine milk was incorrectly revealed in pure buffalo milk by both ELISA tests and also by LFA (Table 3).

Table 1
Results of isoelectric focusing (IEF), ELISA, lateral flow immunoassay (LFA) and real-time PCR (RT-PCR) on cheese samples analysed for the presence of bovine milk, expressed as percentage.[a]

Sample	Label	IEF	ELISA		LFA	RT-PCR
			Ridascreen® Cis	Bio-Shield Cow Cheese		
Pecorino ovine cheese	Ovine	<1	<0.1	<1	−	−
Pecorino ovine cheese	Ovine	<1	<1	<1	−	−
Pecorino ovine cheese	Ovine	<1	<0.1	<1	−	−
Caprine cheese	Caprine	<1	<0.1	<1	−	−
Caprine cheese	Caprine	<1	<1	<1	−	−
Bovine cheese	Bovine	100	>10	>4	+	+
Caciotta mista	Bovine-ovine	50	>10	>4	+	+
Caciotta mista	Bovine-ovine	50	>10	>4	+	+
Mozzarella di bufala (PDO)	Buffalo	<1	<1	<1	+	−
Mozzarella di bufala (Non-PDO)	Buffalo	<1	<1	<1	+	−
Caciocavallo	Buffalo	<1	<1	<1	+	−

[a] Ranges of standard concentrations were 0.1−10% and 0−4% bovine milk for Ridascreen® Cis and Bio-Shield Cow Cheese, respectively; limit of detection for LFA was 0.1% bovine milk in ovine milk.

Table 2
Relative sensitivity, specificity, accuracy and Cohen kappa coefficient of ELISA, lateral flow immunoassay (LFA) and real-time PCR compared with isoelectric focusing (IEF) applied to cheese samples.[a]

Parameter	IEF versus ELISA			IEF versus LFA			IEF versus real-time PCR		
	Observed Value	95% LCL	95% UCL	Observed value	95% LCL	95% UCL	Observed value	95% LCL	95% UCL
Relative sensitivity	100.0	47.3	100.0	100.0	47.3	100.0	100.0	47.3	100.0
Relative specificity	100.0	71.7	100.0	62.5	29.9	86.3	100.0	71.7	100.0
Relative accuracy	100.0	77.9	100.0	72.7	42.8	90.1	100.0	77.9	100.0
Cohen kappa coefficient (κ)	1.00			0.48			1.00		

[a] Values for sensitivity, specificity and accuracy are %. Abbreviations are: LCL, lower confidence limit; UCL, upper confidence limit.

Table 3
Results of ELISA, lateral flow immunoassay (LFA) and real-time PCR (RT-PCR) on samples of pure bovine, ovine and buffalo milk and artificial mixtures of bovine milk in ovine and buffalo milk, expressed as percentage.[a]

Sample	ELISA		LFA	RT-PCR
	Ridascreen® Cis	Bio-Shield Cow Cheese		
Pure bovine milk	>10	>4	+	+
Pure ovine milk	<0.1	0	−	−
Pure buffalo milk	>1	<1	+	−
5% bovine milk in ovine milk	>1	>4	+	+
1% bovine milk in ovine milk	<1	<1	+	+
0.5% bovine milk in ovine milk	<1	<1	+	+
0.25% bovine milk in ovine milk	Not tested	Not tested	+	Not tested
0.1% bovine milk in ovine milk	Not tested	Not tested	+	Not tested
0.05% bovine milk in ovine milk	Not tested	Not tested	+	Not tested
5% bovine milk in buffalo milk	>1	>4	+	+
1% bovine milk in buffalo milk	>1	<1	+	+
0.5% bovine milk in buffalo milk	>1	<1	+	+
0.25% bovine milk in buffalo milk	Not tested	Not tested	+	Not tested
0.1% bovine milk in buffalo milk	Not tested	Not tested	+	Not tested
0.05% bovine milk in buffalo milk	Not tested	Not tested	+	Not tested

[a] Ranges of standard concentrations were 0.1−10% and 0−4% bovine milk for Ridascreen® Cis and Bio-Shield Cow Cheese, respectively; limit of detection for LFA was 0.1% bovine milk in ovine milk. Presence only of test line in RT-PCR gives positive result (bovine milk detected more than 50%).

Bovine milk in ovine and buffalo milk at 5%, 1% and 0.5% was detected by all the methods, while lower concentrations (0.25%, 0.1% and 0.05%) were only tested by LFA (Table 3). All the mixtures of bovine milk in ovine and buffalo milk gave positive results for bovine milk by LFA (presence of both test line and control line in all the replicates). Repeatability of LFA was then 100% for all the artificial mixtures tested.

Pure vegetable drinks (soy, rice, almond and oat) gave negative results for bovine milk by LFA and RT-PCR, while all the mixtures of bovine milk (from 5% to 0.05%) in each type of vegetable drinks resulted positive for bovine milk by LFA. Repeatability of LFA was 100% for all the mixtures tested. Bovine milk samples spiked at 1% and 0.1% concentration in vegetable drinks were detected by RT-PCR in all cases, except for rice.

4. Discussion

Many techniques, e.g., immunological, electrophoretic, chromatographic, spectroscopic or biomolecular methods (Sezer et al., 2018) can be performed for milk adulteration but most of them require long time of analysis, expensive equipment and skilled technicians (Ullah, Khan, Ali, & Bilal, 2020). All the assays carried out in this study show gaps and advantages. ELISA kits are fast and useful for the simultaneous analysis of many samples, but they are more expensive and less sensitive than LFA. The latter gives results that can be visually read and used to test milk before cheese production. Both ELISA and LFA are commercially available as ready-to-use kits and can be carried out in parallel with IEF for the detection of undeclared cow milk in unknown cheese, milk and vegetable samples. The addition of bovine milk in goat, sheep and buffalo milk can be detected at a concentration higher than 1% with ELISA and lower than 1% with LFA and RT-PCR. Moreover, the LFA is cheap, fast and with the highest sensitivity, as it is able to detect also a minimum percentage (0.05%) of bovine milk in beverage drinks and in ovine milk, showing cross-reactivity with buffalo IgG.

According to the results of the cheese samples investigated, there is a good agreement (Cohen kappa value significant and equal to 1) among the two ELISA kits and RT-PCR versus the IEF, while agreement between LFA and IEF is only moderate (Cohen kappa value 0.48) (Landis & Koch, 1977), due to the cross-reaction between anti-bovine IgG antibodies used in the test with buffalo immunoglobulins. In fact, pure buffalo milk, two *mozzarella di bufala* and *caciocavallo*, that tested negative for bovine milk by IEF and RT-PCR, gave positive results for bovine IgG by LFA. Cross-reactions are due to amino acid sequence similarities of buffalo and bovine immunoglobulins (Saini, Maiti, & Kaushik, 2013) and monoclonal antibodies to bovine IgG and IgM bind to buffalo IgG and IgM (Carter, 1998). Ridascreen® Cis shows a cross-reaction between bovine and buffalo IgG, pure buffalo milk resulted positive for bovine IgG, whereas the two tested *mozzarella di bufala* samples and *caciocavallo* are negative. Bio-Shield Cow Cheese did not show any cross-reaction between bovine and buffalo immunoglobulins.

Hurley, Coleman, Ireland, and Williams (2004) reported an indirect competitive and sandwich ELISA able to detect 0.1% adulteration by bovine milk in ovine, caprine, and buffalo milk samples. The detection and quantification limits of the method described by Costa, Ravasco, Miranda, Duthoit, and Roseiro (2008) were ~0.2% adulteration for both bovine and goat milk. A detection limit of 1%, cross-reactivity < 1% and high reproducibility (coefficient of

variation < 10%) were observed by Ren et al. (2014) using the ELISA method. Rodrigues et al. (2012) applied a duplex PCR assay for the detection of bovine milk in goat milk and reported good repeatability, with a detection limit of 0.5%.

In conclusion, all the methods applied in this study show a good diagnostic performance except for ELISA kits. The most sensitive methods, such as real time PCR and LFA, are useful not only for the detection of fraud, but also to highlight any accidental contamination of bovine milk in milk and dairy products of other species and in plant beverages, to protect human health.

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