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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

Table 2
Origin and molecular characterization of isolated VTEC.

Strains ID	Meat type	Virulence genes			Serogroup	Subtype
		vtx1	*vtx2*	*eae*		
1	Raw ground beef 1	−	+	−	O.N.T.[a]	*vtx2g*
2	Raw ground beef 2	−	+	−	O.N.T.	*vtx2c*
3	Raw ground beef 3	−	+	−	O.N.T.	*vtx2a*, *vtx2b*, *vtx2d*
4	Hamburger	+	−	−	O.N.T.	*vtx1c*
5	Beef carpaccio	+	−	−	O.N.T.	*vtx1c*

[a] O.N.T., O not typeable.

using the unweighted pair-group method with arithmetic averages (UPGMA).

3. Results

250 raw beef samples were analysed for the presence of VTEC strains, according to ISO 13136:2012 (ISO/TS 13136, 2012). 229 (91.6%) out of 250 beef samples came out to be negative to verocytotoxin genes, while 21 (8.4%) samples were *vtx*-positive in screening. Of 21 *vtx*-positive enrichment cultures, 1 (4.7%) carried both *vtx2* and *eae*, 3 (14.3%) resulted positive to *vtx1*, and 17 (81%) to *vtx2*. All samples have resulted negative to 13 serogroups tested. VTEC strains have been isolated from 5/250 (2%) raw beef samples, of which 2 (0.8%) positive to *vtx1* and 3 (1.2%) to *vtx2*. *vtx1*-positive (2/5) strains have been isolated from hamburger and beef carpaccio samples, whereas all *vtx2*-positive strains (3/5) from raw ground beef samples (Table 2). None of the VTEC isolates carried *eae* gene and belonged to the serogroups tested.

In addition, two VTEC isolates carrying *vtx1* were positive for *vtx1c* subtype. Among three VTEC isolates carrying *vtx2*, one was positive for *vtx2g*, one for *vtx2c* and one for *vtx2a*, *vtx2b* and *vtx2d* (Table 2). The isolates showed a multi-drug resistant profile. The antibiotic resistance and susceptibility pattern of the strains isolates has been reported in Table 1. Overall, resistance was most frequently observed to amoxicillin-clavulanic acid (100%), ampicillin (100%) and tetracyclin (80%). The isolates were analysed by *Xba*I-PFGE. The dendrogram produced by the UPGMA algorithm study has revealed 5 distinct restriction profiles, indicating their relatively high genetic diversity (Fig. 1).

4. Discussion

Contaminated beef is considered to be a major source of foodborne VTEC infections in humans (Farrokh et al., 2013). Approximately 52% of outbreaks have been associated with bovine products. (Brusa et al., 2012). Since data on VTEC in beef products in Italy are poor, the aim of this study was the monitoring of the VTEC prevalence in several types of raw beef samples (raw ground beef, hamburger) and ready-to-eat meat products (beef carpaccio). In the screening step, the results of this study reported 21 vtx-positive enrichment cultures but only 5 VTEC strains were isolated. The isolation of VTEC from foods is problematic because the bacterium is likely to be present in low numbers, may be sublethally injured and is usually accompanied by large population of competent microflora, including other *E. coli* (Brusa et al., 2012; Farrokh et al., 2013). Failure to isolate VTEC from the vtx-positive samples may due to the loss of Vtx prophages during subculture or the presence of other bacteria carrying vtx (Meng et al., 2014). In addition, the immuno-magnetic separation (IMS) improved the isolation sensitivity of O157 strains at least 100-fold. The isolation of VTEC non-O157 is still a challenge, since non-O157 VTEC strains show great genetic and biochemical diversity, and there is no unique phenotypic marker that can differentiate them from other *E. coli* (Brusa et al., 2012). In this study, VTEC strains were found in 2% of the samples (5/250), according to previous researches in Italy (Bardasi et al., 2015; Conedera et al., 2004; Stampi et al., 2004) and in other countries (Fantelli and Stephan, 2001; Pradel et al., 2000). Although there is a low prevalence rate of VTEC, their occurrence in raw beef and in ready to eat beef products as carpaccio represents an important public health risk. Beef *carpaccio* is a ready to eat product (RTE) and traditionally produced as thin slices from frozen raw or cured beef, which are packed under vacuum or modified atmospheres, and kept at refrigeration temperature until consumption. *Carpaccio* is considered a high-risk food because of the possibility of contamination with pathogenic bacteria from the animal reservoir, such as VTEC, and the minimal process it undergoes (Masana et al., 2015). VTEC can be shed in animal faeces and contaminate the surfaces of raw meat during the slaughter, dressing and packaging. The risk of the product increases when stored at temperature abuse conditions. RTE meat products stored at temperature abuse conditions can result in spoiled or unsafe food relatively early due to faster multiplication of microorganisms (Bravo et al., 2014). In this study, it has been showed the occurrence of VTEC in beef *carpaccio* and this result represents a risk for consumers and a major concern for the food industry. Different strategies for preventing the growth of contaminant pathogens in foods will continue being needed.

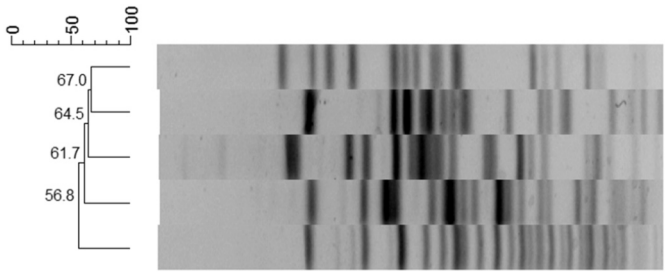

Fig. 1. Pulsed field gel electrophoresis patterns of 5 VTEC strains with *Xba*I. The figure shows 5 distinct restriction pulsed field gel electrophoresis profiles. Lane 1: Raw ground beef 1; lane 2: beef carpaccio; lane 3: Raw ground beef 3; lane 4: Beef hamburger; lane 5: Raw ground beef 2.

2 out of 5 VTEC strains isolated were positive to *vtx1* gene and 3 to *vtx2*. As reported in literature, the *vtx2* gene is clinically the most important Vtx type and the probability of HUS development in infections from strains harbouring *vtx2* is higher than that of strains containing either *vtx1* or both *vtx1* and *vtx2* (Friedrich et al., 2002). The determination of *vtx* subtypes can be an important element in the risk characterization of foodborne VTEC isolates, since the genetic variation of vtx causes changes in its amino acid composition, which may directly influence the virulence of VTEC, resulting in a change in the toxin receptor tropism or toxicity of Verocytotoxigenic pathogenesis and diagnosis of VTEC infections (Paton and Paton, 1998). In this study, *vtx1c*, *vtx2a*, *vtx2b*, *vtx2c*, *vtx2d* were detected. The *vtx1c* subtype is associated with ovine originated VTEC strains (Zhang et al., 2002; Brett et al., 2003), but the high prevalence of *vtx1c* in buffaloes, cattle, and goats has been reported to account for 80% of the *vtx1* subtypes, indicating a wide distribution of *vtx1c* variants in VTEC of bovine origin (Vu-Khac and Cornick, 2008).

In addition, *vtx1c*-producing VTEC is considered a subset of *eae*-negative VTEC, and is responsible for asymptomatic or mild disease (Zhang et al., 2002; Brett et al., 2003; Fitzgerald et al., 2003).

VTEC carrying *vtx2a*, *vtx2c* and *vtx2d* have been associated with severe clinical symptoms, while VTEC carrying *vtx1c* and *vtx2b* have been mainly associated with diarrheal disease (Farrokh et al., 2013). In fact, Karve e Weiss (Karve and Weiss, 2014) showed a stronger link between *vtx2a*, *vtx2c* and *vtx2d* and Gb3 receptors.

Previously, the *vtx2g* variant has been identified from various sources, including cattle, beef or beef-containing products, and humans, suggesting a possible route of exposure of these VTEC types via the food

chain (Beutin et al., 2007; Prager et al., 2011).

All VTEC isolates appeared negative to the genes associated to the tested serogroups. The proportion of non-typable VTEC strains continued to increase in 2014, as reported in the last EFSA report (EFSA and ECDC, 2016).

All isolated strains showed a multi-drug resistance for antimicrobial agents that are often used for therapy of infected humans and animals as well as for prophylaxis and growth promotion of food animals (Kolář et al., 2001). This data are in accord with some previous studies (Nobili et al., 2016; Threlfall et al., 2000). In animals, antimicrobial resistance in zoonotic enteropathogens (e.g., *Salmonella*, *Campylobacter*, *Yersinia*, and some strains of *E. coli*, such as serotype O157:H7) and commensals (e.g., enterococci, most generic *E. coli*) is of special concern to human health because these bacteria are most likely to be transferred through the food chain to humans, or resistance genes in commensal bacteria may be transferred to the zoonotic enteropathogens (Salyers, 1995). Therefore, retail foods, especially meat and meat products, may be an important vehicle for community-wide dissemination of antimicrobial-resistant *E. coli* (Sunde and Norstrom, 2006).

The PFGE results showed a high genetic diversity. This could be explained by the different origin of cattle and possible contamination during the transportation to slaughterhouse and the waiting time before slaughter as a result of cross-infection caused by mixing of animals from different sources (Meyer-Broseta et al., 2001).

Therefore, it is necessary to implement an efficient monitoring plan with a continuous surveillance of VTEC in foodstuffs and animals and a continuing need for training and upgrading of farmers, food-handler and consumers in microbiological food safety criteria. In fact, protection will only occur if all sectors in the chain operate in an integrated way, and food control systems are applied all stages of this chain.

Conflict of interest

The authors have no conflict of interests to declare.

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