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Short communication

Prevalence of Verocytotoxigenic *Escherichia coli* strains isolated from raw beef in southern Italy

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ABSTRACT

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) are a significant foodborne public health hazard, where most human infections are associated with six serogroups (O157, O26, O103, O145, O111 and O104). VTEC was the fourth most commonly reported zoonosis in the EU in 2015, with 5901 confirmed human cases. Ruminant animals, including cattle, are a major reservoir of VTEC. The consumption of VTEC-contaminated animal-derived foodstuffs, especially undercooked ground beef, is an important transmission route. To the best of our knowledge, there are few data available on the contamination of VTEC in meat products in Italy. During 2015 and 2016, 250 raw meat samples were collected from retail markets in southern Italy (Apulia) and analysed for the occurrence of *vtx* genes (*vtx1/vtx2*) at the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata (IZS PB, Italy). In addition, the isolates were characterized by determining the presence of VTEC main virulence factors, the antimicrobial resistance profiles and the genetic relatedness by pulsed-field gel electrophoresis (PFGE). The results have shown that 8.4% (21/250) of the samples were positive for *vtx* genes in the preliminary screening step but VTEC strains were isolated from only 2% (5/250) of overall meat analysed samples, including raw ground beef, beef hamburger and beef carpaccio. 5 isolates displayed a multi-drug resistance phenotype. All VTEC strains were analysed by *Xba*I-PFGE and dendrogram revealed 5 distinct restriction profiles, indicating their relatively high genetic diversity. Although this study demonstrates a low prevalence of VTEC in raw beef marketed in southern Italy, the presence of potentially pathogenic *E. coli* strains points to the need for proper hygiene during meat production to reduce the risk of foodborne illness and transmission of multi-drug resistant organisms via foods to humans.

1. Introduction

VTEC was the fourth most commonly reported zoonosis in the EU in 2015, with 5901 confirmed human cases (EFSA and ECDC, 2016). Verocytotoxin (VT)-producing *Escherichia coli* (VTEC), also referred to as Shiga-toxin producing *E. coli* (STEC), are a group of *E. coli* that carry verocytotoxin (*vtx/stx*) genes encoded on lamboid lysogenic bacteriophage (Duffy et al., 2014). In addition to toxin production, another virulence associated factor expressed by VTEC is a protein called intimin encoded by the *eae* gene and responsible for intimate attachment of VTEC to the intestinal epithelial cells, which causes attaching and effacing (A/E) lesions in the intestinal mucosa (Kaper et al., 1998). VTEC are a group of food and water-borne pathogens associated with a wide spectrum of human diseases, ranging from mild diarrhea to hemorrhagic colitis (HC), thrombo-cytopenia, hemolytic uremic syndrome (HUS), and can also lead to people death (Karmali et al., 2010). VTEC comprises serologically different strains and *E. coli* O157:H7 is the most common cause of VTEC infections (Bai et al., 2015). However, a growing number of non-O157 VTEC strains have been isolated from several clinical cases and outbreaks (Smith et al., 2014). As reported by the European Food and Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), the highest proportions of hospitalized cases have been reported in Italy and other countries. The most common serogroups among HUS cases in 2015 were O157 and O26 (both 27.9%) (EFSA and ECDC, 2016).

Another important characteristic of foodborne *E. coli* infection from the zoonotic perspective is the multidrug resistance (MDR), usually characterized by a complex interaction of different mechanisms conferring resistance to a wide range of antimicrobial compounds (Nagy et al., 2015). Ruminant animals, including cattle, are the major reservoir of VTEC (Caprioli et al., 2005) which can be harboured in, excreted from their gastrointestinal tract and shed in the faeces (Buncic

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Table 1
Antimicrobial resistance of five VTEC strains isolated from raw beef samples.

Antibiotics	Manufacturer[a]	Concentrations	Susceptible breakpoints (mm)	Isolated strains[b]				
				Rraw ground beef 1	Rraw ground beef 2	Rraw ground beef 3	Beef hamburger	Beef carpaccio
Amoxicillin Clavulanic acid	Liofilchem	20/10 μg	≥ 18	R	R	R	R	R
Ampicillin	Liofilchem	10 μg	≥ 17	R	R	R	R	R
Ceftriaxone	BioLab	30 μg	≥ 23	S	S	S	S	S
Cephalothin	Liofilchem	30 μg	≥ 18	R	S	R	I	I
Ciprofloxacin	BioLab	5 μg	≥ 21	S	S	S	S	S
Gentamicin	Liofilchem	10 μg	≥ 15	S	S	R	S	S
Naladixic acid	BioLab	30 μg	≥ 19	S	S	S	S	S
Streptomycine	Liofilchem	10 μg	≥ 15	S	R	R	S	S
Sulfamethoxazole	BioLab	10 μg	≥ 16	S	R	R	I	S
Tetracylcine	Liofilchem	30 μg	≥ 15	R	R	S	R	R

[a] Liofilchem, Roseto degli Abruzzi (Te), Italy; Biolab Inc., Budapest, Hungary.
[b] R = resistant; S = susceptible; I = intermediate.

et al., 2014). These pathogens are transferred from cattle to humans through direct or indirect faecal contamination, further cross-contamination and/or multiplication during production, handling and consumption of beef and products thereof (Buncic et al., 2014). In addition, faecal contamination can be associated with knife entry through the hide into the carcass and also splash back and aerosol deposition of faecal matter during hide removal (Buncic et al., 2014). The consumption of VTEC-contaminated animal-derived foodstuffs, especially undercooked ground beef, is an important transmission route (Erickson and Doyle, 2007). VTEC of various serotypes have been isolated from raw meat samples including beef, mutton, pork, chicken, and wild game meat (Magwedere et al., 2013).

In EU member states, data on VTEC in cattle and beef products are poor (Bonardi et al., 2015). Only a few countries have been monitoring VTEC in cattle and beef meat products, with low isolation rates (1.6% of positive samples for VTEC and 0.2% for VTEC O157, respectively) (EFSA and ECDC, 2016). To the best of our knowledge, there are few data available on the contamination of VTEC in beef products in Italy (Conedera et al., 2004; Dambrosio et al., 2007; Stampi et al., 2004), especially as the surveillance of *E. coli* O157 and non-O157 for meat and products thereof is not currently included in European legislation (Regulation EU No. 2073/2005 and its amendments, Regulation EU No.1441/2007) (Regulation (EC) No. 1441/2007, 2007; Regulation (EC) No. 2073/2005, 2005).

The aim of this study was to determine the prevalence of Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) in raw beef samples and products thereof from retail markets of Apulia (southern Italy) and to explore their pathogenic potential to humans.

2. Material and methods

2.1. Samples

From June 2015 to June 2016, overall 250 raw beef samples and ready-to-eat beef products were collected from retail markets in Apulia (southern Italy). The samples included beef hamburger (n = 100), raw ground beef (n = 100) and beef carpaccio (n = 50). The samples were transported under refrigerated conditions to the laboratories of Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata.

2.2. Detection and isolation of VTEC

Twenty-five grams of each sample was enriched with 225 ml of modified Tryptone-Soy Broth (mTSB) supplemented with novobiocin (16 mg/l) (MICROBIOL DIAGNOSTICI, Sardegna, IT) and incubated at 37 °C for 18–24 h. The samples were analysed according to the ISO/TS 13136:2012 (ISO/TS 13136, 2012). DNA extracts were obtained using PrepSEQ™ Rapid Spin Sample Preparation Kits (Thermo Fisher, Waltham, MA USA) according to manufacturer's instructions and tested by Real Time PCR for the verocytotoxin genes (*vtx1* and *vtx2*) and *eae* gene, using the technology platform 7500 Fast Real-Time PCR (Applied Biosystems, ABI). The identification of O157, O26, O111, O103 and O145 serotypes was performed following the ISO/TS 13136:2012 (ISO/TS 13136, 2012), while the O104:H4 following the procedure European Union Reference Laboratory for *E. coli* (EU-RL for *E. coli*) Method 04 (European Union Reference Laboratory for *E. coli*, 2013a) and the O45, O55, O91, O113, O121, O128 and O146 serotypes according to EU-RL-Method 03 (European Union Reference Laboratory for *E. coli*, 2013b). In addition, conventional PCR for the detection of *vxt1* and *vtx2* gene subtypes was performed as described by Scheutz et al. (2012). VTEC isolates provided by EU RL for *E. coli* were used as reference strains. When one or both *vtx1* and *vtx2* genes were detected in the enrichment broth culture, isolation of VTEC by plating onto solid media [Tryptone Bile X-Glucuronide medium (TBX), Rhamnose McConkey (RMAC), SMAC, CT SMAC, Nutrient Agar (NA)] was attempted, according to ISO 13136:2012 (ISO/TS 13136, 2012).

2.3. Determination of antimicrobial susceptibility of VTEC isolates

All strains isolates were tested for susceptibility to selected antimicrobial agents using a disk diffusion method outlined by the Clinical and Laboratory Standards Institute (CLSI).

The antibiotic disks are reported in Table 1. The results were recorded after 24 h incubation at 37 °C and interpreted according to charts supplied with the discs (CLSI, 2012).

2.4. Molecular characterization

The genetic relationship among the isolated strains was investigated by PFGE in accordance with the PulseNet protocol for *Escherichia coli* O157:H7 (http://www.cdc.gov/pulsenet/pathogens/index.html) and EFSA External Scientific Report about molecular typing of verocytotoxin-producing *E. coli* (Caprioli et al., 2014). VTEC strains were digested with the *Xba*I restriction enzyme (Roche Diagnostics, Monza, MB, IT). The *Salmonella enterica* serovar Braenderup strain H9812 was used as molecular size standard. PFGE was performed in a CHEF MAPPER system (BioRad, Hemel Hempstead, United Kingdom) at 14 °C in 0.5X Tris-borate-EDTA buffer (TBE) (Thermo Fisher Scientific, Waltham, MA USA). The restriction profiles were analysed with GelComparII version 6.6.11 (Applied Maths, Sint-Martens-Latem, Belgium).

The patterns were compared using Dice's similarity coefficient with tolerance and optimization values at 1.5%. The dendrogram was built