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Original Article

Presence of *Escherichia coli* O157, *Salmonella* spp., and *Listeria monocytogenes* in Raw Ovine Milk Destined for Cheese Production and Evaluation of the Equivalence Between the Analytical Methods Applied

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Abstract

Italy is one of the main producers and exporters of cheese made from unpasteurized sheep milk. Since raw milk and its derived products are known sources of human infections, cheese produced from raw sheep milk could pose a microbiological threat to public health. Hence, the objectives of the study were: to characterize the potential risk of the presence of pathogens *Escherichia coli* O157, *Listeria monocytogenes*, and *Salmonella* in raw ovine milk destined for cheese production obtained from all the sheep farms (n = 24) in the Marches region (Central Italy) that directly transform raw milk into cheeses and to evaluate the equivalence between the analytical methods applied. A three-step molecular method (simultaneous culture enrichment, species-specific DNA magnetic isolation, and multiplex real-time polymerase chain reaction) was used for milk (n = 143) and cheese (n = 5) analysis over a 3-year period. *L. monocytogenes* was not detected on any of the farms, while *E. coli* O157 was found on three farms, although only using the molecular method. Four farms tested positive for *Salmonella* spp., and *Salmonella enterica* subsp. diarizonae serovar 61:k:1,5,7 was isolated in one of those cases. This information highlights the need to develop preventative measures to guarantee a high level of consumer safety for this specific product line, and the molecular method could be a time-saving and sensitive tool to be used in routine diagnosis.

Introduction

Sheep milk and derived cheese production are a significant economic sector for Southern European countries, and Italy ranks first among the main exporters of this product (FAOSTAT, 2015). Within Italy, the Marches region (Central Italy) is an area particularly devoted to sheep milk and many types of cheese are made with unpasteurized sheep milk. The so-called ''typical products'', which are often manufactured by the same small farm producing the milk, are popular among consumers and help to preserve local traditions, but can pose a microbiological threat to public health, since they are well-known sources of human infections (Gould *et al.*, 2014).

Considering the high level of consumption in Italy of locally produced raw ovine milk cheeses, our knowledge of the presence of *Escherichia coli* O157 in this product line appears to be inadequate. Goat milk and related dairy products have sometimes been associated with human cases of *E. coli* O157 infection (Caprioli and Tozzi, 1998; Allerberger *et al.*, 2001; McIntyre *et al.*, 2002; Ferens and Hovde, 2011). In the Lazio region, strains of *E. coli* O157 have been isolated from ovine milk with a prevalence of 0.6% (Rubini *et al.*, 1999).

Further epidemiological data indicate that *Listeria monocytogenes* has been found in a high percentage of hard cheeses made from raw or low heat treated sheep milk (EFSA, 2015) in EU countries, including Italy (Ministry of Work, Health and Social Policies, 2009). Moreover, 36 notifications for *Salmonella* and *L. monocytogenes* in milk and milk products were also reported by the RASFF Portal (RASFF, 2015).

A more thorough investigation of pathogen presence in raw ovine milk and related products could therefore provide useful information. Hence, the first objective of this study was to expand the information available about the presence of *E. coli* O157, *Salmonella* spp., and *L. monocytogenes* on ovine milk farms in the Marches region.

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The analytical system plays a main role in epidemiological investigations: recent studies on bovine raw milk highlighted the greater sensitivity of polymerase chain reaction (PCR)-based methods compared to conventional reference ones (Amagliani *et al.*, 2012; Giacometti *et al.*, 2013), with significant time reduction, particularly in case of multiple analysis platforms (Omiccioli *et al.*, 2009; Sjöling *et al.*, 2015).

However, the reliability of PCR detection methods in food depends, in part, on the purity of the target template and the presence of sufficient numbers of target molecules. In the present study, a Magnetic Capture Hybridization assay, which relies on selective isolation of target DNA by hybridization to oligonucleotide probes linked to magnetic nanoparticles, was proposed in a multiplex format (mMCH) for the simultaneous hybrid capture of target sequences from *E. coli* O157, *Salmonella* spp., and *L. monocytogenes*.

A complete three-step multiple platform, consisting of: (1) simultaneous culture enrichment in a specifically formulated medium, (2) species-specific mMCH, and (3) multiplex real-time PCR (m-rtPCR), was used in this work. The second aim of the study was to compare the molecular system with reference methods by first using raw ovine milk artificially contaminated with the three target species and then using milk and cheese field samples collected over a 3-year period.

Materials and Methods

Milk and cheese sampling and microbiological analyses

Samples of raw milk (n = 143), each in five 25 g-sample units (Italian Republic, 2007), were obtained from all 24 ovine dairy farms in the Marches (Central Italy) catchment area of the Istituto Zooprofilattico Sperimentale of Umbria and Marches that directly transform their own produced milk (Carloni *et al.*, 2016). Samples were collected during milking periods over 3 years. In the case of positive results by either microbiological or real-time PCR assays, cheese samples (25 g) made with the same contaminated lot of milk were obtained from the farms and tested with both methods.

Microbiological analyses were carried out by the current recommended reference methods: validated ELFA (Enzyme Linked Fluorescent Assay; BioMérieux, Marcy l'Etoile, France) for *Salmonella* spp. (VIDAS *Salmonella* SLM; AFNOR BIO n. 12/16-09/05), *L. monocytogenes* (VIDAS *L. monocytogenes* II LMO2; AFNOR BIO n. 12/11-03/04), and *E. coli* O157 (VIDAS *E. coli* O157 ECO; AFNOR BIO n. 12/8-07/10). Confirmation of ELFA-positive samples was made according to the following methods: ISO 6579:2002 for *Salmonella* spp., UNI EN ISO 11290-1:1996 for *L. monocytogenes*, and ISO 16654:2001 for *E. coli* O157.

Equivalence evaluation between the analytical methods and statistical analysis

The entire molecular platform was tested for equivalence with standard methods using artificially contaminated samples of ovine milk, as detailed below.

Preparation of artificially contaminated milk samples. Reference strains of the three bacterial target organisms (*Salmonella* Enteritidis ATCC 13076, *L. monocytogenes* ATCC 7644, and *E. coli* O157 ATCC 35150) were separately grown in trypticase soy broth (TSB) to 10^8 CFU/mL, confirmed by standard plate counts. Milk aliquots of 25 g were artificially contaminated with 0-1-10-100 colony-forming unit (CFU) (each level in 30 replicates) of each species simultaneously and diluted 1:10 in Multipathogen Enrichment Medium (MEM; Diatheva, Fano, Italy). After incubation at 35°C for 18 ± 2 h, the bacterial growth was determined by plating on selective media (ALOA agar, CT-SMAC, and Salmonella Chromogenic Medium).

After enrichment, 10 mL-aliquots were processed for DNA isolation by MCH.

Magnetic capture hybridization. SiMAG-Amino-modified magnetic nanoparticles from Chemicell (Berlin, Germany) were covalently conjugated with NH_2-oligonucleotides (capture probes LP, SP, or EP) (Omiccioli *et al.*, 2009) specifically designed for the three target pathogens. Probe attachment and capture efficiency tests were conducted as previously described (Amagliani *et al.*, 2006). Immobilization process yield and efficiency were indirectly estimated by subtraction of unbound probes (measured spectrophotometrically by A_{260} readings and quantified through standard curves) from the starting amount used in the functionalization protocol (5 μmol). Hybridization efficiency was also determined using fluorescently labeled probes with oligonucleotide sequences complementary to LP, SP, and EP (hybridization probes Lhyb, Shyb, and Ehyb). These probes were used in hybridization tests, then detached by denaturation and quantified spectrofluorimetrically.

A mixture of LP-, SP-, and EP-modified nanoparticles was prepared by combining equivalent volumes of each type, and 150 μg per sample was used for the simultaneous specific isolation of DNA of the three bacterial species in an mMCH assay. Briefly, 10 mL-aliquots of milk sample enrichments were centrifuged at 6000 *g* for 20 min at 4°C and bacterial pellets resuspended in 450 μL lysis buffer (0.05 M Tris-HCl pH 8, 0.05 M EDTA pH 8, and 0.5% v/v Triton X-100) with 9 μL RNase A 10 mg/mL and 80 μL Proteinase K 10 mg/mL, for enzymatic digestion at 37°C for 30 min. A subsequent centrifugation at 12,000 *g* for 10 min was required to eliminate the fat layer on the surface. After denaturation at 100°C for 10 min, mMCH was carried out as previously described (Amagliani *et al.*, 2010).

m-rtPCR analysis. DNA-nanoparticle hybrids (5 μL) were then amplified with the Multipathogen FLUO Kit (Diatheva), an m-rtPCR assay for the simultaneous detection of the three target species, with internal amplification control (IAC). The amplification was carried out in a Rotor Gene 6000 (Corbett Research, Sydney, Australia) with the following thermal protocol: 10 min at 95°C, 40 cycles of 20 s at 95°C and 1 min at 63°C. Fluorescence acquisition was set on Green (495–520 nm), Red (647–667 nm), and Orange (590–610 nm) channels for the detection of *Salmonella* spp., *L. monocytogenes*, and *E. coli* O157 respective signals, with Yellow channel (538–554 nm) for the IAC.

Statistical analysis. A comparison of m-rtPCR results with the ELFA method was statistically analyzed. A 2 × 2 table was constructed with positive and negative results obtained through both methods and analyzed by the Cohen's K coefficient to estimate concordance (Mackinnon, 2000).

Molecular analysis of milk and cheese

Milk samples of 25 g were enriched in 225 mL MEM for 18 ± 2 h at 35°C, then 10 mL-aliquots were analyzed as described above for artificially contaminated milk.

When there were discrepancies between ELFA and m-rtPCR results, 1 mL-aliquots of selective enrichment broths (CT-MAC, SX2, Fraser Broth) of VIDAS procedures were used for mMCH and subsequent m-rtPCR.

Cheese samples (25 g) were collected from farms, which tested positive for target pathogens in milk, and analyzed as above.

Results

Magnetic capture hybridization

Capture probe sequences and structures have been previously described and their specificity has already been assessed (Omiccioli *et al.*, 2009). In this study, they were used for nanoparticle functionalization through a well-established protocol (Amagliani *et al.*, 2006). Results of seven immobilization batches indicated good reproducibility and similar values among the three sequences. LP ranged from 690.5 to 788.1 pmol/mg of nanoparticles, SP from 602.4 to 735.5 pmol/mg, and EP from 513.2 to 649.4 pmol/mg.

Hybrid capture proved to be reliable. The efficiency percentage was always considerably higher than 60%, with EP-conjugated nanoparticles being the most efficient, despite showing the smallest amount of immobilized probe (Table 1). Thanks to the similarity of efficiency values, the combination in equal amounts of the three nanoparticle types to be used in an mMCH was possible.

Equivalence evaluation between the analytical methods and statistical analysis

Concentrations of bacterial cultures used for artificial contamination of milk samples were approximately as expected, with *Salmonella* Enteritidis ATCC 13076 being 0.99×10^8 CFU/mL, *L. monocytogenes* ATCC 7644 1.34×10^8 CFU/mL, and *E. coli* O157 ATCC 35150 0.62×10^8 CFU/mL.

Sheep milk samples of 25 g were artificially contaminated with 0-1-10-100 CFU of each bacterial species simultaneously (each level in 30 replicates). To ensure evaluation of the method with unequivocally contaminated samples, after enrichment in MEM, aliquots were plated on selective agar to assess bacterial growth. Bacterial presence of all three species was detected in every replicate of levels 10 and 100 CFU, and in 40% of level 1 CFU. Therefore, only positive replicates of level 1 CFU, along with all replicates of levels 0-10-100 CFU, were used for bacterial DNA isolation by mMCH and subsequent m-rtPCR. Zero-level samples tested negative in m-rtPCR, while all contaminated replicates tested positive for each pathogen. The IAC was correctly amplified, confirming the effective application of mMCH to such a complex matrix as sheep milk.

A comparison of the performance of ELFA and the molecular assay proved their equivalence. Relative accuracy, sensitivity, and specificity were all 100%, demonstrating their complete agreement (Cohen's K coefficient = 1).

As shown in Figure 1, although the m-rtPCR was designed to be qualitative rather than a quantitative assay, a relationship between average Cts and contamination levels can be observed for each pathogen.

Analysis of milk and cheese field samples

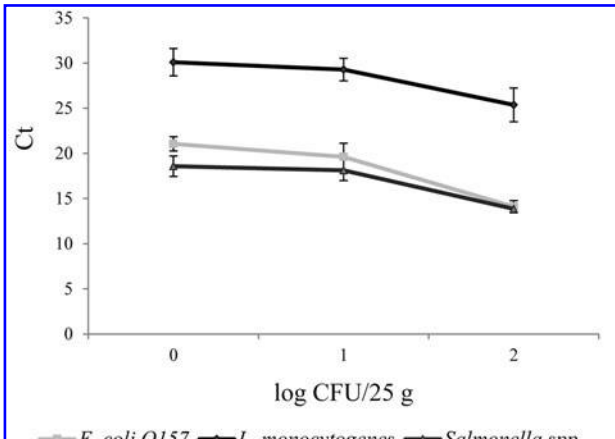
Throughout the 3-year study period, at least four samplings per farm were obtained, although in some cases sampling frequency was higher, thanks to farmer cooperation. A total of 715 25 g-sample units, corresponding to 143 samples in quintuplicate, were examined.

Microbiological analyses of milk. Negative results were always obtained through ELFA assays for *E. coli* O157 and *L. monocytogenes*. *Salmonella* spp. was found on two farms (farm 5, sample 23,600; farm 26, samples 6827, 7820, and 8960; 2.8%), although the microorganism was isolated using reference methods only from farm 26. Serotyping according to the White–Kauffmann–Le Minor scheme, recommended by the EU Reference Laboratory for *Salmonella* spp. and provided by ISO/TR 6579-3:2014, yielded a final identification of *Salmonella enterica* subsp. diarizonae serovar 61:k:1,5,7. These strains, also characterized for antimicrobial

Table 1. Efficiency Tests of Functionalized Nanoparticles

Immobilization process yield	*LP*	*SP*	*EP*
Average ± SD (%)			
Starting amount (5 μmol)	100	100	100
Unbound probe	24.3 ± 2.8	25.6 ± 4.4	35.4 ± 3.0
Covalently coupled probe	75.7 ± 2.8	74.4 ± 4.4	64.6 ± 3.0
Average ± SD (pmol/mg)			
Coupled probe/mg nanoparticles	737.6 ± 36.1	695.7 ± 47.4	596.6 ± 44.9
Total average ± SD			
Coupled probe in 5 mg nanoparticles	3687.9 ± 180.3	3478.4 ± 236.9	2983.0 ± 224.6
Hybrid capture efficiency	*Lhyb*	*Shyb*	*Ehyb*
Average ± SD (%)	68.8 ± 15.1	63.1 ± 6.5	69.5 ± 7.3

Immobilization process yield and hybrid capture efficiency. LP and Lhyb, *Listeria monocytogenes* captures probe and hybridization probe; SP and Shyb, *Salmonella* spp. captures probe and hybridization probe; EP and Ehyb, *Escherichia coli* O157 captures probe and hybridization probe.



Contamination level (log CFU/25 g)	Ct ± SD	
0	*Salmonella* spp.	18.58 ± 1.13
	E. coli O157	21.08 ± 0.79
	L. monocytogenes	30.10 ± 1.51
1	*Salmonella* spp.	18.13 ± 1.14
	E. coli O157	19.60 ± 1.53
	L. monocytogenes	29.28 ± 1.24
2	*Salmonella* spp.	13.85 ± 0.42
	E. coli O157	14.09 ± 0.67
	L. monocytogenes	25.36 ± 1.87

FIG. 1. Molecular assay validation. Average ± SD of Ct obtained through m-rtPCR of artificially contaminated samples (n = 30 for 0-10-100 CFU and n = 12 for 1 CFU of each bacterial target species). Contamination levels are expressed as log CFU/25 g. CFU, colony-forming unit; m-rtPCR, multiplex real-time polymerase chain reaction.

susceptibility according to CLSI (2008 and 2011), were susceptible for each of the tested antimicrobials.

Molecular analyses of milk. Samples were analyzed pooling the five sample units in a single sample. *L. monocytogenes* was never detected. Three samples tested positive for *E. coli* O157 DNA: 7011 (farm 11); 31,401 (farm 6); and 28,487 (farm 26) (2.1%). *Salmonella* spp. DNA was detected in various milk aliquots. *Salmonella* spp. positive samples were as follows: 23,155 (farm 24); 8962 (farm 22); 23,600, 24,800, and 31,242 (farm 5); and 6827, 7820, and 8960 (farm 26) (5.6%). In such cases, corrective actions were suggested to farmers (e.g., milking machine improved sanitation, substitution of milking machine filters, exclusion of diarrhetic cases from milking, and staff training) and shortly after the verification of their application, further samples were collected to check for the possible persistence of the contamination.

The molecular analysis of selective enrichments in CT-MAC or SX2, carried out when discrepancies were observed between ELFA and m-rtPCR (with Ct <34), always yielded positive results.

The discrepancy between ELFA and m-rtPCR may stem from the contamination level, which is reflected by Ct values. Indeed, samples with consistent results always showed Ct values <30, in the range between 20.92 and 29.56 (average 26.57), while discordant results showed Ct ranging from 27.69 to 32.40 (average 30.67) for *Salmonella* spp. and from 27.75 to 36.34 (average 32.80) for *E. coli* O157. It should also be noted that the only sample, from which a bacterial strain was isolated, yielded the lowest Ct (20.92). All results are shown in Table 2.

Cheese samples. All the farms investigated in the present study produce raw milk cheeses, which are sold to consumers at various ripening times. Cheese from farm 11, produced with *E. coli* O157 m-rtPCR positive milk, tested negative for the same pathogen using both ELFA and molecular methods.

Regarding *Salmonella* spp., cheeses from farms 5 and 24 tested negative with ELFA, but positive with m-rtPCR from SX2. The presence of *Salmonella* was confirmed in cheese from farm 26 both with ELFA and subsequent tests according to ISO 6579 and PCR-based assay (Table 2).

Discussion

E. coli O157 infections, salmonellosis, and listeriosis are common illnesses found worldwide and constitute a serious threat to food safety. The presence of even small amounts of pathogenic bacteria in milk destined for the production of raw milk cheeses constitutes a risk for consumers. In particular, the presence of viable *E. coli* O157 is of concern to all producers of raw milk cheeses since it can develop an adaptive tolerance response to acid environments, promoting resistance to gastric acid during the digestive process and the survival of an infective dose of bacteria (Vernozy-Rozand *et al.*, 2005). The need for further investigation of *E. coli* O157 in ovine milk is also justified by a number of experimental studies that have shown the pathogen's ability to survive acidic pH in several fermented dairy products (Gulmez and Guven, 2003), in white brined cheese (Osaili *et al.*, 2014) and in Cheddar cheese after 60 days of ripening (Schlesser *et al.*, 2006). The growth of *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 has recently been detected in cheese during extended storage at 25°C (Leong *et al.*, 2014). It is therefore imperative to ensure that milk used in cheese production is of the highest bacteriological quality.

The aim of this investigation was to broaden our knowledge of the presence of *E. coli* O157, *Salmonella* spp., and *L. monocytogenes* in ovine milk in Central Italy and to evaluate the equivalence between the analytical methods.

The recovery of bacteria from foods, including milk, is often complicated by the very low level of bacterial contamination. The use of a proper enrichment medium can therefore improve the sensitivity and reproducibility of the test. The medium MEM ensured the concurrent growth of the mentioned species and was able to enrich a test sample with multiple pathogens.

The subsequent phase of the molecular protocol was the mMCH. The immobilization process is highly standardized and provided materials suitable for the intended downstream application with food matrix enrichments. Apart from our group's work, the application of MCH for bacterial DNA detection in food has never been reported. However, MCH

Table 2. Summary of Results of Sheep Milk and Cheese Field Sample Analyses with Both ELFA and the Molecular Assay

		Salmonella *spp.*			Escherichia coli *O157*			Listeria monocytogenes		
			m-rtPCR (Ct)			*m-rtPCR (Ct)*			*m-rtPCR (Ct)*	
Farm No.	*Sample No.*	*ELFA*	*MEM*	*SX2*	*ELFA*	*MEM*	*CT-MAC*	*ELFA*	*MEM*	*FB*
5	23,600	+	29.21	n.d.	—	—	n.d.	—	—	n.d.
	24,807 (C)	−	—	—	—	—	n.d.	—	—	n.d.
	24,800	−	31.33	27.12	—	—	n.d.	—	—	n.d.
	31,242	−	27.69	19.66	—	—	n.d.	—	—	n.d.
	33,070 (C)	−	—	24.78	—	—	n.d.	—	—	n.d.
6	31,401	−	—	—	—	34.29	n.d.	—	—	n.d.
11	7011	−	—	n.d.	—	27.75	—	—	—	n.d.
	12,364 (C)	−	—	n.d.	—	—	—	—	—	n.d.
22	8962	−	32.40	35.00	—	—	n.d.	—	—	n.d.
24	23,155	−	31.25	29.04	—	—	n.d.	—	—	n.d.
	24,945 (C)	−	—	35.06	—	—	n.d.	—	—	n.d.
26	28,487	−	—	—	—	36.34	n.d.	—	—	n.d.
	6827[a]	+	20.92	n.d.	—	—	n.d.	—	—	n.d.
	9083 (C)	+	27.56	20.24	—	—	n.d.	—	—	n.d.
	7820	+	25.59	n.d.	—	—	n.d.	—	—	n.d.
	8960	+	29.56	n.d.	—	—	n.d.	—	—	n.d.

Only results of positive samples are reported.
[a]Isolation of *Salmonella enterica* subsp. diarizonae 61:k:1,5,7.
(C), cheese; CT-MAC, MacConkey broth with Cefixime and Tellurite; ELFA, Enzyme Linked Fluorescent Assay; FB, Fraser Broth; m-rtPCR, multiplex real-time polymerase chain reaction; MEM, Multipathogen Enrichment Medium; n.d., not determined; SX2, Salmonella Xpress 2.

proved highly effective when directly applied to milk samples (Amagliani *et al.*, 2006) and in multiplex format for the specific and simultaneous isolation of *E. coli* O157, *Salmonella* spp., and *L. monocytogenes* DNA in artificially and naturally contaminated bovine milk (Omiccioli *et al.*, 2009) and seafood (Amagliani, *et al.*, 2010). In this study, it has been used for bacterial DNA isolation from raw ovine milk and cheese. Results proved the equivalence of ELFA validated systems and the multiplex molecular assay. Hence, the molecular assay appears to be suitable for routine use and to support ISO culture methods for preliminary screening in accordance with the EU Regulation 2073/2005 recommendations concerning alternative methods. The molecular assay is a platform that could also be applied to other food product lines.

In the present study, the analysis of field samples of raw sheep milk revealed the absence of pathogens or their presence at very low levels. Previous investigations of the same farms (Carloni *et al.*, 2016) showed good hygienic conditions and the proper application of good farm management practices.

After a 3-year sampling period involving 715 sample units from 24 raw sheep milk farms, only one farm tested positive (ELFA and m-rtPCR with pathogen isolation) for *S. enterica* subsp. diarizonae 61:k:1,5,7 in milk and derived cheese. This result confirmed that sheep in Central Italy can be a potential reservoir for *S. enterica* subsp. diarizonae 61:k:1,5,7, in accordance with previous investigations (Davies *et al.*, 2001; Alvseike *et al.*, 2004; Zweifel *et al.*, 2004). However, although foodborne infections in humans caused by this serovar from domestic animals such as sheep have occasionally been described (Davies *et al.*, 2001), it is not included among the five serovars with public health significance (Commission Regulation [EC] No. 1003/2005), and there were no reports of its presence in food or animals in the last EFSA Report on zoonoses (EFSA, 2015).

Concerning *E. coli* O157, results of the molecular approach indicate occasional pathogen presence at very low levels. Hence, sheep milk and cheese producers must remain vigilant with this food matrix, paying particular attention to hygienic aspects of production. However, positive results obtained only by the molecular method should be considered with caution, since the results of biomolecular diagnoses should always be confirmed by pathogen isolation to prove its vitality (UNI EN ISO 20838:2006). m-rtPCR analysis of cheese samples produced with milk, which tested PCR positive and ELFA negative for *E. coli* O157, did not confirm the presence of the pathogen.

These results may be accounted for by the fact that the cheese was analyzed after 20 days of ripening, and pathogen growth might have been hampered by unfavorable conditions occurring in that matrix due to acidification and aging, especially if we consider the low starting concentration of the pathogen. Indeed, factors influencing pathogen survival and growth in cheese are numerous and include acidity, preservatives, temperature, competing flora, water activity, and salt concentration (FDA, 2015).

The implementation of food quality assurance systems along the ovine chain, as previously described for bovine milk (Cusato *et al.*, 2013), would provide clear benefits in term of hygienic quality. The steps involved should include a diagnosis of the prerequisites, training of the food handlers, and implementation of good manufacturing practices (GMPs), sanitation standard operating procedures (SSOPs), and hazard analysis and critical control point (HACCP) principles (Costa Dias *et al.*, 2012).

Conclusions

Results of this study suggest the presence of *Salmonella* spp. and *E. coli* O157 at very low levels and the absence of *L. monocytogenes* in sheep raw milk and cheese in Central Italy. Such data can inform the National Public Health Service of the need to implement an effective control strategy to ensure the safety of consumers, especially as regards the possible environmental contamination during and after milking.

Thanks to its intrinsic properties of high specificity and sensitivity, along with its rapidity, molecular diagnosis through validated methods could provide a very useful tool in early warning systems, thus offering improved and cost effective public health protection.

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Disclosure Statement

No competing financial interests exist.

References

Allerberger F, Wagner M, Schweiger P, Rammer HP, Resch A, Dierich MP, Friedrich AW, Karch H. *Escherichia coli* O157 infections and unpasteurised milk. Euro Surveill 2001;6: 147–151.

Alvseike O, Vardund T, Lindstedt B, Heir E, Eriksson E, Kapperud G. Molecular epidemiology and population genetics of *Salmonella* subspecies diarizonae in sheep in Norway and Sweden. Epidemiol Infect 2004;132:253–261.

Amagliani G, Omiccioli E, Brandi G, Bruce IJ, Magnani M. A multiplex magnetic capture hybridisation and multiplex Real-Time PCR protocol for pathogen detection in seafood. Food Microbiol 2010;27:580–585.

Amagliani G, Omiccioli E, del Campo A, Bruce IJ, Brandi G, Magnani M. Development of a magnetic capture hybridisation-PCR assay for *Listeria monocytogenes* direct detection in milk samples. J Appl Microbiol 2006;100:375–383.

Amagliani G, Petruzzelli A, Omiccioli E, Tonucci F, Magnani M, Brandi G. Microbiological surveillance of a bovine raw milk farm through multiplex real-time PCR. Foodborne Pathog Dis 2012;9:406–411.

Caprioli A, Tozzi AE. Epidemiology of Shiga toxin–producing *Escherichia coli* infections in continental Europe. In: *Escherichia coli O157:H7 and Other Shiga Toxin–Producing E. coli Strains*. Kaper JB, O'Brien AD (eds.). Washington, DC: American Society for Microbiology, 1998, pp. 38–48.

Carloni E, Petruzzelli A, Amagliani G, Brandi G, Caverni F, Mangili P, Tonucci F. Effect of farm characteristics and practices on hygienic quality of ovine raw milk used for artisan cheese production in central Italy. Anim Sci J 2016; 87:591–599.

Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Approved standard, 3rd ed*. CLSI Document M31-A3 (ISBN 1-56238-659-X). Wayne, Pennsylvania: Clinical and Laboratory Standards Institute, 2008.

Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-First Informational Supplement*. CLSI Document M100-S21. Wayne, Pennsylvania: Clinical and Laboratory Standards Institute, 2011.

Commission Regulation (EC) No 1003/2005 of 30 June 2005 implementing Regulation (EC) No 2160/2003 as regards a Community target for the reduction of the prevalence of certain salmonella serotypes in breeding flocks of Gallus gallus and amending Regulation (EC) No 2160/200.

Costa Dias MA, Sant'Ana AS, Cruz AG, de Assis F. Faria J, de Oliveira CAF, Bona E. On the implementation of good manufacturing practices in a small processing unity of mozzarella cheese in Brazil. Food Contr 2012;24:199–205.

Cusato S, Gameiro AH, Corassin CH, Sant'Ana AS, Cruz AG, de Assis F. Faria J, de Oliveira CAF. Food safety systems in a small dairy factory: Implementation, major challenges, and assessment of systems' performances. Foodborne Pathog Dis 2013;10:6–12.

Davies RH, Evans SJ, Preece BE, Chappell S, Kidd S, Jones YE. Increase in *Salmonella enteric* subspecies *diarizonae* serovar 61:k:1,5,(7) in sheep. Vet Rec 2001;149:555–557.

EFSA (European Food Safety Authority). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2013. EFSA J 2015;13: 3991 [162 p.].

EU (2005). Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Available at: http://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32005R2073, accessed December 1, 2015.

FAOSTAT, FAO Statistic Division, Available at: http://faostat.fao.org, accessed July 2015 and at: http://faostat.fao.org/site/342/default.aspx, accessed November 27, 2015.

FDA, Food and Drug Administration, 2015. Food and Drug Administration. Evaluation and Definition of Potentially Hazardous Foods—Chapter 3. Factors that Influence Microbial Growth. Available at: www.fda.gov/Food/FoodScience Research/SafePracticesforFoodProcesses/ucm094145.htm, Page Last Updated: 03/19/2015 accessed November 4, 2015.

Ferens WA, Hovde CJ. *Escherichia coli* O157:H7: Animal reservoir and sources of human infection. Foodborne Pathog Dis 2011;8:465–487.

Giacometti F, Bonilauri P, Serraino A, Peli A, Amatiste S, Arrigoni N, Bianchi M, Bilei S, Cascone G, Comin D, Daminelli P, Decastelli L, Fustini M, Mion R, Petruzzelli A, Rosmini R, Rugna G, Tamba M, Tonucci F, Bolzoni G. Four-year monitoring of foodborne pathogens in raw milk sold by vending machines in Italy. J Food Protect 2013;76:1902–1907.

Gould LH, Mungai E, Behravesh CB. Outbreaks attributed to cheese: Differences between outbreaks caused by unpasteurized and pasteurized dairy products, United States, 1998–2011. Foodborne Pathog Dis 2014;11:545–551.

Gulmez M, Guven A. Survival of *Escherichia coli* O157:H7, *Listeria monocytogenes* 4b and *Yersinia enterocolitica* O3 in different yogurt and kefir combinations as prefermentation contaminant. J Appl Microbiol 2003;95:631–636.

International Organization for Standardization, ISO 6579:2002 Microbiology of food and animal feeding stuffs—Horizontal method for the detection of *Salmonella* spp.

International Organization for Standardization, ISO/TR 6579-3:2014 Microbiology of the food chain—Horizontal method for the detection, enumeration and serotyping of Salmonella—Part 3: Guidelines for serotyping of *Salmonella* spp.

International Organization for Standardization, ISO 16654:2001 Microbiology of food and animal feeding stuffs—Horizontal method for the detection of Escherichia coli O157.

Italian Republic. *Conferenza Permanente per i rapporti tra lo Stato, le Regioni e le Province Autonome di Trento e Bolzano, Provvedimento n. 5/CRS del 25 gennaio 2007 in materia di vendita diretta di latte crudo per l'alimentazione umana*. GU Serie Generale n.36 del 13-2-2007- Suppl. Ordinario n. 36.

Leong WM, Geier R, Engstrom S, Ingham S, Ingham B, Smukowski M. Growth of *Listeria monocytogenes*, *Salmonella* spp., *Escherichia coli* O157:H7, and *Staphylococcus aureus* on cheese during extended storage at 25°C. J Food Protect 2014;77:1275–1288.

Mackinnon A. A spreadsheet for the calculation of comprehensive statistics for the assessment of diagnostic tests and inter-rater agreement. Comput Biol Med 2000;30: 127–134.

McIntyre L, Fung J, Paccagnella A, Isaac-Renton J, Rockwell F, Emerson B, Preston T. *Escherichia coli* O157 outbreak associated with the ingestion of unpasteurized goat's milk in British Columbia, 2001. Can Comm Dis Report 2002;28:6–8.

Ministry of Work, Health and Social Policies. (2009). *Listeria monocytogenes* in cheeses. Opinion n. 3 of 22/04/2009. Available at: www.salute.gov.it/imgs/C_17_pagineAree_1212_listaFile_itemName_3_file.pdf, accessed December 1, 2015.

Omiccioli E, Amagliani G, Brandi G, Magnani M. A new platform for Real-Time PCR detection of *Salmonella* spp., *Listeria monocytogenes* and *Escherichia coli* O157 in milk. Food Microbiol 2009;26:615–622.

Osaili TM, Al-Nabulsi AA, Olaimat AN, Shaker RR, Taha M, Holley RA. Survival of *Escherichia coli* O157:H7 during manufacture and storage of white brined cheese. J Food Sci 2014;79:1750–1755.

RASFF (Rapid Alert System for Food and Feed). (2015). Available at: https://webgate.ec.europa.eu/rasff-window/portal/?event=searchResultList, accessed December 1, 2015.

Rubini S, Cardeti G, Amiti S, Manna G, Onorati R, Caprioli A, Morabito S. Verocytotoxin-producing *Escherichia coli* O157:H7 in sheep milk. Vet Rec 1999;9:56.

Schlesser JE, Gerdes R, Ravishankar S, Madsen K, Mowbray J, Teo AY. Survival of a five-strain cocktail of *Escherichia coli* O157:H7 during the 60-day aging period of cheddar cheese made from unpasteurized milk. J Food Protect 2006;69:990–998.

Sjöling Å, Sadeghipoorjahromi L, Novak D, Tobias J. Detection of major diarrheagenic bacterial pathogens by multiplex PCR panels. Microbiol Res 2015;172:34–40.

UNI EN ISO 11290-1:1996 Microbiology of food and animal feeding stuffs. Horizontal method for the detection and enumeration of *Listeria monocytogenes*. Part 1: Detection method.

UNI EN ISO 20838:2006. (2006). Microbiology of food and animal feeding stuffs–Polymerase chain reaction (PCR) for the detection of food-borne pathogens—Requirements for amplification and detection for qualitative methods.

Vernozy-Rozand C, Mazuy-Cruchaudet C, Bavai C, Montet MP, Bonin V, Dernburg A, Richard Y. Growth and survival of *Escherichia coli* O157:H7 during the manufacture and ripening of raw goat milk lactic cheeses. Int J Food Microbiol 2005;105:83–88.

Zweifel C, Zychowska MA, Stephan R. Prevalence and characteristics of Shiga toxin–producing *Escherichia coli*, *Salmonella* spp. and *Campylobacter* spp. isolated from slaughtered sheep in Switzerland. Int J Food Microbiol 2004; 92:45–53.

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