International Journal of Food Microbiology 423 (2024) 110846

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Emerging of Shiga toxin-producing *Escherichia coli* O177:H11 and O177:H25 from cattle at slaughter in Italy

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ARTICLE INFO

Keywords:
STEC O177
dairy cattle
carcass
hide cleanliness
antimicrobial resistance

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic pathogens frequently carried by cattle, responsible in humans of mild to bloody diarrhoea, haemolytic uraemic syndrome (HUS) and even death. In 2023–2024, a study on STEC contamination of hide and carcasses of dairy cattle at slaughter was planned in Emilia-Romagna region (northern Italy). When the study was still in progress and 60 animals were sampled, the detection of STEC O177 isolates reached high rates and gained our attention. A total of five O177 STEC strains were detected, namely four from three carcasses (5.0 %) and one from a hide sample (1.7 %). The isolates were typed by WGS as following: *1)* STEC O177:H11 sequence type (ST) 765 (*stx2a*$^+$, *eae*$^+$), detected from one carcass; *2)* STEC O177:H25 ST659 (*stx2c*$^+$, *eae*$^+$) detected from three carcasses and one hide sample. One carcass was contaminated by both STEC serotypes. The isolates carried other virulence determinants often found in STEC strains associated with HUS, namely the *exha, astA* and *espP* genes, together with genes for adhesion to the epithelial cells of the gut (*lpfA, fdeC, fimH)* and non-Locus for Enterocyte Effacement (LEE) effector protein genes *(nleA, nleB)*. The STEC O177:H11 isolate harboured antimicrobial resistance (AMR) genes to β-lactams ($bla_{\text{TEM-1A}}$), aminoglycosides (*aadA1*, *aph(3″)-Ib*, *aph(6)-Id*), trimethoprim (*dfrA1*), sulphonamides *(sul1*, *sul2),* tetracyclines *(tetA)*, *(tetB),* streptothricin *(sat2),* and quaternary ammonium compounds (*qacEdelta1)*. On the contrary, the STEC O177:H25 isolates carried no AMR genes. Persistent carriage of STEC O177:H25 ST659 (*stx2c*$^+$, *eae*$^+$) at farm level was assessed by testing animals of the same herd sent to slaughter.

Interestingly, the colonies of STEC O177:H11 and STEC O177:H25 had different morphology on CHROMagar™ STEC plates, being mauve and colourless, respectively. Since mauve is the colour STEC colonies commonly have on the CHROMagar™ STEC medium, our findings can help microbiologists in the selection of uncommon serotypes.

To the best of our knowledge, this is the first detection of STEC O177 from carcasses and hides of dairy cattle at slaughter. Noteworthy, the STEC-positive hide was classified as "very dirty" thus stressing the need of clean animals entering the slaughter chain, as required by Regulation (EC) No 853/2004. Since STEC O177 has been responsible of HUS in Europe, our data could add information on the source of uncommon serogroups in human infections.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are zoonotic pathogens responsible for severe human infections which can be transmitted by food ingestion, direct animal contact, and human-to-human contact (EFSA, 2020). The most recent and comprehensive source attribution analysis available for STEC underlined that "bovine meat and products thereof" and "milk and milk products" were the vehicles most frequently implicated in STEC infections in the EU in the last ten years (EFSA and ECDC, 2023). Clinical illnesses in humans can vary from uncomplicated non-bloody diarrhoea to severe diseases like haemorrhagic colitis (HC) and life-threatening haemolytic uremic syndrome (HUS) (Karmali et al., 2010). Pathogenicity of STEC for humans can be enhanced by antimicrobial resistance (AMR), which has been reported globally in isolates

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https://doi.org/10.1016/j.ijfoodmicro.2024.110846
Received 12 April 2024; Received in revised form 24 July 2024; Accepted 26 July 2024
Available online 27 July 2024

from animal and human origin and can be responsible for treatment failure in the early stages of infection (Mir and Kudva, 2018).

STEC infections ranked fourth among zoonotic diseases in the European Union (EU) in 2022, with 7117 confirmed cases and a notification rate of 2.1 cases per 100,000 population (EFSA and ECDC, 2023). Unlike other Member States, STEC surveillance in humans in Italy is sentinel and almost limited to the HUS cases. For this reason, the number of reported cases per year is lower than in other EU countries where STEC surveillance system covers all population. In Italy, 65 and 118 confirmed human STEC cases were reported in 2021 and 2022, respectively (EFSA and ECDC, 2023).

The acronym STEC refers to an *E. coli* strain that has acquired the capacity to produce Shiga toxins (Stxs). There are two major Stx types (Stx1 and Stx2) which include several subtypes, namely 4 for Stx1 (a, c, d and e) and 14 for Stx2 (a-m; o) (EFSA, 2020; Lindsey et al., 2023). Many STEC harbour the intimin encoding *eae* gene in the Locus of Enterocyte Effacement (LEE), a 35 kb pathogenicity island whose encoded genes cause histological Attaching and Effacing (A/E) lesions on the surfaces of intestinal epithelial cells responsible for diarrhoea in humans and calves (Fakih et al., 2017; Tozzoli and Scheutz, 2014). STEC which carry the LEE with the *eae* gene are called LEE-positive, to distinguish them from LEE-negative STEC, which carry genes encoding for different adhesion factors (EFSA, 2020; Kaper et al., 2004).

STEC reported as responsible for human diseases are mostly restricted to a small number of serogroups, *i.e.* the so-called "top 5" O26, O111, O103, O145 and O157 in Europe or "top 7" (adding O121 and O45) in the USA. Other serogroups frequently reported include O80 and O91 (Dewsbury et al., 2022; EFSA, 2020; Ekong et al., 2015). In 2022, the most common serogroup notified in the EU was O26 (over 50 % of the reported HUS cases), followed by O157, O80 and O145. However, non-serotyped STEC isolates represented the third most reported "STEC serogroup" in 2022, probably because only a reduced panel of serogroups (such as the "top 5" or the "top 7") are routinely tested or because laboratories ignore the serotyping procedure (EFSA and ECDC, 2023).

Cattle are considered the main animal reservoir for STEC, shedding with faeces a very high number of strains which are pathogenic to humans. The "top 7" serogroups can be found in cattle faeces, together with other highly pathogenic STEC, as the serotypes O104:H4 and O80:H2 (Browne et al., 2018; Cabal et al., 2015; De Rauw et al., 2019; Galarce et al., 2021; Hussein and Bollinger, 2005; Karama et al., 2019).

In the framework of an ongoing project focusing on STEC detection in cattle at slaughter, the recurrent isolation of STEC O177 has gained our attention because, despite being reported in human cases of HUS in Europe, it is not among to the most common serogroups. The main goals of this paper are focused on *i)* virulotyping and antimicrobial resistance of STEC O177 isolated from hides, carcasses and faecal matter of dairy cattle at slaughter; *ii)* information on colonies morphology of this uncommon STEC serogroup, and *iii)* utility of Whole Genome Sequencing as surveillance tool to characterize rare STEC serotypes that could cause foodborne infections.

2. Materials and Methods

2.1. Sample collection

Since April 2023, a project focusing on STEC detection in cattle at slaughter level has been implemented in Emilia-Romagna region, northern Italy. Up to February 2024, 60 culled dairy cows were randomly selected and swabbed on the hide surface after stunning and on the carcass after dehiding and evisceration by using sterile sponges moistened with 10 ml of Buffered Peptone Water (BPW, Oxoid, Basingstoke, UK). Environmental sampling of surfaces at lairage as well as in processing areas was not allowed by the food business operator (FBO) and therefore not included in the study.

A five-step evaluation system was used to assess the level of cleanliness of cattle, which can be classified into five categories: Category 1 - clean and dry; Category 2 - slightly dirty; Category 3 - dirty; Category 4 - very dirty; Category 5 – very dirty and wet (Food Standard Agency, 2002). Before hide sampling and immediately after animal exsanguination, cattle carcasses were assigned to one of the cleanliness categories. The swabbed areas included the hide surface (400 cm^2 in the areas of the brisket and as many in the hind legs, corresponding to a total of 800 cm^2) and the half carcass before refrigeration (400 cm^2 covering the areas of neck, brisket, flank, and thigh). Two sponges for animal were used, one for the hide and the other for the skinned carcass. After swabbing, the sponges were separately placed in sterile bags and transported to the laboratory under refrigeration conditions. In addition, the farms of origin were recorded to trace back the herds of STEC-positive animals and retest cattle of the same farm sent to slaughter to assess the possible persistence of shedding animals. For retesting, rectal swabs were collected from the animals entering the slaughterhouse.

2.2. Sample testing by ISO 13136:2012

The samples were tested within two hours after collection. Following the ISO/TS 13136:2012 method (ISO, 2012) each sponge was put into a sterile bag containing 90 ml of modified Tryptone Soya Broth (mTSB; Oxoid) added with novobiocin (Oxoid), homogenised in a stomacher blender for 2 min and incubated at 37 °C ± 1 °C for 18 to 24 h. After enrichment, a Real-Time PCR was conducted as described in the ISO method using the iQ-Check STEC VirX kit (Biorad, Hercules, CA, USA). For samples showing a positive signal for *stx1/2* determinants (with or without a positive signal for the *eae* gene) 10 µl of their enrichment broth were plated onto three selective media, *i.e.,* Tryptone Bile X-GLUC Agar (TBX; Oxoid) recommended by ISO/TS 13136:2012, plus Cefixime Rhamnose Sorbitol MacConkey Agar (CR-SMAC; Oxoid) and CHROMagar™ STEC (CHROMagar, Paris, France) selected by the laboratory. The plates were incubated at 37 °C ± 1 °C for 20–24 h, except for TBX (41.5 °C ± 1 °C for 20–24 h). Following the manufacturers' instructions, suspect colonies should have the following appearance: Blue green on TBX (presumptive *E. coli* colonies), straw coloured on CR-SMAC (presumptive *E. coli* O157 colonies) and mauve on CHROMagar™ STEC (presumptive STEC colonies). Up to 50 suspect colonies per sample were selected, point-inoculated in Tryptone Soya Agar (TSA; Oxoid) plates and incubated at 37 °C ± 1 °C for 20–24 h. After growth, the single colonies were pooled in sterile distilled water up to seven per pool and tested by PCR. If a pool was positive for the *stx1* and/or *stx2* genes, with or without the *eae* gene, the individual colonies forming the pool were retested by PCR to select the positive ones. *E. coli* species identification was performed by the API ®20E (bioMérieux, Marcy l'Etolie, France) microsubstrate system and the STEC isolates were frozen at −80 °C before further testing.

2.3. Antimicrobial susceptibility testing

The STEC isolates were tested for susceptibility to antimicrobials by using the Sensititre™ EUVSEC3 plates (Thermofisher Scientific, East Grinsted, UK) following the manufacturer's instruction. Clinical categorization of the isolates was performed based on the clinical breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2024). Each Sensititre™ EUVSEC3 plate is customized with 15 antimicrobials (AMs) belonging to 12 different classes: a) aminoglycosides: amikacin, gentamicin; b) β-lactams: ampicillin; c) third generation cephalosporins: cefotaxime, ceftazidime d) carbapenems: meropenem; e) macrolides: azithromycin; f) quinolones: nalidixic acid; g) fluoroquinolones: ciprofloxacin; h) phenicols: chloramphenicol; i) polymixin: colistin; j) tetracyclines: tetracycline; k) glycylcyclines; tigecycline; l) folate pathway antagonists: sulfamethoxazole, trimethoprim. *E. coli* ATCC 25922 was used as quality control microorganism.

2.4. Whole genome sequencing

The isolates were tested for subtyping and characterization by Whole Genome Sequencing (WGS). The genomic DNA of the STEC isolates was extracted on EZ2 connect using EZ1&2 DNA Tissue Kit (Qiagen, Hilden, Germany). Libraries were prepared using Illumina DNA Prep Kit and Nextera DNA CD Indexes (Illumina, San Diego, CA, USA), following the manufacturer's instructions, and WGS was performed on MiSeq Illumina System (Illumina, San Diego, CA, USA). The raw reads quality was verified using FastQC (Galaxy Version 0.72 + galaxy1), and adapters and low-quality reads were trimmed with Trimmomatic (Galaxy Version 0.38.1) using the following quality filter: a minimum quality of Q25, a window size of 20 with Q25 as the average quality, and a minimum length read of 36 bp. Then, the trimmed reads were assembled *de novo* in contigs using SPAdes (Galaxy Version 0.38.1). Firstly, the contigs were analysed with the tool *E. coli* Serotyper (Galaxy Version 1.2) for O and H typing. Then, sequence type (ST) was assigned using the MLST tool (Galaxy Version 2.16.1) (ARIES, 2024). To detect the virulence genes, such as genes encoding Shiga toxins, and the antimicrobial resistance genes, VirulenceFinder and ResFinder tools were applied (Center for Genomic Epidemiology, 2024). The diversity of the strains was assessed through single nucleotide polymorphisms (SNPs) typing using the CFSAN pipeline (https://snp-pipeline.readthedocs.io/en/latest/). The results were plotted in a maximum-likelihood phylogenetic tree produced with the RAxML (Stamatakis, 2014) software and visualized with iTOL (https://itol.embl.de/).

3. Results

Six STEC isolates belonging to serogroup O177 were detected from different sample types. Four isolates were detected from three bovine carcasses, being carcass No 27 contaminated by two different strains (O177:H11 ST765 and O177:H25 ST659). The other two carcasses (No 39 and No 46) were positive for STEC O177:H25 ST659, which was isolated also from the hide of cattle No 60, assigned to category 4 of cleanliness. Prevalence of STEC O177-contaminated carcasses was 3/60 (5.0 %; 95 % CI 1.7–13.7), while prevalence on hide surface was 1/60 (1.7 %; 95 % CI 0.3–8.9). Farm traceability for cow No 27 gave us the possibility of testing another animal (A2) arriving from the same herd, whose rectal swab was positive for STEC O177:H25 ST659. The time interval between sampling was three months.

On the CHROMagar™ STEC medium the colonies of STEC O177:H11 ST765 and STEC O177:H25 ST659 were different: The former had the typical mauve appearance and were positive for the *stx2a*, *eae* and *exha* genes, and the latter were colourless and positive for the *stx2c*, *eae* and *exha* genes.

The complete set of virulence genes harboured by the STEC O177 isolates is shown in Table 1. Beyond *stx2a* or *stx2c* and *eae*, they carried toxin (*astA, ehxA, hylE*) and adhesion genes (*lpfA, fdeC, fimH)*. The presence or the absence of virulence genes was displayed with a heatmap using Seaborn Python libraries (Fig. 1). For the construction of the graphic representation, only genes with a percent identity >90 % and which cover the full-length reference gene were included. A perfect alignment is 100 % and must cover the entire length of the virulence gene in the reference database. In our study, all genes found have a percentage of identity in the alignment between the best matching virulence gene in VirulenceFinder and the corresponding sequence in the input genome higher than 98 %.

In addition, the STEC O177:H11 strain carried several genes encoding for resistant factors to β-lactams ($bla_{\text{TEM-1A}}$), aminoglycosides (*aadA1*, *aph(3″)-Ib*, *aph(6)-Id*), trimethoprim (*dfrA1*), sulphonamides *(sul1*, *sul2),* tetracyclines *(tetA, tetB),* streptothricin *(sat2),* and quaternary ammonium compounds (*qacEdelta1),* while STEC O177:H25 did not.

Consistently, MIC testing revealed breakpoints values indicative of resistance to ampicillin, tetracycline, tigecycline, trimethoprim and sulphamethoxazole for STEC O177:H11 only, while STEC O177:H25 strains were sensitive to all the AMs tested.

Finally, the genetic relationship between STEC O177:H25 strains was evaluated by SNPs analysis (Fig. 2 a). Using SNPs distance matrix results, a phylogenetic tree has been constructed with maximum likelihood method (Fig. 2 b). The figure shows that all the strains are genetically different and with the exception of the isolates A2 and 27C/2, which were isolated from dairy cows reared in the same farm, that having only 17 different SNPs can be considered phylogenetically closely related (Fig. 2).

4. Discussion

As pointed out in the Introduction section, our paper is focused on STEC O177 only, regardless of other STEC serogroups detected on carcasses and hide surfaces and irrespective of the ending of the project, because information on a highly pathogenic and poorly investigated STEC serogroup should deserve rapid dissemination to the scientific community. To the best of our knowledge, our study reports the first isolation of STEC O177 from bovine meat in Europe. These data are important because serogroup O177 was responsible for HUS cases in Italy, as well as in other European countries (Italian National Institute of Health, 2022; Rodwell et al., 2023). Since it does not belong to the most commonly identified STEC serogroups, namely STEC-5 or STEC-7, its description in terms of virulence, cultural traits and animal niche is of the greatest importance.

The two serotypes O177:H11 and O177:H25 differed for the Stx2 variant, antimicrobial resistance and prevalence, with STEC O177:H11 found on one carcass only (1.7 %) and STEC O177:H25 found on three carcasses (5.0 %) and one hide surface (1.7 %). As shown in Fig. 1, all the isolates harboured a core of 24 virulence genes, including the *stx2, eae*, *ehxA,* and *espP* genes, which are associated with severe clinical outcomes in humans (Boerlin et al., 1999), together with haemolysis, adhesion, and other virulence genes frequently carried by strains associated with HUS (Naseer et al., 2017). The main difference in their virulence consisted in the Stx2 variant. Indeed, the strain O177:H11 ST765 harboured the *stx2a* gene encoding Stx2a, which is significantly associated with the risk of HC and HUS, being more potent than other Stx2 subtypes on human renal epithelial cells and Vero cell monolayers (Fuller et al., 2011). On the contrary, all O177:H25 strains harboured *stx2c*, a subtype also associated with infections ending with HC and HUS (Alotaibi and Khan, 2023). Although all isolates with serotype O177:H25 presented the same Stx2 subtype (*stx2c*) and belonged to the same sequence type (ST659), they showed slight differences in the virulence genes profile. An explanation could be that *E. coli* virulence factors are found on mobile genetic elements, such as plasmids, bacteriophages, transposons, pathogenicity islands (PAIs), and insertion sequence elements. These elements can be lost and transferred from bacterium to bacterium leading to a different virulence genes profile between strains (FAO/WHO, 2019).

Additional differences between the two serotypes laid down in their sensitivity to antimicrobials (AMs), since STEC O177:H11 carried resistant genes to six classes of AMs (β-lactams, aminoglycosides, trimethoprim, sulphonamides, tetracyclines, and streptothricin) and to quaternary ammonium compounds (CARD, 2023; Paulsen et al., 1993), while the STEC O177:H25 strains carried no AMR genes. Phenotypical resistance was assessed by the MIC test, with the STEC O177:H11 strain resistant to four classes of AMs (β-lactams, tetracyclines, sulphonamides and diaminopyrimidines) and therefore classified as multidrug resistant (Magiorakos et al., 2012). Indeed, a wider set of AMR genes were identified using WGS analysis, as $bla_{\text{TEM-1A}}$, *aadA1*, *aph(3″)-Ib*, *aph(6)-Id*, *dfrA1, sul1*, *sul2, tetA* and *tetB*, which are frequently harboured by STEC isolates (Mir and Kudva, 2018), together with unusual determinants, as *sat2* and *qacEΔ1*. Interestingly, the *sat2* gene encodes for streptothricin-acetyltransferase which confers resistance to streptothricin, a nucleoside antibiotic (Tietze and Brevet, 1990), whose resistance was not tested

Table 1
Virulence genes harboured by the STEC O177 strains of bovine origin, their functions, and references.

Virulence genes	O177 serogroup						Virulence factors	Functions	References
	O177:H11	O177:H25							
	27C/3	27C/2	39C/3	46C/2	60H/2	A2			
astA	+	+	+	+	+	+	Enteroaggregative heat-stable enterotoxin 1 (EAST1)	Activation of an intra-cellular cascade mechanism	Mainil, 2013 Savarino et al., 1993
anr		+	+	+	+	+	Anaerobic transcriptional regulator	Expression of physiological functions under oxygen-limiting or anaerobic conditions	Winteler and Haas, 1996
cia	+						Colicin-Ia	Membrane depolarization	Yao et al., 2022
cif	+	+	+	+	+	+	T3SS effector Cif	Citotoxicity	Garmendia et al., 2005
csgA	+	+	+	+	+	+	Curlin major subunit CsgA	Amyloid forming fibrils	Szulc et al., 2021
eae	+	+	+	+	+	+	*E. coli* A/E gene	Intimin encoding	Jerse et al., 1990 Mainil, 2013
ehxa	+	+	+	+	+	+	Plasmid-encoded enterohaemolysin	Hemolytic activity	Mainil, 2013 Schmidt et al., 1994
espA	+	+	+	+	+	+	T3SS translocator EspA	T3SS structural protein	Mainil, 2013
espB	+	+	+	+	+	+	T3SS translocator EspB	T3SS structural protein	Mainil, 2013
espF	+						T3SS secreted effector EspF	Citotoxicity	Garmendia et al., 2005
espI	+	+	+	+	+	+	T3SS secreted effector EspI	Disruption of protein trafficking	Clements et al., 2013
espJ	+						T3SS secreted effector EspJ	Clearance dynamics regulation	Garmendia et al., 2005
espP	+	+	+	+	+	+	Serine protease EspP	Limitation of effector translocation to host cells	Cameron et al., 2018 In et al., 2013
fdeC	+	+	+	+	+	+	Factor adherence *E. coli*	Adhesion	Nesta et al., 2012
fimH	+	+			+	+	Type 1 fimbriae D-mannose specific adhesin	Adhesion	Magala et al., 2020
fyuA	+						Ferric yersiniabactin uptake receptor	Biofilm formation	Hancock et al., 2008
gad		+		+	+	+	Glutamate decarboxylase	Acid resistance system enzyme	Castanie-Cornet et al., 1999
hlyE	+	+	+	+	+	+	Hemolysin E	Lysis of mammalian cells	Ristow and Welch, 2016
iha		+	+	+	+	+	IrgA homologue adhesin	Confers adherence capability	Tarr et al., 2000
	27C/3	27C/2	39C/3	46C/2	60H/2	A2			
irp2	+						Iron uptake system	Siderophore encoding	Smati et al., 2017
iss	+	+	+	+	+	+	Increased serum survival gene	Complement resistance	Biran et al., 2021
iucC		+	+	+	+	+	Iron uptake chelate	Aerobactin synthetase reaction	de Lorenzo and Neilands, 1986
iutA		+	+	+	+	+	Iron uptake transport	Receptor for iron-aerobactin	de Lorenzo et al., 1986
katP		+	+	+	+	+	Bifunctional catalase-peroxidase family member	Catalase-peroxidase activity	Brunder et al., 1996
lpfA	+	+	+	+	+	+	Type 1 fimbrial protein	Adhesion	Valat et al., 2012
nleA	+	+	+	+	+	+	non-LEE-encoded T3SS secreted effector NleA	Disruption of intestinal tight junctions	Thanabalasuriar et al., 2010
nleB	+	+	+	+	+	+	non-LEE-encoded T3SS secreted effector NleB	Inhibition of TNFα pathway	Newton et al., 2010
nleC	+		+	+	+	+	non-LEE-encoded T3SS secreted effector NleC	Blocking of the inflammatory response	Pearson et al., 2011
nlpI	+	+	+	+	+	+	New lipoprotein I	Outer membrane-anchored lipoprotein	Schwechheimer et al., 2015
ompT	+	+	+	+	+	+	Omptin family outer membrane protease OmpT	Cleavage of antimicrobial peptides	McCarter et al., 2004
stx2a	+						Shiga Toxin 2a	Protein synthesis inhibition	Melton-Celsa, 2014
stx2c		+	+	+	+	+	Shiga Toxin 2c	Protein synthesis inhibition	Melton-Celsa, 2014
tccp			+				Tir-cytoskeleton coupling protein	Direct role in A/E lesion formation and actin polymerization	Garmendia et al., 2005
terC	+	+	+	+	+	+	Tellurium resistance protein TerC	Transmembrane protein eccoding	Turkovicova et al., 2016
tir	+	+	+	+	+	+	T3SS translocate intimin receptor Tir	Intimin receptor (Tir) encoding	Mainil, 2013

	27C/3	27C/2	39C/3	46C/2	60H/2	A 2			
traJ	+						F plasmid positive regulator protein TraJ	Plasmid transfer during bacterial conjugation	Gubbins et al., 2002
traT	+	+	+	+	+	+	Complement resistance protein precursor TraT	Outer membrane protein	Binns et al., 1981
yehA	+	+	+	+	+	+	putative fimbrial adhesin YehA	*yeh* operon - biofilm formation	Korea et al., 2010
yehB	+		+	+			putative fimbrial usher protein YehB		
yehC	+	+	+	+	+	+	putative fimbrial chaperone YehC		
yehD	+	+	+	+	+	+	putative fimbrial protein YehD		

because not included in the panel of antimicrobials prioritized by Decision (EU) 2020/1729 (European Commission, 2020). The *qacEΔ1* is an integron-encoded resistant gene to quaternary ammonium compounds, frequently found in *E. coli* (Sajeev et al., 2023). Its identification is of concern because reduced sensitivity to disinfectants commonly used in the food industry may promote STEC food-borne diseases (Tong et al., 2021).

Concerning the isolation procedure, the ISO/TS 13136:212 method requires that *stx*-positive enrichment broths must be plated onto TBX agar plates and, eventually, other selective media to pick up *E. coli* resembling colonies. In our study, *stx2*- and *eae*-positive enrichment broths of carcass, hide surface and faecal samples gave the following outcomes once plated onto the solid media: 1) TBX agar (Oxoid): The selection of suspect *E. coli* blue-green colonies grown in the medium proposed by the ISO method was not useful to identify STEC O177 colonies because they could not be distinguished from common *E. coli* ones, thus resulting in time-consuming and unsuccessful confirmation tests; 2) CR-SMAC (Oxoid): Since the medium specifically targets straw-coloured colonies of serogroup O157, the selection of non-target pink colonies was not useful to identify *stx*-carrying cultures; 3) CHROMagar™ STEC (CHROMagar): This was the medium of choice for the detection of the STEC O177 strains, but great attention should be paid to the selection of suspect colonies. Indeed, following the manufacturer's instructions, both blue and colourless colonies should be discharged. On the contrary, excluding the abundant blue ones (presumptive *Enterobacterales* colonies), both the mauve (suspect STEC colonies) and colourless colonies (presumptive *Enterobacterales*) were tested by PCR and found to be positive for *stx2* and *eae* genes. Since the manufacturer clarifies that the mauve colonies could belong to the "most common" STEC serotypes, we strongly recommend of testing also colourless ones, which could belong to serotypes not included in the validating procedure (https://www.chromagar.com/en/product/chromagar-stec/).

In conclusion, the chromogenic selective medium used in the study can give better results than the TBX and CR-SMAC media in terms of colonies identification, the morphology of which, however, might vary among serotypes. After STEC isolation, we strongly recommend WGS as the best tool to identify not only virulence traits and AMR determinants, but also a much larger number of serotypes if compared to the method proposed by the ISO/TS 13136:2012 and restricted to the STEC-5.

In our study, STEC O177 were isolated both from carcasses and hide surface of dairy cows. Noteworthy, the positive hide was classified as "very dirty" (Category 4) thus stressing the need of clean animals entering the slaughter chain, as required by Regulation (EC) No 853/2004 (European Commission, 2004). The higher occurrence of STEC O177 from carcasses than hides could be related to environmental contamination of equipment and working areas by the microorganisms entering the slaughterhouse *via* contaminated animals. Since we had no agreement to assess the environmental contamination by STEC at the slaughterhouse, our hypothesis will need further studies. In any case, it is not surprising because the persistence of STEC on food processing equipment, as well as in the farm environment, can be favoured by the formation of biofilm (Blankenship et al., 2020; Wang et al., 2012).

The role of STEC O177:H11 for human health has been reported in England, where STEC O177:H11 ST29 was responsible for 3 % the HUS cases notified in 2014–2021 (Rodwell et al., 2023). In Italy, according to the Italian Haemolytic Uraemic Syndrome Registry, STEC O177 was reported in 2 % of 78 HUS cases notified between 1st August 2021 and 31st July 2022, with 76 (97 %) cases diagnosed in the paediatric population (< 15 years) and two cases in adult patients (3 %). Among the paediatric population, >70 % of the HUS cases affected children <4 years (median age: 37 months). In 58 cases (74 %) information on serogroups was available, with O26 largely prevalent (52 %), followed by O157 (19 %), O145 (10 %) and O111 (5 %). One patient (2 %) was infected by O177, which was also reported in HUS cases of the previous 10 years. Unfortunately, the sources of human infections have remained unknown, as the Italian National Institute of Health commonly tests biological samples (faeces, serum) collected from hospitalized patients in different regions of the country, in absence of any link to food items (Italian National Institute of Health, 2022).

Some information on *E. coli* belonging to serogroup O177 is available from literature. In cattle population, STEC O177:H11 ST765 carrying *stx2c* and *eaeβ* was isolated from healthy cattle in Belgium, together with Enteropathogenic *E. coli* (EPEC) O177:H11 ST765 strains (carrying the *eae*-gene but lacking the *stx* genes) in healthy and diarrhoeic calves as well as in healthy cattle. These bovine STEC and EPEC isolates shared several virulence genes with a human STEC O177:H11 ST29 strain carrying *stx1a* and *eaeβ* isolated in the same country, thus suggesting a continuous evolution of STEC and EPEC strains in the bovine reservoir and a likely connection with human sources in the same geographical area (Habets et al., 2022). From healthy cattle, STEC O177:H25 carrying a *stx2c* variant gene identical to the *stx2c* of a human clinical isolate was reported from China (Sheng et al., 2018). Interestingly, STEC O177:H25 was isolated from two patients (St. Olav176 and St. Olav178 strains) in Norway (Gabrielsen et al., 2015) and was included among the 21 most relevant serogroups for human infections in Spain (Sánchez et al., 2015).

In our study, the persistence of STEC O177:H25 at herd level was confirmed by testing the faecal matter of a dairy cow (A2) slaughtered three months after the first detection on the carcass No 27. Indeed, having only 17 different SNPs, as inferred by the SNPs analysis, these isolates (A2 and 27C/2) can be considered highly related. This result confirmed that STEC O177:H25 detected on the carcass No 27 in November 2023 was still shed by cattle of the same farm in February 2024, in line with the studies on persistent shedding of STEC by cattle (Blankenship et al., 2020).

Since the STEC O177 isolates of the study were detected from culled dairy cows and not from calves or beef cattle, we could not exclude the likelihood of milk contamination, especially because persistence of STEC on udders and teats has been demonstrated (Fremaux et al., 2006). As human infections are frequently caused by ingestion of raw or undercooked bovine meat as well as raw milk and cheeses made from raw milk (EFSA, 2020), our findings could be helpful for tracing STEC human cases of O177:H11 and STEC O177:H25 in the bovine food chain.

Finally, although very challenging, on-farm monitoring of STEC shedding by bovines prior to slaughter could allow food handlers to manage the risk during the slaughter practices. As proposed by EFSA (2013), the STEC status of bovines to be slaughtered within one month could be assessed by testing pooled faeces on farm. These results should be integrated in the Food Chain Information - required by Regulation (EC) No 853/2004 (European Commission, 2004) - to classify the cattle sent to slaughter into high or low risk categories and allow Food

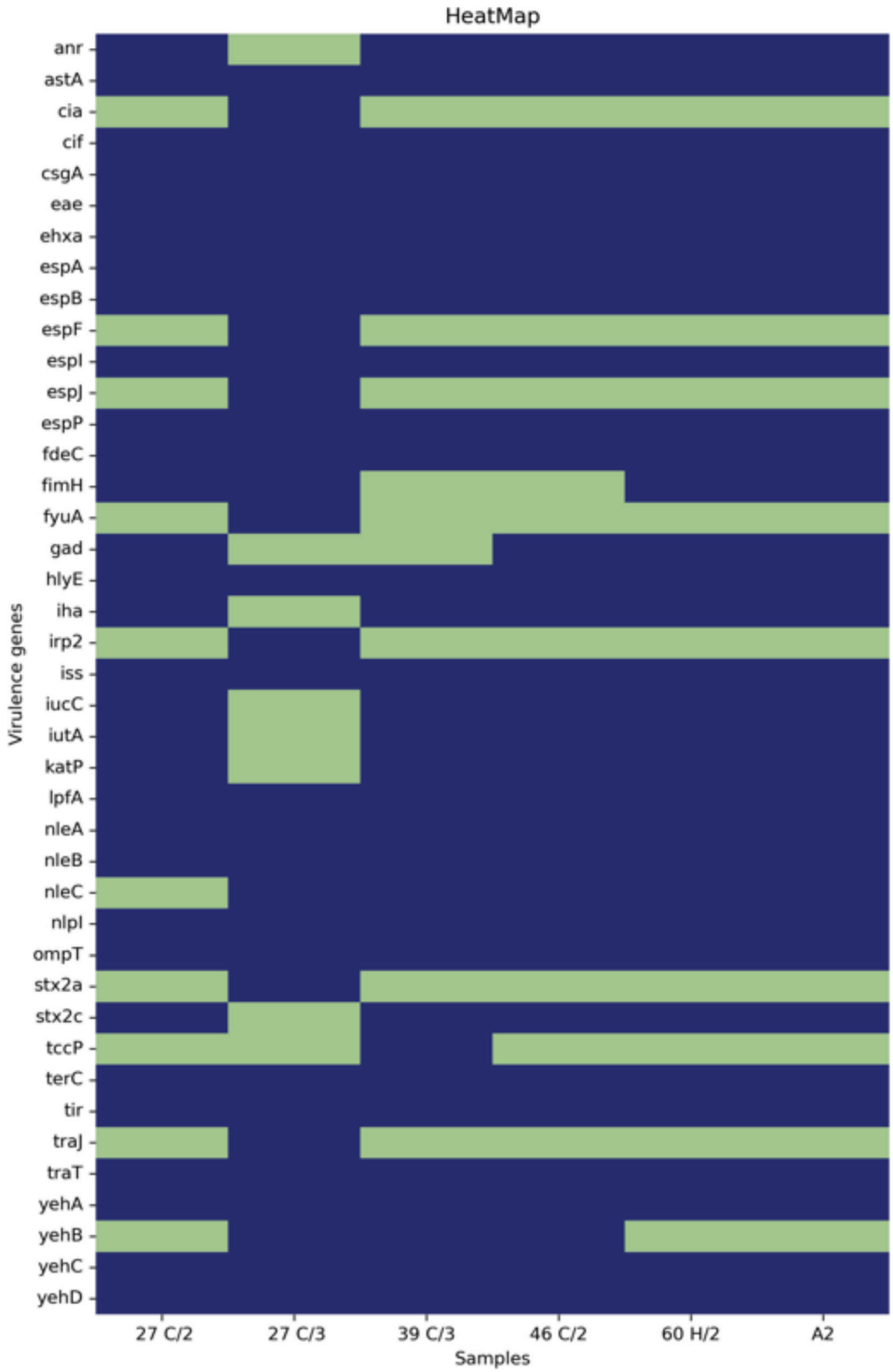

Fig. 1. Heatmap showing the presence/absence of virulence genes (y-axis) within the STEC isolates identified in this study (x-axis). Presence of virulence genes shown in blue, while absence in light green.

Business Operators to select the most appropriate slaughter process, including logistic slaughtering of STEC-positive animals. As an alternative, STEC testing of bovine carcasses, while not yet covered by Regulation (EC) No 2073/2005 (European Commission, 2005), could be a less expensive tool to improve process controls at slaughter.

Funding

This work was supported by the University of Parma [FIL 2022, DR 418–2023].

a)

	27 C/2	39 C/3	46 C/2	60 H/2	A2
27 C/2	0	560	567	44	17
39 C/3	560	0	147	558	559
46 C/2	567	147	0	564	565
60 H/2	44	558	564	0	45
A2	17	559	565	45	0

b)

Fig. 2. a) SNPs distance matrix results of the STEC O177:H25 strains using A2 as reference.
b) Phylogenetic tree based on the maximum likelihood method of the STEC O177:H25 strains. The A2 isolate was used as outgroup to root the tree.

CRediT authorship contribution statement

Silvia Bonardi: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Mauro Conter:** Writing – original draft, Investigation, Data curation. **Laura Andriani:** Writing – original draft, Formal analysis. **Cristina Bacci:** Supervision, Data curation. **Giulia Magagna:** Writing – original draft, Formal analysis. **Martina Rega:** Formal analysis. **Luca Lamperti:** Data curation. **Carlo Loiudice:** Investigation, Data curation. **Marco Pierantoni:** Supervision. **Virginia Filipello:** Writing – original draft, Supervision, Data curation.

Declaration of competing interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the study reported in this paper.

Data availability

The data that has been used is confidential.

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