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Short communication: Isolation of Shiga toxin-producing *Escherichia coli* in raw milk and mozzarella cheese in southern Italy

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are a significant food-borne public health hazard in Europe, where most human infections are associated with 5 serogroups (O157, O26, O103, O145, and O111). In 2015, 95 food and environmental samples were examined for the presence of Shiga toxin genes (*stx1* and *stx2*). The STEC were isolated from 2 raw milk and 1 mozzarella cheese samples that were collected in the period between June and September. To the best of our knowledge, this finding represents the first report of STEC isolation from mozzarella cheese produced in Italy, and it suggests that both the quality of raw milk used to produce mozzarella and the thermal inactivation treatment associated with the curd-stretching step should be carefully monitored.
Key words: Shiga-toxigenic *Escherichia coli*, raw milk and mozzarella cheese, PFGE, real-time PCR

Short Communication

Shiga toxin-producing *Escherichia coli* (**STEC**) can cause human infections ranging from uncomplicated diarrhea to severe diseases as hemorrhagic colitis and life-threatening hemolytic uremic syndrome (**HUS**; Melton-Celsa et al., 2012). Human STEC infections are acquired through direct or indirect fecal-oral contact with human or animal feces and ruminants have been identified as a major reservoir of STEC (Caprioli et al., 2005). As reported in the literature, contaminated raw milk and raw milk products are among the main risk factors considered STEC vectors (Baylis, 2009), as also reported in several studies in Italy (Trevisani et al., 2014a). A typical Italian cheese is cow milk mozzarella cheese, mainly manufactured in limited geographical areas of southern Italy using traditional protocols. Apulia is one of the main Italian regions where this food commodity is produced. The main features of the production process include the use of raw milk, natural whey starter cultures, stretching in hot water at 90°C (temperature of the curd 58–65°C), and storage at 0 to 4°C for 4 d before consumption as fresh. In Italy, previous experimental studies revealed the STEC contamination in raw milk products, although with a low prevalence (Conedera et al., 2004).

In a recent outbreak in southern Italy, with 22 cases of HUS due to O26 STEC, the infection was related to a suspect contamination of raw milk products or vegetables distributed locally in Apulia region. Given the increase of HUS cases in the Apulia region in recent years, the aim of our study was to report the results of a 1-yr monitoring program and the prevalence of STEC in food and environmental samples and to assess the risk of food contamination with STEC.

In 2015, specifically in the period between June and September, 95 food and environmental samples were sent to Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (**IZS PB**), a public veterinary institute that conducts prevention, control, and research activities in animal health and welfare, food safety, and environmental protection. In particular, 32 cheese samples (33.7%), 6 raw milk samples (6.3%), 27 specimens of meat and meat products (28.4%), 10 vegetable samples (10.5%), 17 samples of water (17.9%), and 3 fecal samples were analyzed (3.1%). The samples were collected from local retail stores and farmers' markets in the Apulia region by the local health services and transferred under refrigerated conditions to the laboratories of IZS PB.

The samples were analyzed according to ISO (2012). The DNA extracts were obtained using PrepSEQ Rapid Spin Sample Preparation Kits (Thermo Fisher

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Scientific, Waltham, MA) according to manufacturer's instructions. The DNA were tested by real-time PCR for the Shiga toxin genes (*stx1* and *stx2*) and *eae* gene using the technology platform 7500 Fast Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific). The identification of O157, O26, O111, O103, and O145 serotypes was performed following ISO (2012), whereas O104:H4 identification followed the procedure European Union Reference Laboratory for *E. coli* (2013a) and O45, O55, O91, O113, O121, O128, and O146 serotypes according to European Union Reference Laboratory for *E. coli* (2013b). In addition, *stx* gene subtypes were determined according to the protocol described by Scheutz et al. (2012). The STEC isolates provided by European Union Reference Laboratory for *E. coli* were used as reference strains.

Presumptive positive samples were subjected to the microbiological isolation according to ISO (2012). All strain isolates were tested for susceptibility to selected antimicrobial agents using a disk diffusion method outlined by the Clinical and Laboratory Standards Institute (CLSI, 2012). The antimicrobials assayed are reported in Table 1. The results were recorded after 24 h of incubation at 37°C and interpreted according to charts supplied with the discs (CLSI, 2012).

The isolates were subjected to molecular typing by pulsed-field gel electrophoresis (**PFGE**) according to the CDC PulseNet protocol (Ribot et al., 2006) and multilocus sequence typing (**MLST**) according to the *E. coli* MLST website (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli) using 7 housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*).

Out of 95 samples analyzed, 7 enrichment cultures (7%) were positive for *stx* genes, but STEC strains were isolated from only 3 (3%) of them: 2 from raw milk samples and 1 from mozzarella cheese. The contaminated mozzarella cheese was made from curd purchased from the dairy farm where the 2 STEC-positive raw milk samples had been collected. All isolates were positive for the *stx2* gene and negative for the *stx1* and *eae* genes and for all the serogroup-associated genes tested. As reported in the literature (Farrokh et al., 2013), the virulence gene *stx2* is the most clinically important Stx type. It has been shown that the probability to develop HUS upon STEC infection is higher when STEC-producing Stx2 are involved. In addition, subtyping of *stx* genes showed the presence of both *stx2c* and *stx2d* subtypes, those most commonly associated with severe human disease (Farrokh et al., 2013). The 3 isolates exhibited a multidrug resistance profile characterized by resistance to ampicillin, amoxicillin-clavulanic acid, cephalothin, streptomycin, and tetracycline. These data are in accordance with previous studies (Schroeder et al., 2002), suggesting the use of these drugs has been a key factor in the emergence of antimicrobial-resistant *E. coli* and the risk of transfer the antimicrobial resistance from food animals to humans through the spread of genetic elements. Two isolates were also resistant to ciprofloxacin and 1 to sulfamethoxazole (Table 1).

The genetic relatedness of the 3 STEC strains isolated from raw milk and mozzarella cheese samples was investigated by PFGE, which showed a 100% similarity (Figure 1). The MLST confirmed the strict correlation of the 3 STEC isolates, which showed the same sequence type (ST1611). This sequence type could be assigned to the phylogenetic group B1 (Doumith et al., 2012). Interestingly, the STEC strain isolated from mozzarella cheese was of the same sequence type, virulence genes profile, and PFGE pattern of the STEC isolates from raw milk samples collected at the farm that had pro-

Table 1. Antimicrobial resistance of 3 Shiga toxin-producing *Escherichia coli* strains

Antibiotic	Manufacturer[1]	Concentration (μg)	Susceptible breakpoint (mm)	Isolated strains[2]		
				Raw milk 1	Raw milk 2	Mozzarella cheese
Amoxicillin/clavulanic acid	Liofilchem	20/10	≥18	R	R	R
Ampicillin	Liofilchem	10	≥17	R	R	R
Ceftriaxone	BioLab	30	≥23	S	S	S
Cephalothin	Liofilchem	30	≥18	R	R	R
Chloramphenicol	Mast Diagnostics	30	≥18	S	S	S
Ciprofloxacin	BioLab	5	≥21	R	S	R
Gentamicin	Liofilchem	10	≥15	S	S	S
Naladixic acid	BioLab	30	≥19	S	S	S
Streptomycin	Liofilchem	10	≥15	R	R	R
Sulfamethoxazole	BioLab	10	≥16	S	R	S
Tetracycline	Liofilchem	30	≥15	R	R	R

[1]Liofilchem, Roseto degli Abruzzi (Te), Italy; Biolab Inc., Budapest, Hungary; Mast Diagnostics Ltd, Merseyside, UK.
[2]R = resistant; S = susceptible.