Prevalence and molecular characterisation of Shiga toxin-producing *Escherichia coli* in raw milk cheeses from Lazio region, Italy

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Abstract

In recent years, the incidence of foodborne diseases caused by Shiga toxin-producing *Escherichia coli* (STEC) has increased globally. For this reason, within the specific regional control plan for the detection of STEC in food products in Italy, the presence of STEC in unpasteurised milk cheeses was investigated. In total, 203 samples obtained from March 2011 to December 2013 were analysed, with two standard methods (ISO 16654:2001 and ISO 13136:2012). Two strains of *E. coli* O157 were isolated (2/161, 1.2%) but did not carry any virulence-associated genes and 22 *stx*-positive samples (22/146, 15.1%) were detected in enrichment cultures, mostly from ovine cheeses. Only two strains isolated from different ovine cheeses carried *stx* gene and none of these was *eae*-positive. This study confirms the presence of *stx*-positive *E. coli* and suggests that this type of food cannot be excluded as a potential vehicle of STEC.

Introduction

Several clones of *Escherichia coli* (*E. coli*) are potentially pathogenic by virtue of a variety of infective and toxin-producing mechanisms. Shiga toxin-producing *E. coli* (STEC), also called verocytotoxic producing *E. coli* (VTEC), can produce one or more cytotoxins, named Shiga toxin 1 (stx_1) and Shiga toxin 2 (stx_2) (Farrokh *et al*., 2013) and can harbour additional virulence factors that are involved in the development of the disease in human. Shiga toxin-producing *E. coli* represents a significant risk to public health and has recently emerged as a major cause of foodborne illness (Perelle *et al*., 2007). The symptoms associated with STEC infection in humans vary from mild to severe, the latter being characterised by a bloody diarrhea, which is often accompanied by abdominal cramps, usually without fever. Shiga toxin-producing *E. coli* infections can also result in haemolytic-uremic syndrome (HUS). Humans most frequently become infected with STEC by ingestion of contaminated food (meat, especially undercooked beef hamburgers; raw milk; cheese; ready-to-eat sausages; sprouts) or water or by direct contact with animals (Gyles, 2006).

Escherichia coli O157:H7 causes the majority of human cases of STEC infection and HUS worldwide but in recent years there has been an increase in the incidence of disease caused by non-O157 STEC (Farrokh *et al*., 2013). In 2011 a large outbreak of STEC O104:H4 occurred within the EU, primarily in Germany with seed sprouts identified as the probable source of infection. In 2012, a total of 5671 confirmed human cases were reported in Europe. In the past five years the trand in confirmed STEC infections in the EU has continued to rise even when the 2011 *E. coli* O104:H4 outbreak was not considered (EFSA, 2014).

The ruminant digestive system is the primary reservoir for STEC: milk being contaminated by bacterial cells excreted in the faeces that taint cattle udders, teats and hides and the general farm environment (Nastasijevic *et al*., 2008). Shiga toxin-producing *E. coli* prevalence in raw milk usually ranges between 0 and 2% (Farrokh *et al*., 2013); recently, within the EU, 56 units (2.1%) out of a total of 2610 cheeses and other dairy products tested were found to be STEC-positive (EFSA, 2014).

As a result of the increase in the number of confirmed STEC infections reported in Europe, the Lazio Region and the Institute for Experimental Veterinary Medicine of Lazio and Tuscany M. Aleandri implemented a three-year monitoring plan for the detection of STEC in raw milk dairy products. In this study we report on the prevalence of STEC in raw milk dairy products in the Lazio region and identify the number of samples found positive for the two virulence-associated genes (*stx* and *eae*).

Materials and Methods

From March 2011 to December 2013, the local Veterinary Services obtained a total of 203 cheese samples from both small and large retail stores distributed across the region of Lazio; culturing and molecular methods, these samples were analysed for the presence using both of STEC.

Culturing methods

The presence of *E. coli* O157 was evaluated in 161 samples, using a culture method based on the International Organization for Standardization (ISO) 16654:2001 (ISO, 2001) preceded by immunoassay screening (VIDAS ECO BioMérieux validated by AFNOR BIO 12/8-07/00; BioMérieux, Marcy l'Etoile, France). Briefly, the method consisted of pre-enriching a portion of a test sample, in Modified Tryptic Soy Broth (mTSB) (Biolife, Milan, Italy) incubated for 24 h at 41.5°C, followed by immunomagnetic separation using Dynabeads® anti-*E. coli* O157 (Dynal; Invitrogen, Carlsbad, CA, USA); after screening with VIDAS ECO, a second immunoseparation using Dynabeads was performed and cultured in solid selective media [Cefixime Tellurite Sorbitol McConkey Agar (CT-SMAC; Biokar, Allonne, France), and Sorbitol McConkey Agar (SMAC; Biokar)]. After incubation, the presence of *E. coli* was confirmed using biochemical tests, with the identity of *E. coli* O157 estabilished using Latex Test (Oxoid, Basingstoke, UK) agglutination (latex test, Oxoid). Subsequently, a standardised polymerase chain reaction (PCR) method provided by the EU Reference Laboratory (Italian National Institute of Health, Rome), was used to test all the positive *E. coli* O157 samples for the presence of the Shiga-toxin genes stx_1 and stx_2 and the *eae* (intimin) gene responsible for the attaching/effacing lesions.

Molecular methods

In 2012, the ISO issued a horizontal method for the detection of STEC and for determining the presence of O157, O111, O26, O103 and O145 serogroups (ISO 13136:2012; ISO, 2012), extended subsequently to include also the O:104:H4 serogrup. In total, 146 samples were screened for the presence of STEC using the real-time PCR method that is linked to ISO 13136:2012. Briefly, the method included an 18-24 h enrichment step at 37±1°C in Buffered

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Peptone Water (BPW; Oxoid), from which the total DNA was purified using InstaGene reagent (Bio-Rad, Hercules, CA, USA) and was used as template for the stx_1 and stx_2 and the *eae* real-time PCRs. The real-time PCRs were performed in a Stratagene Mx3005P (Agilent Technologies, Santa Clara, CA, USA) instrument and the PCR mixes and protocols were set following the ISO 13136:2012 guidelines. When one or both stx_1 and stx_2 genes were detected in the enrichment broth culture, isolation of the *E. coli* strains by plating onto solid media [Tryptone Bile X-Glucuronide medium (TBX), Rhamnose McConkey (RMAC), SMAC, CT SMAC, Nutrient Agar (NA)] was attempted. All samples positive for the presence of one or both *stx1* and *stx2* genes were further analysed for the presence of the *eae* gene. In case of positivity for the *eae* gene, the characterisation of the *E. coli* serogroups O26, O103, O111, O145 and O157 was performed first from the enrichment culture and then from the isolated colonies. All samples that were *stx*-positive but *eae*-negative, were then screened for the O104:H4 serogroup.

Results

Culturing yielded only two strains of *E. coli* 0157 from the 161 samples analysed. None of them carried the *stx1*, *stx2* and *eae* virulence genes.

The ISO 13136:2012 (ISO, 2012) molecular method, when applied to the 146 enriched broth cultures, revealed the presence of 22 *stx*-positive samples (22/146, 15.1%). Of these, six were *stx1* positive, ten *stx2* positive, and six both *stx1* and *stx2* positive combined. Ten of the same 22 samples were also positive for the *eae* gene. The results showed a higher prevalence of virulence-associated genes in raw sheep's milk dairy products compared to those of other species (Table 1).

The two *E. coli* isolated from these 22 stx-positive samples and derived from two ovine cheese samples, possessed only one or bith of the two *stx* genes and not the *eae* gene. One strain, belonging to the O103 serogroup, was both *stx*1 and *stx*2 positive, while the other isolate was only *stx*1 positive however this isolate did not belong to any of the 6 serogroups that were investigated (O157, O111, O26, O103, O104:H4 and O145).

Discussion

In this study, by using culturing, the *stx*-positive strain of *E. coli* O157 was not isolated from any of the samples of raw milk dairy products tested. This is in agreement with other studies and would seem to underpin the very low prevalence of this STEC serotype in raw milk cheeses (Baylis, 2009).

The prevalence of *stx* genes by PCR following enrichment (15.1%) is similar to the rate reported in previous studies. Vernozy-Rozand *et al*. (2005) found these genes to occur in 13.1% of raw milk cheeses tested in France, while Zweifel *et al*. (2010) reported a 5.7% prevalence rate in 1502 raw milk cheeses sampled at factories in Switzerland. The higher prevalence in raw milk sheep cheeses may be linked to the method of production. In fact, in the region of Lazio, sheep owners commonly use an old-fashioned breeding system and traditional, artisanal, cheese-making procedures, sometimes under conditions that are unhygienic.

Despite the significant prevalence rate, we isolated only two strains of *E. coli* O:157 harbouring *stx* genes. A similarly low recovery rate of STEC strains from PCR-positive samples has been reported upon previously (Pradel *et al*., 2000; Fach *et al*., 2001; Vernozy-Rozand *et al*., 2005).

Both *stx*-positive isolates did not possess the *eae* gene. Again, our finding seems to match that previously made, during which it was noted that only a minority of STEC strains found in raw milk and raw milk cheeses possessed the *eae* gene encoding for intimin (Baylis, 2009). It was reported by Boerlin *et al.* (1999) that STEC strains carrying the *eae* gene have the ability to cause attaching and effacing lesions on epithelial cells, which seems to be a characteristic associated most frequently to human disease. However, not all STEC strains linked to human foodborne outbreaks are *eae*-positive, as was noted during the *E. coli* O104:H4 outbreak in Germany in the summer of 2011. This underlines the potential importance of alternative pathogenic mechanisms and involving colonisation and aggregation of the bacteria in the human digestive tract (Neill, 1997; Baylis, 2009).

The presence of *stx*-positive *E. coli* in raw milk dairy products suggests that this type of foodstuff cannot be excluded as a potential vehicle for STEC and, therefore, represents a safety concern with regard to human health. However, the very low number of positive samples detected during our survey is cause for comfort; indeed, only two strains isolated from different ovine cheeses carried the *stx* gene, neither of which was *eae*-positive.

Conclusions

In conclusion, given the relatively high number of *stx*-positive samples detected, and the increase in reported outbreaks of foodborne STECs (EFSA, 2014), a specific control plan, concerning raw and thermolised milk cheeses, is now being planned for Lazio region (2014).

Table 1. Samples of raw milk cheeses analysed for the presence of *Escherichia coli* O157 using culturing and Shiga toxin-producing *Escherichia coli* polymerase chain reaction.

Milk origin	*E. coli* O157		STEC			
	Samples analysed (n) (ISO 16654:2001)	Presence of *E. coli* O157 (n)	Samples analysed (n) (ISO 13136:2012)	Presence of *stx* gene° (n)	Presence of *eae* gene# (n)	Presence of *stx* and *eae* genes§ (n)
Ovine	54	2 (3.7)^	43	8 (18.6)	2 (4.7)	7 (16.3)
Cow	63	0	61	3 (4.9)	8 (13.1)	1 (1.6)
Water buffalo	24	0	29	0	2 (6.9)	0
Goat	6	0	5	1 (20)	2 (40)	1 (20)
Mixed milk cheese$	8	0	5	0	2 (40)	0
Unknown	6	0	3	0	1 (33.3)	1 (33.3)
Total	161	2 (1.2)	146	12 (8.2)	17 (11.6)	10 (6.8)

STEC, Shiga toxin-producing *Escherichia coli*; ISO, International Organization for Standardization. Values in parenthesis are expressed as percentage. °Presence of *stx* genes, detection of stx_1 or stx_2 gene (or both) in DNA extracted from enrichment broth; #presence of *eae* gene, detection of *eae* gene in DNA extracted from enrichment broth; §presence of *stx* and *eae* genes, detection of stx_1 or stx_2 gene (or both) and *eae* gene in DNA extracted from enrichment broth; ^both isolated strains did not carry any virulence-associated genes (*eae*, stx_1 and stx_2); $mixed milk cheese, cheese made from mixtures of milk obtained from two species.

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