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Escherichia coli and food safety: occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in Triveneto (Northern Italy)

Escherichia coli e sicurezza alimentare: presenza di *Escherichia coli* produttori di tossine Shiga (STEC) nell'area del Triveneto.

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Summary

Abstract

Shiga toxin-producing *Escherichia coli* (STEC) is currently the third most common cause of foodborne disease in Europe, after *Campylobacter* and *Salmonella*. A global estimation made by WHO reported 2.5 million cases (including 1.2 million cases foodborne), with 3.330 cases of haemolytic uremic syndrome (HUS) and 269 deaths in 2010. Despite the relevance of STEC for public health, a precise estimate of its occurrence in foodstuffs is not available. This is because the only existing regulatory limit following a microbiological criterion in Europe concerns sprouts, even though several data, including those of the Triveneto about the occurrence of STEC in certain food categories, show that the main food categories involved in STEC outbreaks are meat and dairy products (especially of raw milk origin). The lack of regulation means that monitoring plans among Member States (MSs) are not harmonised and therefore a correct estimation of STEC presence in time and space is prevented. Therefore, an EU regulation regarding the monitoring of STEC in foodstuffs involved is needed. Moreover, the methods currently used to identify STEC do not estimate the pathogenicity of isolates, because a pathogenicity marker shared by all STEC strains does not still exist. Nevertheless, several MSs are implementing Whole Genome Sequencing (WGS) techniques that allow human isolates to be fully typed by identifying the genes most frequently associated with severe illness. In this sense, it will be possible to reformulate the current methods of STEC detection and characterization to better identify the pathogen in food matrixes.

Riassunto

Gli *Escherichia coli* produttori di tossine Shiga (STEC) rappresentano ad oggi la terza causa di infezione zoonotica a trasmissione alimentare in Europa, dopo *Campylobacter* e *Salmonella*. Una stima globale del WHO dell'anno 2010 ha riportato 2.5 milioni di casi (di cui 1.2 trasmessi da alimenti) con 3,330 casi di sindrome emolitico-uremica (SEU), e 269 morti. Nonostante la sua rilevante importanza per la salute pubblica, non è possibile avere una stima precisa sulla presenza di questo microrganismo negli alimenti da esso contaminati. Questo perché l'unico limite microbiologico europeo (criterio di sicurezza alimentare) riguarda i germogli, ma i dati riportati nell'elaborato, tra cui la presenza di STEC in alcune categorie alimentari del Trivento, dimostrano che i maggiori alimenti coinvolti nei focolai da STEC sono altri, in particolare la carne e i prodotti lattiero-caseari (latte crudo, nello specifico). La mancanza di una regolamentazione per queste categorie di alimenti fa sì che i piani di monitoraggio dei vari stati europei non siano armonizzati e di conseguenza non è possibile formulare l'incidenza corretta di STEC nel tempo e nello spazio. Si rende quindi necessaria una regolamentazione a livello europeo per quanto riguarda il monitoraggio e la sorveglianza degli STEC negli alimenti implicati. In più, i metodi attualmente utilizzati per identificare gli STEC non permettono di stimare il grado di patogenicità dell'isolato e questo perché ad oggi non esiste ancora un marker di patogenicità per questo patogeno. Tuttavia, sempre più paesi stanno implementando le tecniche basate sul sequenziamento del genoma (Whole Genome Sequencing; WGS) che permettono di tipizzare completamente gli isolati umani individuando geni o combinazioni di essi più frequentemente associati alle forme di malattia grave. Grazie a questi sviluppi, sarà possibile riformulare i metodi attuali di identificazione e caratterizzazione per ottenere una maggiore comprensione di questo patogeno.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a defined as a pathogenic variant (pathotype) of *E. coli* (Croxen et al., 2013). STEC represents one of the most common foodborne diseases causing gastrointestinal symptoms globally (FAO/WHO, 2018) and it ranked third among the human zoonoses in the EU during 2019, following *Salmonella* and *Campylobacter* (EFSA and ECDC, 2021).

STEC is characterized by the production of toxins either termed Shiga toxins (Stx), because of the similarity with the toxin produced by *Shigella dysenteriae* (O'Brien et al., 1982), or Verocitotoxins (VT) because of their activity on Vero cell monolayers (Konowalchuk et al., 1977). Human STEC infection can cause severe illnesses, such as hemorrhagic colitis and hemolytic uraemic syndrome (HUS), especially among young children and elderly (Ochoa & Cleary, 2003).

Ruminants represent the main reservoir of STEC, harboring them in the gut. Thus, human infection usually occurs through contaminated food or water with cattle feces (Gyles, 2007).

To date, contaminated milk and bovine meat represent both the major cause of STEC outbreaks in EU (EFSA BIOHAZ Panel et al., 2020) and the major STEC source (EFSA and ECDC, 2021). Despite this, the only existing regulatory limit following a microbiological criterion in Europe concerns sprouts (Regulation (EC) No 2073/2005).

In occasion of my internship period in the public health laboratory 'Istituto Zooprofilattico delle Venezie', headquarters of Trento, I had access to the data concerning the detection of STEC in certain food categories. Most of the self-monitoring plans of Triveneto concerns raw milk and raw milk products because of the importance of cheese-manufacturing in this area. The data of Triveneto area are compared with the occurrence of STEC in foodstuffs, especially milk and milk products, presented in the EFSA and ECDC 2019 zoonoses report, as well as the number of outbreaks where raw milk was implicated in EU (2012-2017) and the number of RASFF notifications concerning these products during 2020-2021.

These data show that raw milk and raw dairy products represent a relevant source of STEC in the EU, especially in those areas where raw milk is widely used for dairy products. Thus, the harmonization including monitoring plans along the high-risk food chains is needed, in order to provide an accurate estimation of the occurrence of STEC in those food categories (EFSA BIOHAZ Panel et al., 2020).

1. *Escherichia coli*

Escherichia coli is a Gram-negative bacillus, oxidase-negative, within the Enterobacteriaceae family. It is an aerobe or facultative anaerobe bacterium, non-spore-forming, non-motile or motile thanks to its peritrichous flagella. Mesophilic like all the other Enterobacteriaceae, it represents the major part of the normal microflora in warm blooded animals intestinal tract, since its optimum growing temperature is 35-40°C, with some strains able to grow at 46°C. Other environmental factors, such as pH and water activity (a_w) affect the growth and survival of *E. coli*. The optimum pH is 6-7, with a range of 4.4-10.0, while the minimum value of a_w is 0.95 (Table 1) (Desmarchelier PM & Fegan N, 2003; ICMSF, 1996).

According to the Kauffman classification scheme, *E. coli* can be classified by serotypes on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens. A specific combination of O, H and sometimes K antigens, defines a serotype (Kaper et al., 2004; Nataro & Kaper, 1998). The somatic antigen (O) is represented by the polysaccharide portion of cell wall lipopolysaccharide (LPS) and it is thermostable, while the flagellar antigen (H) has a proteinaceous nature and it is thermolabile. Currently, there are more than 188 O antigens and 56 H antigens (EFSA BIOHAZ Panel et al., 2020).

	Minimum	**Optimum**	**Maximum**
Temperature (°C)	7–8	35–40	46
pH	4.4	6–7	10.0
Water activity	0.95	0.995	–

Table 1: Limits for growth of E. coli when other conditions are near optimum.

Through gain and loss of genetic material *E. coli* has acquired virulence attributes, causing three general clinical syndromes: enteric/diarrheal disease, urinary tract infections and sepsis/meningitis (Kaper et al., 2004). A growing number of serogroups, based only on O antigens, are more frequently associated with pathogenic *E. coli* and thus with human disease: for instance, O157, O26, O111, O103 and O145 are defined as “top five” serogroups causing illness in humans (EFSA BIOHAZ Panel et al., 2020). However, serogroups and serotypes have a critical role in the epidemiological investigations, because they do not define the virulence of the microorganism, being O/H surface antigens only (EFSA BIOHAZ Panel et al., 2020). In fact, not all serotypes have been implicated in human infections (FAO/WHO, 2018). Moreover,

E. coli virulence genes are often present on mobile genetic elements which can be lost or transferred, and the same serotype often carries different virulence genes and hence can cause different diseases (EFSA BIOHAZ Panel et al., 2020).

(EFSA BIOHAZ Panel et al., 2020), virulence characteristics and mechanisms of pathogenicity of the microorganism define the classification by pathotypes:

1. Enteropathogenic *E. coli* (EPEC);
2. Enterotoxigenic *E. coli* (ETEC);
3. Enteroaggregative *E. coli* (EAEC);
4. Enteroinvasive *E. coli* (EIEC);
5. Diffusely Adherent *E. coli* (DAEC);
6. Adherent Invasive *E. coli* (AIEC);
7. Shiga toxin-producing *E. coli* (STEC).

EPEC was the first identified *E. coli* pathotype after infant diarrhoea large outbreaks in the United Kingdom during 1940s and 1950s (Bray, 1945). As mechanism of action, EPEC produces a localized adherence to the intestinal mucosae through their bundle-forming pili, which enable it to bind together the cells forming a network (Giron et al., 1991). This step triggers the signal for the attaching and effacing (A/E) lesion, which is the hallmark of EPEC pathogenesis (Croxen et al., 2013). A/E lesion is characterized by an intimate adhesion between the microorganism and the small-intestinal epithelial cells with altered cytoskeleton, which is ensured by the intimin protein, encoded by the *eae* gene. This results in the characteristic 'pedestal-like' lesion, produced through secretion of conserved bacterial proteins via a type III secretion system (T3SS) (Kenny, 2002) which leads to the dissolution of the intestinal brush border causing watery diarrhea in the host.

ETEC differs from the other pathotypes thanks to its capacity of secreting heat-labile toxins (LTs) or heat-stable toxins (STs). This pathotype represents the major cause of the so-called "traveler's diarrhoea" and it is endemic in most developing countries with significant mortality rates in children (EFSA BIOHAZ Panel et al., 2020).

EAEC can adhere to the intestinal mucosa in a pattern known as auto-aggregative, in which bacteria adhere to each other forming a biofilm on the mucosa. This mechanism is followed by the secretion of enterotoxins and cytotoxins and leads to a mucous diarrhoea which has the most severe effect on the colon (Kaper et al., 2004).

EIEC is capable of invading the epithelial cells of the intestine, resulting in lesions by migrating into adjacent cells (Kaper et al., 2004).

DAEC is characterized by the ability to induce a cytopathic effect; microvilli extension after adhering the mucosa could be the mechanism that results in diarrhoea (Kaper et al., 2004).
AIEC colonizes the intestinal mucosa of patients with Crohn's disease and is capable of invading the epithelial cells as well as replicating within macrophages. AIEC uses type I pili to adhere to the intestinal cells and long polar fimbriae that contribute to invasion (Croxen et al., 2013).
STEC is characterized by the production of Shiga toxin (Stx), which invade the bloodstream and mostly affect the micro-circulation of colon and kidneys. Most of STEC share with EPEC the A/E lesion, which enables them to attach the intestinal mucosa of the gross intestine through the intimin, resulting in watery diarrhoea. The pathotype Enterohaemorrhagic *E. coli* (EHEC) was previously defined as STEC subgroup associated with haemorrhagic colitis. Nevertheless, EHEC has been substituted with STEC terminology, since the term EHEC was based on the overcome opinion that only certain types of STEC were highly pathogenic to humans and homogeneously identified by the presence of the *eae* gene and specific LPS (EFSA BIOHAZ Panel et al., 2020)**.**

However, this scheme does not take in account the emergence of cross-pathotypes, caused by genes transfer between organisms. This process brings to the creation of new strains harboring pathogenicity genes associated with more than one pathovar, e.g. the EAEC O104:H4 strain acquiring *stx2a* genes isolated in the German outbreak during 2011(Brzuszkiewicz et al., 2011) and for this reason called enteroaggregative hemorrhagic *E. coli* (EAHEC). To date, techniques based on Whole Genome Sequencing (WGS) are useful both for serotyping and identification of virulence genes, improving the detection of new cross-patotypes (Lindsey et al., 2016).

2. Shiga toxin-producing *Escherichia coli* (STEC)

STEC strains are an important cause of foodborne disease (WHO, 2018). An *E. coli* strain is defined as STEC if it is able to produce at least one type of *E. coli* Shiga-toxins (Stx) which are encoded in prophages integrated into the bacterial chromosome (Gyles, 2007). The name 'Shiga' derive from the similarity to a cytotoxin produced by *Shigella dysenteriae* serotype 1 (O'Brien et al., 1982), but Stx can also be called Verotoxins (VT), based on their cytotoxicity for Vero cells (Konowalchuk et al., 1977). Stxs exists in two major types, Stx1 and Stx2, each of them including variants. Stx1 is structurally identical to the Shiga toxin of *S. dysenteriae*, but for one amino acid. In contrast, Stx2 share less than 60% amino acid sequence with Stx1 (O'Brien et al., 1982). A study published in 2014 estimated the existence of to over 1000 serotypes producing any one of the Stx1 and Stx2 subtypes, or combinations of them (Bettelheim et al., 2014)

The most well-known and studied STEC serotype is O157:H7 (Lim et al., 2010). Recognised for the first time as a cause of bloody diarrhea in the USA in 1982, which involved at least 47 people from Oregon and Michigan who consumed undercooked beef patties belonging to the same food-chain restaurant (Riley et al., 1983). O157:H7 serotype is currently the most frequently linked to foodborne illness (WHO, 2018). Nevertheless, many non-O157 serogroups have been recognised and associated with human disease from the 1990s onwards (Lothar Beutin, 1998). For instance, serotype O103:H2 infected 9 German children after the consumption of raw-milk during a school-trip to Austria in 2017 (Mylius et al., 2018). Another relevant serogroup is O26, which has been recently detected in a French outbreak which involved 13 children after the consumption of raw cow's milk cheeses (Jones et al., 2019). Overall, non-O157 serogroups have a various distribution across different countries (FAO/WHO, 2018).

2.1. Growing and resistance features

STEC strains share with the Enterobacteriaceae family most of their growing (Table 1 in section 1) and stress-resistance features that have been well studied for STEC O157:H7. The a_w parameter is particularly important, considering that STEC can survive during drying process of foodstuff where a_w gradually decreases. A study made by the Istutito Zooprofilattico delle Venezie (Northern Italy) during 2018-2019, found that some raw milk cheeses where still positive for STEC after 7 (a_w=0,94), 8 and 12,3 (a_w=0,91) months of ripening (IZSVe, 2019). Two other main characteristics are critical in favouring STEC pathogenicity: acid and low temperature resistance. Both O157 and non-O157 STEC strains (G.-H. Kim et al., 2016) are

more tolerant to acid than commensal *E. coli*; in addition, the capacity to grow down to pH of 4.4 (WHO, 2018) enables STEC to survive and grow in foodstuffs like yogurt (Massa et al., 1997) and juices (Linton et al., 1999). STEC acid resistance is obviously useful to its survival in the host's stomach, thus favouring the intestinal infection (Yuk et al., 2008). STEC is able to effectively contaminate refrigerated food thanks to its cold resistance (Lekkas et al., 2006). The minimum temperature for *E. coli* O157:H7 growth is reported to be 7°C (ICMSF, 1996). However, STEC frozen resistance has been assessed. Two studies by Strawn and Danyluck (2010a; 2010b) demonstrated that *E. coli* O157:H7 can show frozen resistance in different fruits. STEC frozen resistance was also observed in meat, for example during an outbreak involving twelve cases associated with the consumption of frozen beef burgers in UK in 2017. For these reasons, to inactivate STEC completely, pasteurization or cooking at 70 ºC at the core of the products is necessary (Byrne et al., 2020).

STEC resistance features are crucial for viability in the host as well as in the environment. It has been demonstrated that, excreted via animal faeces, STEC can contaminate manure and therefore soil and water and resist thanks to biofilm formation (Vogeleer et al., 2014). This results in the contamination of fresh produce such as lettuce or crops. Moreover, the environmental contamination involves food processing plants, compromising food safety. For instance, contamination of beef carcasses occurs at different stages during processing, and this is often caused by the formation of STEC biofilms on the surface of slaughtering equipment (Vogeleer et al., 2014). As a matter of fact, it has been shown that different STEC strains can form biofilms on different food or food contact surfaces, as observed in two studies carried out in Argentina. The first one reported the presence of STEC in carcasses and cuts of meat during slaughter. Interestingly, for meat samples contamination rates varied among the different cuts (chuck: 12.2%; rump toast: 12.2%; minced beef: 40.74%) (Etcheverría et al., 2010). The second study reported the presence of STEC non-O157 in carcasses, cuts, and trimmings from eight beef slaughterhouses (Brusa et al., 2017). Resistance to sanitizers has also been demonstrated for STEC, thanks to its capacity to form biofilms. A case study illustrates that polystyrene and glass surfaces showed a sanitizer resistance which was strain dependent (Wang et al., 2012).

2.2. Pathogenicity

STEC virulence determinants are reported to be integrated in the chromosome, but they can also be found as mobile genetic elements, such as plasmids, bacteriophages and pathogenicity islands PAIs (Bolton, 2011). Currently, the most relevant and known virulence factors of STEC are the

stx and the *eae* genes, even if STEC uses also other mechanisms to invade the host and cause infection (EFSA BIOHAZ Panel et al., 2020).

After being ingested, STEC's acid and bile resistance enables it to survive the stomach and the small intestine (Large et al., 2005). Genes involved in this phase could be *ure* (involved in urease transport) (Bolton, 2011; Yin et al., 2009), *ecf* (encoding enzymes that enhance membrane structure), *katP* (encoding a catalase peroxidase) and *stcE* (encoding anesterase inhibitor) (Bolton, 2011). Secondly, the adhesion/colonization of the mucosa of colon is enabled by fimbrial adhesins encoded by *hcp* (a type IV pilus involved in cell invasion), *ecp* (pilus involved in adherence and colonisation) and *efa* (fimbrial adhesins) genes (Bolton, 2011; EFSA BIOHAZ Panel et al., 2020). In most STEC strains, the initial adhesion triggers the attaching and effacing (A/E) lesion through the expression of the *eae* gene. The *eae* gene is located on a PAI called locus of enterocyte effacement (LEE), which encodes a protein translocation system of type III (T3SS). T3SS is an adherence system consisting of an outer membrane protein called intimin or Eae (*E. coli* attaching and effacing protein) and its receptor, the translocated intimin receptor (TIR), and other effectors that are translocated by the secretion system (Gyles, 2007). The type III secretion apparatus has a syringe-like structure that transports effector proteins from the bacterium into the host cells. These effectors perform a series of actions including invasion, haemolysis, repression of the host lymphocyte response, inhibition of phagocytosis, cytotoxicity and iron transportation (EFSA BIOHAZ Panel et al., 2020). Significantly, a rearrangement of the intestinal epithelial cell architecture is initiated; a pedestal structure is formed on the cell surface, where the microvilli disappear, and the accumulation of modified cytoskeletal proteins (actin) maintains this formation beneath the adherent bacteria. The TIR protein, encoded by *tir/espE* gene, represents the main promoter of the adhesion being inserted into the host cell membrane through the T3SS, and it acts as the receptor for intimin on the bacterial surface (EFSA BIOHAZ Panel et al., 2020). After the disruption of microvilli caused by the A/E lesion, the absorption of nutrients is impeded, resulting in watery diarrhoea (Gyles, 2007).

It should be noted that not all STEC cause the A/E lesion, because they lack the LEE. Pathogenic LEE-negative STEC strains use alternative attachment mechanisms, as observed for the STEC O104:H4 strain isolated during the German outbreak in 2011 that carries the *aggr* gene located in the virulence plasmid pAA (Kaper et al., 2004). The gene *aggr* regulates the expression of aggregative fimbriae which allows the microorganism to adhere and translocate the Stx (Boisen et al., 2014). Other virulence factors encoding adhesins have been identified thanks to molecular characterization, such as *paa* (porcine A/E lesion-associated protein), *efa1* (LEE-gene encoding adhesin), *ompA* (outer membrane protein which binds the brain micro-

vascular endothelial receptor glycoprotein) (Kaper et al., 2004) and *IpfA* (long polar fimbriae) (Bolton, 2011; Kaper et al., 2004). In a recent study, a PAI named Locus of Adhesion and Autoaggregation (LAA) has been discovered through WGS. It seems that this PAI is exclusively present in a subset of emerging LEE-negative strains causing severe illness, and therefore its role in the attachment could be crucial in the pathogenesis (Montero et al., 2017). Currently, the role of factors other than *eae* gene in the attachment of LEE-negative STEC is still not clear, but they have to be considered in the pathogenicity assessment of STEC since they have been associated with severe diseases in humans (Newton et al., 2009).

Shiga toxins secretion represents the following step after the attachment of STEC to the intestinal epithelium. Shiga toxins are encoded by bacteriophages (Stx phages) which carry the *stx* gene and have the capability to lysogenise non-pathogenic bacterial strains and convert them into STEC, like the Stx-producing EAEC O104:H4 strain mentioned above (EFSA BIOHAZ Panel et al., 2020)**.** This feature makes the genomes of STEC strains highly variable, which has to be taken into account for the pathogenicity assessment of the pathotype. Stx proteins consist of five identical B subunits and a A subunit with enzymatic activity. Once released in the colon, toxins translocate across the intestinal epithelium and travel by the bloodstream to reach their target cells. B subunits are responsible for binding the toxin to the glycolipid globotriaosylceramide (Gb3) receptor on the surface of target cells. After that, the complex AB5 is internalised within an endosome, which traffics to the Golgi apparatus and then to the endoplasmic reticulum (ER). In the ER the complex is splitted into the A1 subunit and the A2B5 portion. The A1 chain, thanks to it enzymatic activity, enters the cytosol and remove a specific adenine base from the 28 S rRNA. This results in the prevention protein synthesis (Gyles, 2007; Melton-Celsa, 2014). The presence of Gb3 receptors on renal endothelial cells make renal cells one of Shiga toxins' targets. The action of toxins causes the cell death, leading to an occlusion of the micro-vasculature at this level. This damage potentially culminates in the severe form of the disease, the hemolytic uremic syndrome (HUS), which is characterized by haemolytic anaemia, thrombocytopaenia and possibly fatal acute renal failure. Other Stx target cells are represented by the colon's microvasculature endothelial cells rich of Gb3 receptors, resulting in bloody diarrhoea (BD), haemorrhagic colitis, necrosis and intestinal perforation (Kaper et al., 2004) and in the central nervous system (CNS), resulting in its failure (Obata, 2010). CNS failure represents the final stage of Stx damage which have previously caused oedema, hypoxic-ischemic changes and micro-haemorrhages, as showed by autopsies and magnetic resonance imaging (Obata, 2010).

Stx exists in two major types, Stx1 and Stx2 with, currently, four Stx1 subtypes (Stx1a, Stx1b, Stx1c, Stxd) and seven Stx2 subtypes (Stx2a-Stx2l) (EFSA BIOHAZ Panel et al., 2020). Stx1structure is almost identical to that of *Shigella* (O'Brien et al., 1982), while Stx2 shares approximately 55% of amino acid homology with Stx1 (Kaper et al., 2004). Overall, Stx2 is currently been associated with the most severe cases of illness (Boerlin et al., 1999; EFSA BIOHAZ Panel et al., 2020).

Among Stx1 group, a relevant subtype is *stx1a*, which is associated with hospitalisation and BD (Brooks et al., 2005). In contrast, *stx1d* and *stx1c* are less associated with human illness, although being frequently isolated in animals (Brandal et al., 2015; Buvens et al., 2012; Fierz et al., 2017). Among Stx2 group, subtypes *stx2a* and *stx2d* are significantly associated with severe illness (Buvens et al., 2012; De Rauw et al., 2019; Marejková et al., 2013). As a matter of fact, the cytopathic effect on Vero cells and on primary renal proximal tubule epithelial cells, is 25 times more potent in these two variants than *stx2b* and *stx2c* (Fuller et al., 2011). Subtype *stx2b* is, in fact, more frequently associated with mild illness than severe one (Buvens et al., 2012; Fierz et al., 2017). On the other hand, *stx2e* subtype is rarely found as cause of human disease. Instead, it is associated with severe disease in pigs, called oedema disease (Lothar Beutin et al., 2008). A limited association with diseases in humans has also been found for *stx2f* and *stx2g* subtypes (Amézquita-López et al., 2018).

To undertake a pathogenicity assessment of STEC, the European Food Safety Authority (EFSA) was asked to compare both literature and information from The European Surveillance System (TESSy) data (2012-2017). The TESSy data included the presence of specific gene/gene combinations and/or *stx* subtypes and severe illness expressed as HUS, hospitalizations, or bloody diarrhoea (BD). The virulence profile of the isolates was available for 3,942 cases out of 29,945 human STEC cases reported in the EU/EEA[1] from 2012 to 2017. TESSy data firstly shows that *stx2a* was associated with the highest rates of HUS, hospitalisations and BD, alone or in combination with other *stx* subtypes. Secondly, *Stx2d* as well had a significative HUS rate, but in absence of *eae.* Moreover, most of subtypes were associated with HUS and hospitalisations and, importantly, all subtypes were associated at least BD. Finally, the presence of the *eae* gene was defined as an aggravating factor, since the majority of STEC isolates associated with HUS, hospitalisation and/or BD, carried that gene. EFSA Opinion 2020 concluded that intimin (*eae*) or Stx toxin subtype could not be used to predict clinical outcome since intimin was present in the majority but not severe illness cases and all STEC subtypes were associated with at least one of severe illness outcomes. Thus, all STEC subtypes may be hazardous for human health. As a

[1] The European Union countries (27) plus the European Economic Area (Iceland, Liechtenstein and Norway).

consequence, the serogroup can't be considered as a virulence marker, as previously argued, since the same serogroup often carries different virulence genes (EFSA BIOHAZ Panel et al., 2020). In view of the above, a specific pathogenicity marker of STEC does not exist, thus preventing an association between isolates and health risk.

For this reason, many studies aim to discover other possible virulence genes implicated in the pathogenicity of STEC. For instance, well known is the role of enterohaemolysine which releases haemoglobin from red blood cells to provide a source of iron for the bacterial cells (L Beutin et al., 1989)**,** but also of proteases (Burland, 1998), catalases (Brunder et al., 1996) and esterase inhibitor (Lathem et al., 2002). Furthermore, genes encoding a pilus involved in invasion and formation of biofilm (*hcp)* have been discovered (Xicohtencatl-Cortes et al., 2007), as well as enterotoxins encoded by the *set* gene (Afset et al., 2006). Moreover, WGS technologies allowed the identification of new PAIs**,** encoding non-LEE effector proteins (*nle*) (Naseer et al., 2017) and more recently, Gardette and colleagues (2019) discovered 13 metabolism genes encoded during the infectious process (Gardette et al., 2019). These and other virulence genes, together with the main ones, could constitute the combination of genes which can be used as pathogenicity marker for STEC. In this respect, WGS technologies result helpful since they allow to identify at the same time all the virulence genes harboured by an isolate.

2.3. Pathogenesis and clinical signs

The pathogenesis of STEC require its ingestion, usually throughout contaminated food or water. As previously argued, STEC firstly reaches and attaches to the gross intestinal mucosa causing mild non-bloody diarrhea. Secondly, Stx secretion induces the development of the intestinal and extraintestinal complications caused by the vascular damage (Croxen et al., 2013): bloody diarrhoea (BD), haemolytic uraemic syndrome (HUS), which often includes acute kidney failure and CNS failure. A huge percentage of patients are hospitalized, some develop end-stage renal disease (ESRD) that can be followed by death (FAO/WHO, 2018).

The potential of STEC to induce severe disease is not only due to virulence factors; the dose response to STEC and human factors must be considered (EFSA BIOHAZ Panel et al., 2020). Data about the dose response are estimated, especially from the amount of contaminated food consumed by people who did or did not become ill (EFSA BIOHAZ Panel et al., 2020). As instance, less than 10 cells in beefburgers have been held responsible for an outbreak in Wales during 1994 (Willshaw et al., 1994), or less than 50 cells in a dry fermented salami in an USA outbreak (Tilden et al., 1996). Moreover, a synergistic effect with intestinal microbiota has been observed. Goswami and colleagues (2015) showed that STEC O157:H7 strains increase Stx2

production by co-culturing them with commensal *E. coli* (Goswami et al., 2015). *Cryptosporidium* spp. and *Campylobacter* spp. were also attributed as co-infectors in a survey of 1,800 STEC infections (Luna-Gierke et al., 2014). For what extent human factors, age is highly associated with the occurrence of severe illness; children less than 5 years old and adults more than 75 are the more exposed (EFSA BIOHAZ Panel et al., 2020). Furthermore, the proportion of infection among children less than 5, was mostly caused by Stx2, especially Stx2a (Friedrich et al., 2002). Underlying diseases, such as cases reported with diarrhoea caused by *Clostridium difficile* or influenza A , are considered to be predisposing factors (Thomas et al., 1994). Also, patients under immunosuppressive therapy post transplantation are more exposed, as showed by a fatal case which developed HUS after a STEC infection (Fasel et al., 2014).

Considering those variating factors, almost 75% of individuals exposed to STEC will remain free of any symptoms (Travert et al., 2021). The incubation period, which is important to identify the potential source of contamination, seems to vary in different reports. A recent review reports a mean incubation time which ranges from 3.5 to 8.1 days and identifies the patient age and the attack rate as influencing factors of the incubation period (Awofisayo-Okuyelu et al., 2019). The disease usually begins with watery diarrhoea, which is the result of the A/E lesion and it can be accompanied by fever, crampy abdominal pain or vomiting. After that, haemorrhagic colitis may occur in the following days as a consequence of the vascular damage to the level of colon. In this phase as well, patients may experience vomiting, abdominal cramps and rarely fever (Cleary, 2004). In severe cases, faecal specimens are described as “all blood and no stool” (Nataro & Kaper, 1998) to underline the severity of symptoms. BD can last for more than a week (Cleary, 2004). After that, 90% of patients recover, but in 5-10% of them (particularly young children and the elderly) the infection may lead to a life-threatening disease, such as the haemolytic uraemic syndrome (HUS) which occurs between days 5 and 13 after the initial onset of diarrhoea (Tarr et al., 2005; WHO, 2018). HUS belongs to the group of thrombotic microangiopathies (TMA) which all share a common pathologic description of arterial, intra-renal or systemic micro-vascular occlusion, resulting from endothelial aggression accompanied by the formation of platelet aggregates. In the case of HUS the lesion affects mostly the kidney, resulting in the triad of mechanical haemolytic anaemia, platelet activation/aggregation leading to thrombi and thrombocytopenia, and kidney failure (Bruyand et al., 2019; Travert et al., 2021). To understand the impact of STEC disease, a recent study estimated that STEC causes 2,801,000 acute illnesses worldwide annually which leads to 3,890 cases of HUS (0,14%), 70 cases of end-stage renal disease (ESRD) (0,002%), and 230 deaths (0,008%) (Majowicz et al., 2014). Long-term sequelae can also develop from HUS (affecting 20

to 40% of patients), including hypertension, proteinuria, chronic kidney disease, end-stage kidney disease, but also extra-renal sequelae such as cardiac complications, colonic strictures, neurological disorders, cognition and behaviour changes, and diabetes mellitus (Spinale et al., 2013).

Unfortunately, the notification of STEC cases is often underestimated since several countries notify only severe cases. Italy represents an example of STEC cases underestimation because only HUS cases are reported. The Italian Haemolytic Uraemic Syndrome Registry has been active since 2005 and overall data reported almost 40 HUS cases annually, 70% of which are caused by STEC. The age range is 0-15 years old with a median of 25 months. The prevalent serogroups identified are: O26, O157, O111 and O103. A recent summary reported that, between March 2020 and February 2021 54 cases were recorded in 15 regions, with 94% of the cases belonging to the pediatric population. During the year, the average notification rate for people under the age of 15 was 0.61 per 100,000 inhabitants, with significant variations in different regions. Valle d'Aosta reached the highest rate with 5.8 cases per 100,000 inhabitants, while in Umbria, Liguria, Veneto, Lombardy, Calabria and the Autonomous Province of Bolzano the notification rate was greater than national average (0.61/100,000 inhabitants) (Figure 1). (Italian National Institute of Health, https://www.epicentro.iss.it/en/hus/epidemiology-italy)

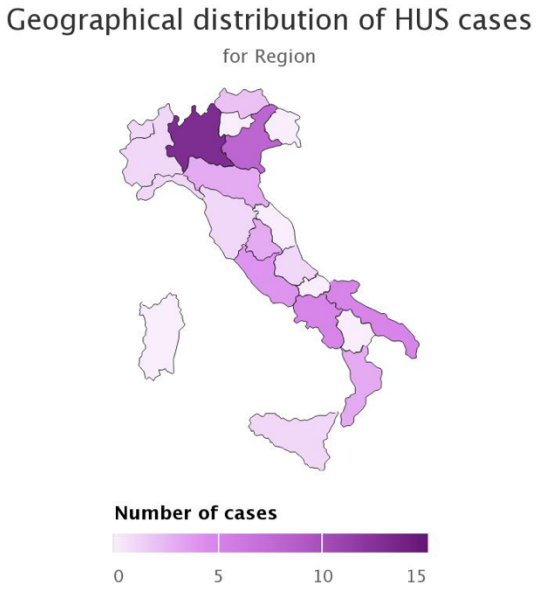

Figure 1: Geographical distribution of HUS cases in Italy for Region from 1 March 2020 to 28 February 2021, Italian Haemolytic Uraemic Syndrome Registry.

3. Diagnosis: methods to detect and characterize STEC

Different methods for the detection and characterization of STEC are available. First of all, the Gold Standard is the detection by the Vero cell assay (VCA) where the Vero cells represent a continuous line of African green monkey kidney cells (Konowalchuk et al., 1977). Considering that the only feature of STEC that distinguishes it from non-pathogenic *E. coli* is the production of Shiga toxins, the VCA identifies STEC thanks to the cytopathic effect caused by toxins to a monolayer of Vero cells after 48-72 h (To & Bhunia, 2019). Unfortunately, this technique requires specific skills, and it is associated with high costs, thus its use is restricted to reference laboratories only.

To date, STEC are commonly detected by molecular methods. The current international standard to identify STEC in food, feed and environmental samples is the ISO/TS 13136:2012 method, which is based on Real-time PCR. The Real-time PCR reaction can present up to five different fluorophores to identify the major virulence genes *stx* and *eae* genes, as well as the genes encoding the top-five serogroups associated with HUS in the EU (O157, O111, O26, O103, and O145). Moreover, since the importance of the major German outbreak caused by *E. coli* O104:H4 during 2011, the detection of this serotype has been integrated in the ISO/TS 13136:2012 (ISO, 2012). When the Real-time PCR identifies *stx1* and/or *stx2* genes, the isolation of the strain by cultural methods is performed. The isolation is needed since *stx* phages or STEC DNA can be present in the samples in absence of viable cells, resulting in false-positive results. Overall, results are as follows:

- Negativity to *stx* genes: absence of STEC;
- Positivity to *stx* genes in absence of isolation: presumptive detection of STEC;
- Positivity to *stx* and *eae* in absence of isolation: presumptive detection of STEC causing attaching/effacing lesion;
- Positivity to *stx*, *eae* and genes associated with serogroups in the absence of isolation: presumptive detection of the most pathogenic STEC strains;
- Positivity to *stx* genes followed by isolation: STEC detection;
- Positivity to *stx* and *eae* followed by isolation: detection of STEC causing attaching/effacing lesion;
- Positivity to *stx, eae* and genes associated with serogroups followed by isolation: detection of the most pathogenic STEC strains (ISO, 2012).

The presumptive positivity resulting from the Real-time PCR analysis and the impossibility of strain isolation can occur for three reasons:

1. Presence of free DNA in the enrichment culture from lysed and/or non-viable STEC strains;
2. Presence of not-integrated bacteriophages in the enrichment culture, in absence of STEC cells;
3. Presence of STEC cells below the detection limit.

While presence of free DNA or bacteriophages do not represent a risk of infection in humans, undetected STEC cells might be, due to the lack of scientific data on infectious dose levels for STEC, apart from O157 e O111 which infectious dose has been estimated around 10 cells (Italian Ministry of Health, 2017).

Nevertheless, other methods are used to identify STEC. Up to date, a selective and differential medium able to specifically identify all STEC strains does not exist except for O157 serogroup. The specifically detection of *E. coli* O157 is described by the EN ISO 16654:2001 method, which was the first reference method for the detection of STEC in food and animal feeding stuffs. This method was based on an immune-magnetic-based procedure, followed by a plating step onto a selective agar medium. The immuno-magnetic concentration consisted in the concentration of *E. coli* O157 grown in an enrichment broth and kept in contact with magnetic beads coupled with antibody against the O157 lipopolysaccharide (LPA). Thereafter, the plating step on a cefixime and potassium tellurite supplemented MacConkey agar containing sorbitol (CT-SMAC) ensured the isolation of *E. coli* O157 (EFSA BIOHAZ Panel et al., 2020; ISO, 2001). Immunological methods can also be employed, providing indirect evidence of STEC presence. In contrast of other methods, these tests detect Stxs, although not distinguishing between Stx1 and Stx2. They are available as kit ELISA, but they are mostly used in clinical diagnosis (K.A. Bettelheim & Beutin, 2003; EFSA BIOHAZ Panel et al., 2020).

In order to perform epidemiological investigation during outbreaks, characterization, serotyping and subtyping of STEC strains are crucial. It is important to notice that serological typing is widely used but it is known that the serotype alone does not describe the pathogenicity of STEC, and a single serotype may carry different virulence factors. Nevertheless, techniques for STEC serotyping include: the traditional phenotypic serotyping, Real-time PCR methods and WGS. The latter has the best advantages, since it can compare all database genes with each test strain and the output is the predicted O and H serotype. Once implemented, WGS is faster than traditional methods, and problems, such as antisera cross-reaction and novel O-groups, are almost resolved. Furthermore, the implementation of WGS in STEC molecular typing turns out

to be essential for exhaustive studies of outbreaks. Indeed, this high-throughput technique allows to correlate the presence of specific virulence genes with the possible onset of severe illness symptoms (EFSA BIOHAZ Panel et al., 2020).

4. Epidemiology

E. coli O157:H7 was the first STEC serotype recognized during a food-borne outbreak in the USA in 1982 (Riley et al., 1983). This was followed by a multistate outbreak in 1993, when *E. coli* O157 was recognized as a pathogen with public health significance (Bell, 1994). Hence, *E. coli* O157 became a nationally (USA) notifiable infection in 1994, and by 2000 reporting was mandatory in 48 states (Rangel et al., 2005). Currently, the FoodNet program provides active surveillance of food-borne illnesses in the United States. In 2016, 52 public health laboratories reported 5,441 cases of culture-confirmed STEC infections, including 2,323 O157 and 3,104 non-O157 cases (Marder et al., 2017). Compared with 2016–2018, preliminary data from 2019 report an increment of 34% in STEC incidence. In particular, the incidence of STEC O157 infections decreased by 20% and the incidence of non-O157 infections increased by 35%. Despite this, O157 remains the first common serogroup in the USA, with an incidence of 23% during 2019 (397 among 1,725 STEC isolates) (Tack et al., 2020).

A global estimation of STEC incidence has been published by WHO in 2018. This study was conducted by the Foodborne Disease Burden Epidemiology Reference Group (FERG) which estimated that in 2010 2.5 million new STEC cases occurred worldwide (1.2 million of which are estimated to be foodborne), resulting in 3,330 HUS cases, 200 end-stage renal disease, 269 deaths and 27,000 DALYs (Disability Adjusted Life Years) (FAO/WHO, 2018).

According to Zoonoses Directive 2003/99/EC, the reporting of foodborne disease outbreaks caused by STEC is mandatory in Europe. From data collected by the European Center of Disease Prevention and Control (ECDC) and the European Food Safety Authority (EFSA), it emerges that between 2015 and 2019 STEC infection was the third most reported foodborne zoonosis in humans (after *Campylobacter* and *Salmonella*) (as shown in Figure 2) (EFSA and ECDC, 2021), with an increasing trend throughout the years (Figure 3) (ECDC, http://atlas.ecdc.europa.eu/public/index.aspx).

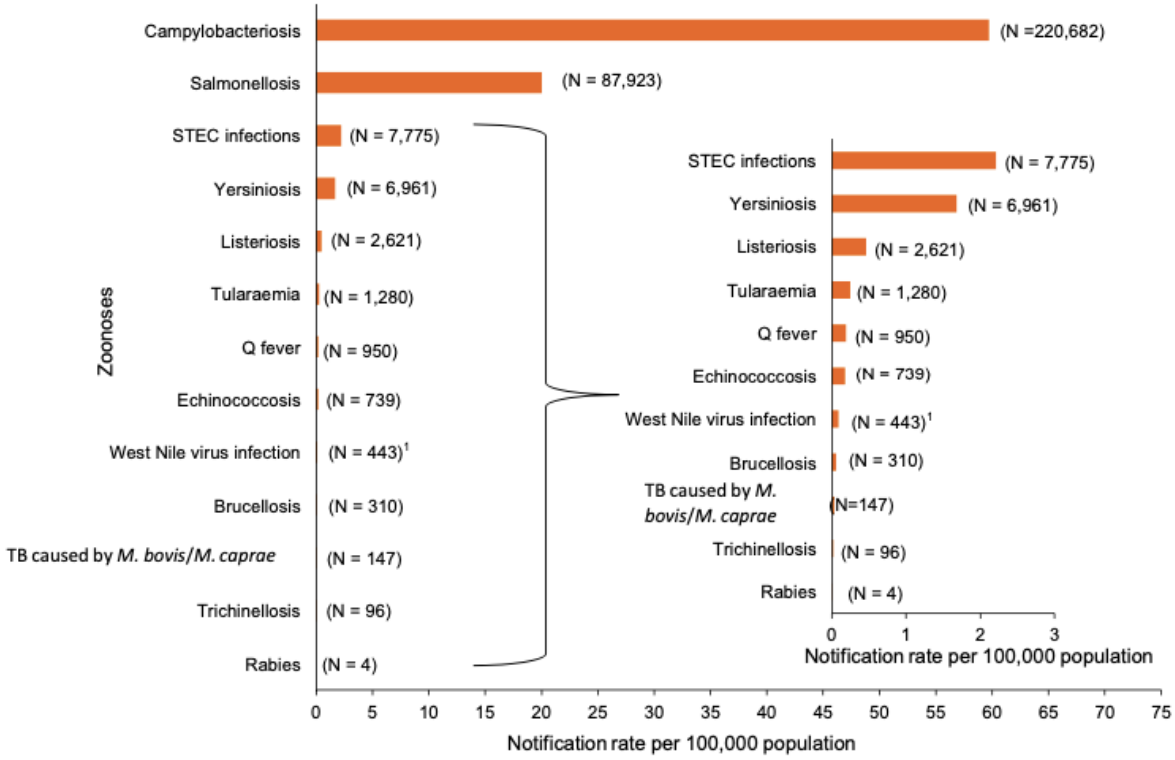

Figure 2: Reported numbers and notifications rates of confirmed human zoonoses in the EU, 2019.

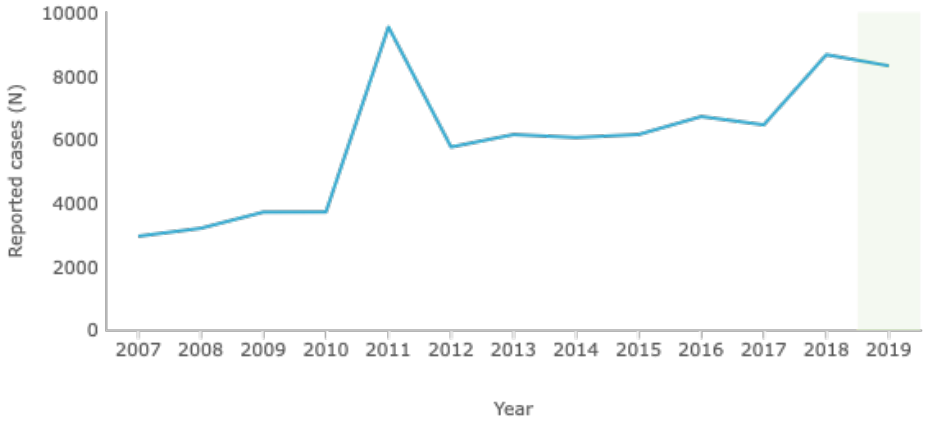

Figure 3: Total number of confirmed cases from 2007 to 2019.

In 2019, 29 out of 32 EU/EEA countries reported STEC infections data. It is important to underline that for six Member States (MSs) notification is either voluntary (Belgium, France, Luxembourg, and Spain), or based on another type of system (Italy and the United Kingdom). Moreover, the surveillance systems for STEC infections have national coverage in all EU/EEA countries except for three: France, Italy, and Spain. Therefore, no estimate for population coverage was provided, and no notification rates could be calculated for these three countries. In France, STEC surveillance is based on pediatric haemolytic uraemic syndrome (HUS)

surveillance, and in Italy it is primarily based on the abovementioned national registry for HUS (ECDC, 2021). Overall, 29 EU/EEA countries reported 8,313 confirmed cases of STEC infection during 2019. The notification rate was 2.2 cases per 100,000 population, which is about

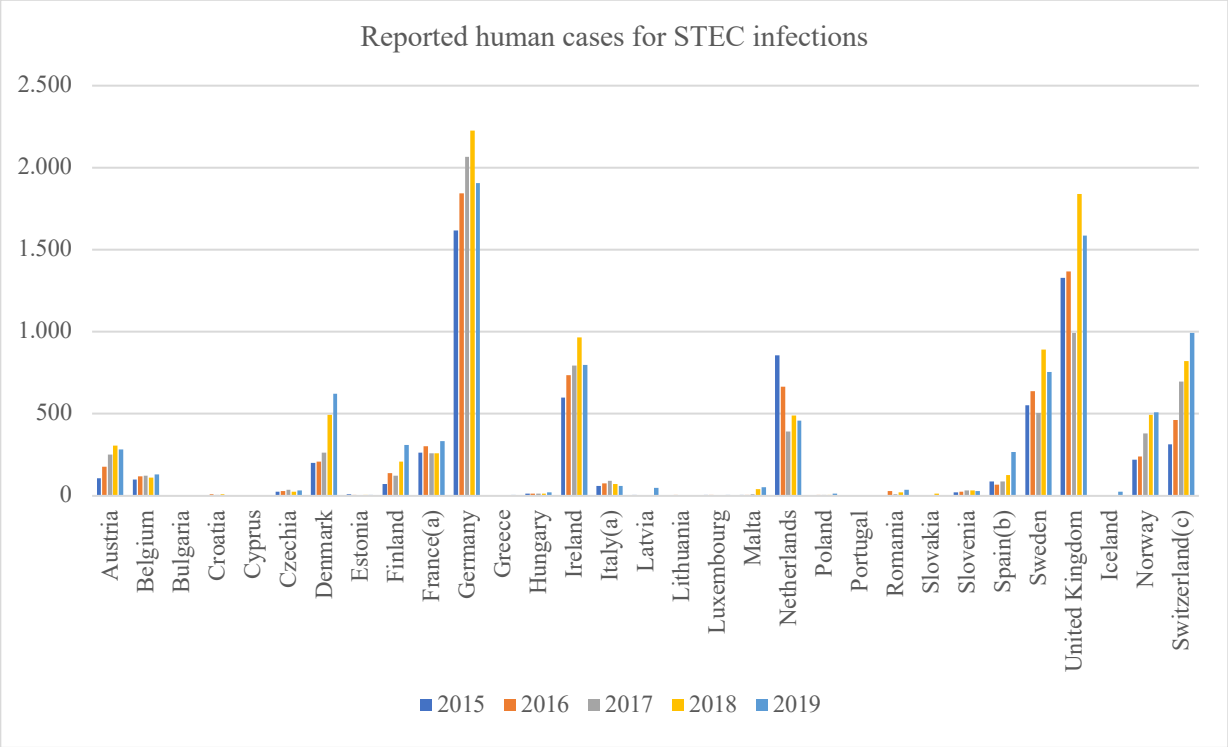

Figure 4: Reported human cases for STEC infections by country and year, 2015-2019.

(a) : Sentinel surveillance; mainly cases with HUS are notified

(b) : Sentinel surveillance; no information or estimated coverage. So, notification rate cannot be estimated

(c) Switzerland provided the data directly to EFSA. The human data for Switzerland includes data from Liechtenstein.

the same level as in 2018, but higher if compared to the previous four years. The highest numbers of confirmed cases were reported by Germany and the United Kingdom, which together accounted for 42% of all reported cases in the EU/EEA. Data about reported human cases by country and year are shown in Figure 4 (EFSA and ECDC, 2021).

The highest rate of confirmed cases was observed in 0–4-year-old children (10.3 cases per 100,000 population) as shown in Figure 5 (ECDC, http://atlas.ecdc.europa.eu/public/index.aspx).

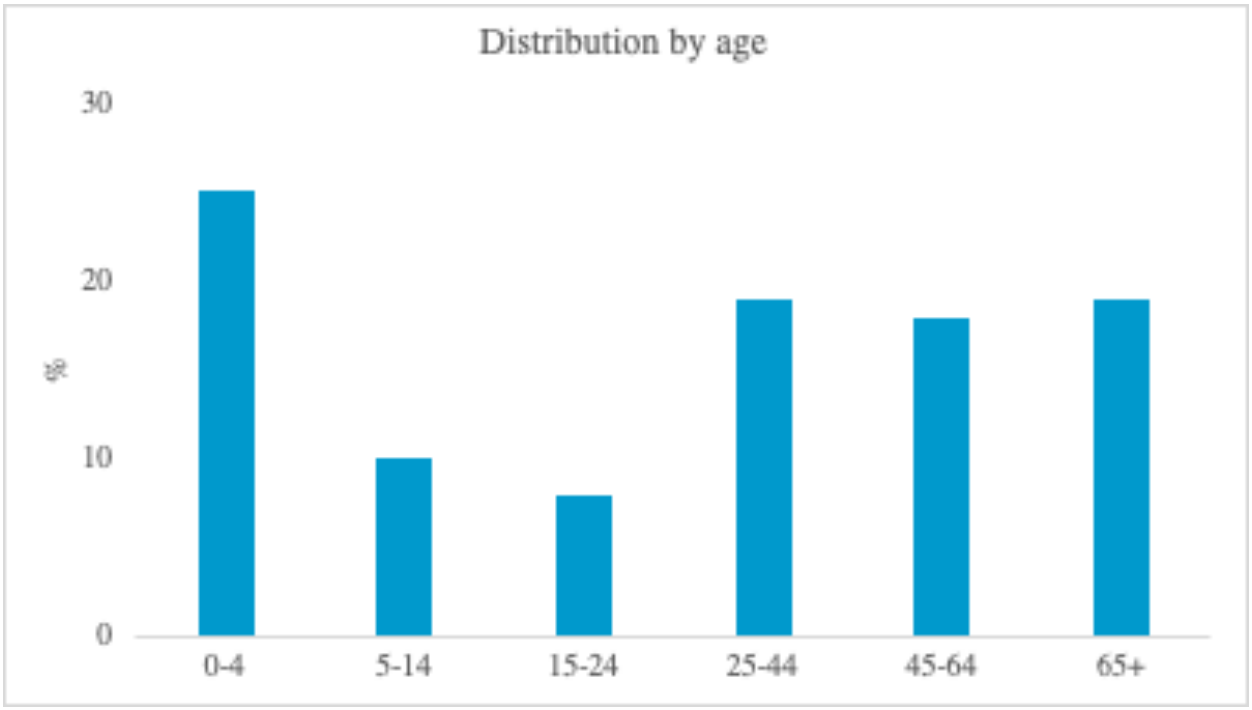

Figure 5: Distribution of age in confirmed cases, 2019.

Moreover, a clear seasonal trend was identified in confirmed STEC cases between 2010 and 2019, with more cases reported during the summer months (June–September) (Figure 6) (ECDC, 2021).

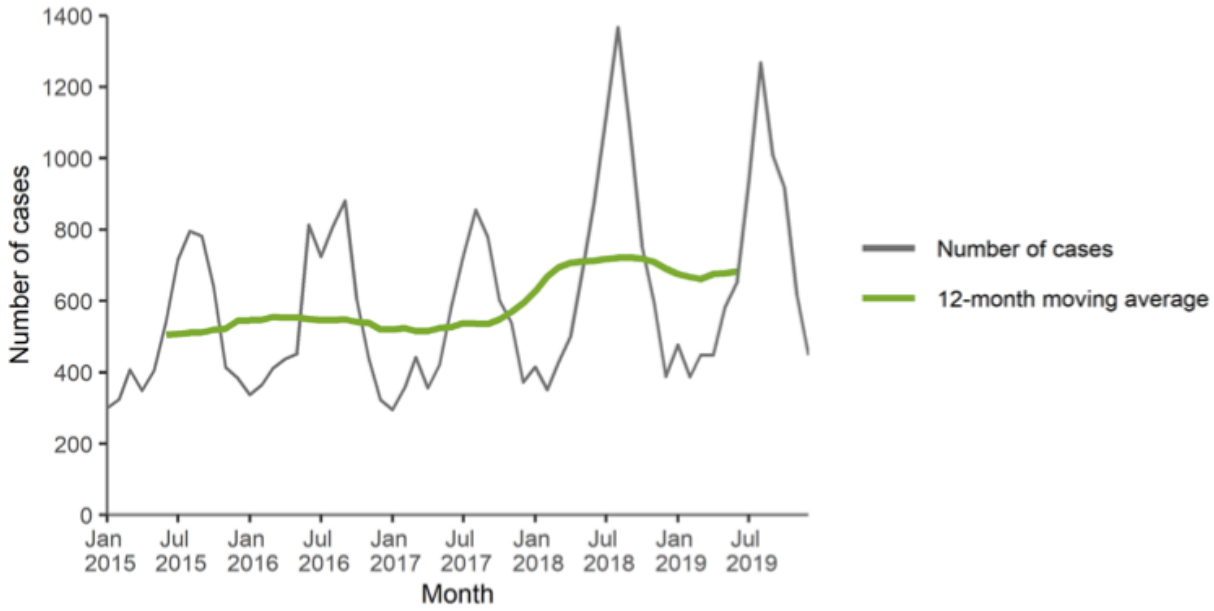

Figure 6: Distribution of confirmed STEC infection cases by month, EU/EEA, 2015-2019

In 2019, 35% of 3,410 STEC cases were hospitalised (cases with known information on hospitalisation), 12 of 5,099 cases with known outcome were reported to have died, resulting in an EU case fatality rate of 0.2%. The number of HUS cases was the same as in 2018. illustrates Among the 409 HUS cases, the highest proportion of patients was reported in the youngest age groups from 0–4 years (69%) to 5–14 years (18%) (Figure 7) (EFSA and ECDC, 2021).

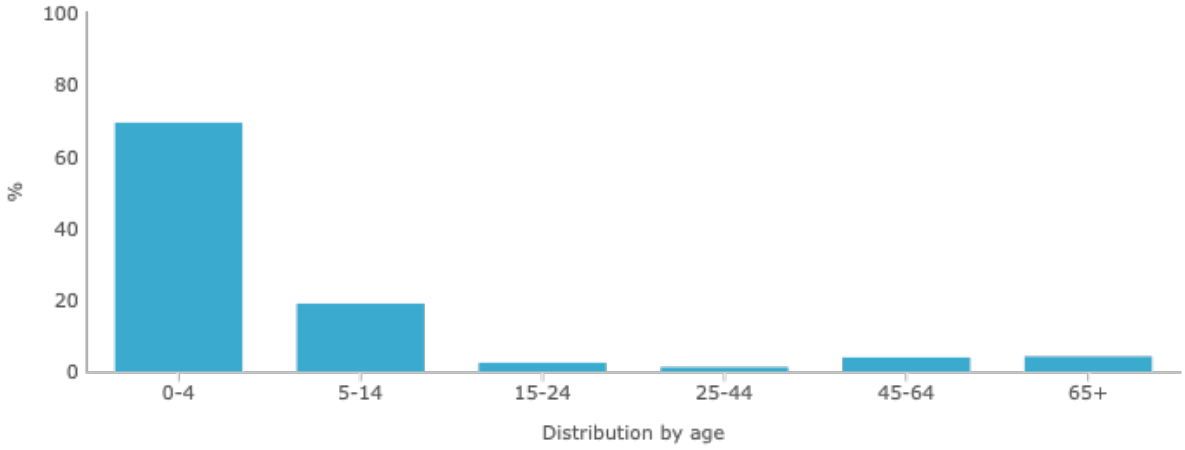

Figure 7: Distribution by age in HUS cases, 2019.

However, the highest number of fatal cases was reported in the age groups >25 years (60%), half of which were caused by HUS (Figure 8) (ECDC, http://atlas.ecdc.europa.eu/public/index.aspx).

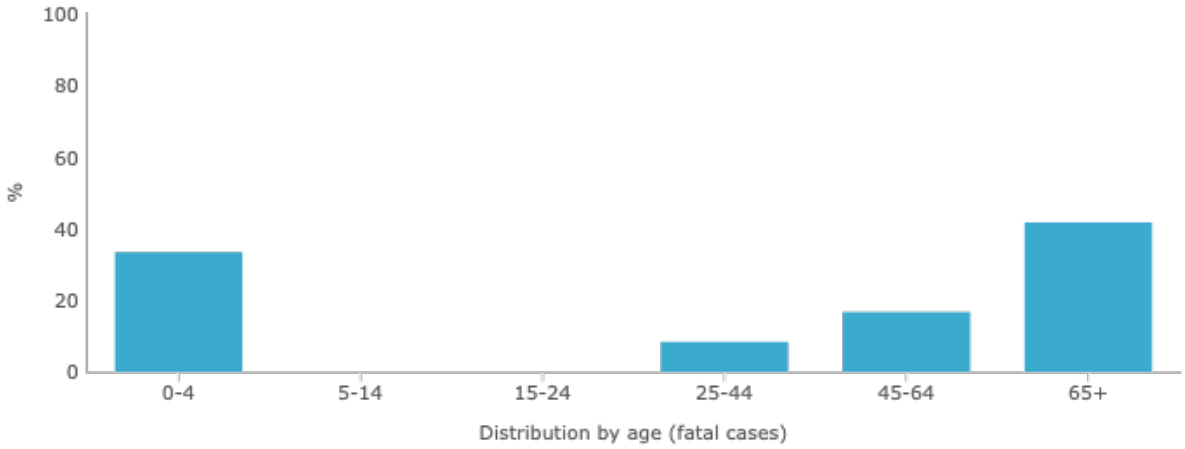

Figure 8: Distribution by age in fatal cases, 2019.

Considering that the identification of the serogroups is currently used for epidemiological tracking, in 2019 the five most reported serogroups in the EU were O157, O26, O146, O103, and O91 (serogroup data were available for 57.9% of human cases). Even if O157 ranked first among serogroups, its proportion has been decreasing from 54.9% in 2012 to 26.6% in 2019, in line with the emergence of other serotypes and updated research techniques. For example, O26 serogroup has continuously rising from 11.6% in 2012 to 16.0% in 2019. Overall, the proportion of serogroups other than O157 increased by 9.2% compared with 2018 (EFSA and ECDC, 2021).

As previously discussed, a specific marker for the pathogenicity of STEC still not exist. However, an association of certain virulotypes (combination of virulence genes) with severe human cases has been made. In 2019 the most frequently reported were *stx1*-/*stx2*+/*eae*+ and *stx1*+/*stx2*+/*eae*+. Unexpectedly, *stx1a* was mostly associated with severe illness in 2019, thus surpassing *stx2a* which had always been associated with the most severe cases (EFSA, 2021).

4.1 Animals as reservoir of STEC

Animals represent the major source of STEC and among them ruminants are the main natural reservoir (Gyles, 2007), harbouring STEC mainly in the recto-anal junction (Naylor et al., 2003). Especially cattle are considered to be the most important source of STEC O157; adults are usually asymptomatic carriers, instead of calves which may experience diarrhoea (Caprioli et al., 2005). The lack of symptoms in cattle, as other ruminants, is due to the absence of vascular receptors for Shiga toxins (Gb3), especially those of intestinal vasculature (Pruimboom-Brees et al., 2000). Lowest rates of shedding occur in calves before weaning, as they are not functionally ruminants yet and therefore STEC do not survive in the abomasum during milk digestion. The shedding period varies from 3 to 24 months (Menrath et al., 2010) and the excretion is mostly intermittent (Persad & LeJeune, 2014). The prevalence of STEC in ruminants is also influenced by seasons; a higher rate of faecal shedding has been reported by several studies during warmer months as well as for clinical cases in humans (ECDC, 2021; Merialdi et al., 2014). This phenomenon can be explained because STEC, as mesophilic microorganisms, are less likely to be isolated during colder months, since their survival in the environment is generally shorter during this period, resulting in a reduction of cases of infection among animals. Animals shedding more than 10^4 CFU/g feaces are defined as “super-shedders” which are certainly responsible of the majority of environmental contamination (Chase-Topping et al., 2008).

Small ruminants (sheep and goats) as well play a critical role as STEC reservoir, especially in Australia, Norway (Persad & LeJeune, 2014) and Scotland (Evans et al., 2011),

where their breeding is widespread. As cattle, they shed STEC O157, but also STEC O26. Higher shedding during warmer months has been demonstrated as well. Other ruminants has been identified as shedders of STEC, like water buffalo, deer, elk and bison (Persad & LeJeune, 2014).

STEC can also be detected in wild ruminants (Caprioli et al., 2005), especially deer from which STEC O157 has been frequently isolated (Renter et al., 2001). A recent study performed in the Central Italian Alps tested 201 free-ranging red deer faecal samples (*Cervus elaphus)* founding out that 40 (19.9%) of them were positive for STEC. WGS was used to characterise 31 isolates. The most detected serotype was O146:H28 (n=10, 32.3%). Furthermore, virulotyping showed that *eae* lacked in all the isolates and *stx* subtypes were present in different combinations (Lauzi et al., 2021). The study also underlines that manipulation of deer meat requires the greatest attention, since their role as reservoir. Moreover, deer contribute to the environmental contamination of soil and water sources. Some studies confirmed that consumption of deer meat can be associated with human infections, as for example the STEC O157:H7 infection of a child who developed HUS in the USA (Rabatsky-Ehr et al., 2002).

On the other hand, monogastric animals do not represent an important source of STEC. Equine are considered spillover hosts; although transmitting STEC, they are unable to maintain infection in absence of repeated exposure (Persad & LeJeune, 2014). Human clinical cases caused by direct contact with horses have been reported (Chalmers et al., 1997). Swine, unlike ruminants, possess Stx-sensitive vascular receptors causing oedema disease after the intestinal colonization. Stx2e is the most frequent Stx detected from pigs and only a few human cases are associated with the consumption of pork meat. Outbreaks linked to pork meat consumption occurred in Canada (MacDonald et al., 2004; Trotz-Williams et al., 2012), Italy (Conedera et al., 2007) and Australia (Paton et al., 1996). Companion animals also contribute to the epidemiology of STEC; they can be spillover hosts and, through their close interaction with humans, the transmission of STEC can occur (J.-S. Kim et al., 2020).

Wild animals, such as rats, pigeons and flies, are assuming a more important role in causing outbreaks associated to consumption of fruits and vegetables contaminated with their faeces. In fact, they can live in close proximity with livestock and therefore transmit STEC to farmed animals (J.-S. Kim et al., 2020).

Recent studies reported also the presence of STEC in fresh fish and shellfish in consequence of water contamination; in fact these animals are not considered reservoir, but rather dead-end hosts (Persad & LeJeune, 2014).

4.2 Transmission routes and sources of infection

Food of animal origin, water, vegetables and fruits represent sources of STEC infection for humans. Food contamination occurs predominantly through faecal contamination of products during human activities such as slaughtering or milking. Furthermore, infection occurs also by ingestion of contaminated water as well as fruit or vegetables contaminated by animal manure or non-potable water (Gyles, 2007). STEC direct transmission to humans can also occur, as for instance in children in didactic farms or zoos, but also in veterinarians or farm operators (Croxen et al., 2013). Finally, direct contact human-to-human can be responsible for STEC transmission, because of its low infectious dose (Gyles, 2007). Transmission routes are shown in figure 9 (Franz, 2007).

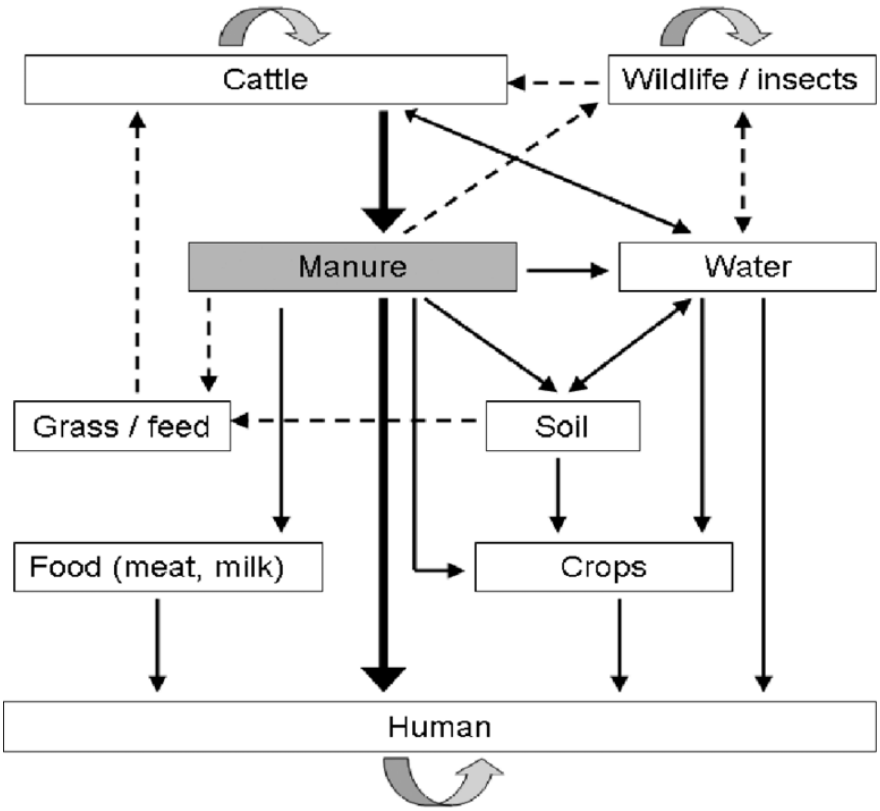

Figure 9: Reservoirs and modes of transmission of STEC. Solid lines represent direct or indirect transmission routes between cattle and humans, dashed lines represent transmission lines back to cattle (Franz, 2007).

By virtue of the growth and resistance characteristics of STEC, in 2003 the Scientific Committee On Veterinary Measures Relating To Public Health (SCVPH) identified the following foodstuff categories for which STEC pose a danger to public health:

- raw or undercooked beef and possibly meat from other ruminants;
- minced and/or fermented beef, and products thereof;
- raw milk and raw milk products;
- fresh produce, in particular sprouted seeds and unpasteurized fruit and vegetable juices;
- water.

Despite the existence of so many foodstuffs at risk, the only regulatory microbiological criterion for STEC is for sprouts, as set in the Commission Regulation No 209/2013 amending Commission Regulation (EC) No 2073/2005. This criterion is based on the absence of STEC O157, O26, O111, O103, O145 and O104:H4 in 25 grams of sprouts placed on the market during their shelf-life. The samples must be tested following the analytical method CEN ISO TS 13136: 2012.

Monitoring of STEC along the food chain by MSs in mandatory under Directive 2003/99/EC. In addition, following Regulation CE No 178/2002 (European Parliament and Council, 2002) food shall not be placed on the market if it is unsafe. Hence, producers of food at risk are required to include STEC in their own-check plans to assess hygiene and safety of their products. In this respect, the guidance on the application of Regulation CE No 178/2002 for STEC imposes different restrictive measures based on the risk profile of the types of food (European Commission, 2014). Specifically, two type of risk profile has been determined, *i.e.* food profile 1 and food profile 2. Food profile 1 include the foods at highest risk for human health, especially those which are usually consumed without cooking or another treatment able to eliminate or reduce to an acceptable level the risk of infection by STEC. Food profile 2 includes contaminated food which are destinated to be cooked before consumption or to another treatment able to eliminate or reduce to an acceptable level the risk of infection by STEC. Importantly, the information about the required treatment must be clear to the consumer, reporting it on the label. In addition, any other information concerning the prevention of specific harmful effect from a particular food or food category must be reported to the consumers (European Commission, 2014)

Risk management measures are needed when the detection of STEC is confirmed, thus at least a *stx* gene has been detected. For food with a risk profile 1, corrective actions should be

implemented if there is evidence of STEC contamination, regardless of the serogroup or the presence of *eae* gene (*i.e.* isolation of *E. coli* harbouring *stx* genes).

Instead, for food with a risk profile 2, corrective actions should be triggered only for STEC strains belonging to the serogroups most frequently associated with severe illnesses (*i.e.* serogroups O157, O26, O103, O145, O111, O104). Moreover, corrective actions should differ depending on whether the food at risk has already reached the retail level or not. Food which is already placed on the market should be withdraw or recalled according to Article 19 of Regulation (EC) No 178/2002. Food not yet placed on the market can be submitted to further processing, such as a treatment eliminating the STEC hazard (European Commission, 2014).

5. Data on STEC in Triveneto area (Northern Italy)

During my internship period at the Istituto Zooprofilattico delle Venezie, territorial headquarters of Trento (Northern Italy), I had access to the data concerning the occurrence of STEC in certain food categories in the Triveneto area (Table 2). In the Triveneto area food companies carry out self-monitoring plans to detect STEC in foodstuffs: milk and milk products, meat and meat products and sprouts. This activity is mainly carried out on dairy product, especially raw milk origin, even if controls in meat products are increasing.

Data were available from 2018 to the end of July 2021. A total of 1,595 samples were tested in the study period, with 77 (5%) positive for STEC, 1,323 negative (83%) and 193 presumptive-positive (12%) (Table 2). Among positive samples, 73 (94.8%) were from milk and milk products and 4 (5.2%) from meat and meat products. The distribution of positive sample throughout 2018-2021 is shown in Figure 10.

Food category	**Positive**	**Negative**	**Presumptive**	**N samples units**
Milk and milk products	**73**	**1259**	**188**	**1520**
Butter	0	19	4	23
Curd	0	149	26	175
Hard cheese	12	203	25	240
Fresh cheese	54	748	95	895
Raw milk	3	46	6	55
Raw cream	4	89	34	127
Gastronomic food preparation	0	5	0	5
Meat and meat products	**4**	**64**	**5**	**73**
Meat	1	13	1	15
Packed meat	1	4	1	6
Frozen meat	1	2	1	4
Minced meat	0	5	0	5
Fresh sausage	0	1	0	1
Seasoned sausage	1	4	0	5
Muscle	0	6	1	7
Meat based preparation	0	28	1	29
Meat based product	0	1	0	1
Total (%)	**77 (5%)**	**1323 (83%)**	**193 (12%)**	**1593**

Table 2: Distribution of positive, negative, and presumptive-positive samples in food categories, 2018-2021.

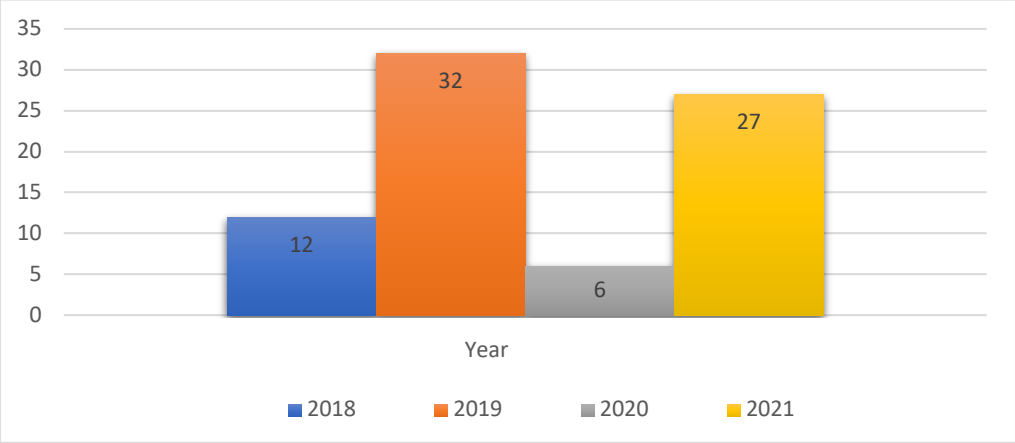

Figure 10 Distribution of STEC-positive samples in the Triveneto area, 2018-2021

In the Triveneto area, the most frequently detected STEC-positive samples belonged to milk and milk products. A total of 73 out of 1,520 samples (4.8%) resulted contaminated by STEC. The main subcategories involved were fresh and hard cheeses, with 54 (3.5%) e 12 (0.8%) positive samples respectively. Fresh cheese positive samples were represented by 5 cheeses made from goat's milk and 49 cheeses made from cow's raw milk. All hard cheese positive samples were made from cow's raw milk. The largest distribution of STEC-positive samples among cheeses may be explained by the largest number of tested samples in comparison with the other categories (butter, curd, raw milk, raw cream, gastronomic food preparation). For what extent meat and meat products, 4 samples out of 73 (5.5%) resulted positive. All positive samples were from wild ruminants, respectively two from deer meat and two from roe deer. Although data of 2021 are not completed, they are higher than those reported for the years 2018 and 2020. Data for virulence factors were available only for the strains collected during the years 2019, 2020 and 2021. On a total of 65 isolates collected in this period, 60 (92.3%) were tested for *stx* and *eae* genes (Table 3). Most isolates were *stx1*+ and *eae*+ (40; 66.7%), 12 were *stx2*+ *and eae* – (20%), six were *stx2*+ and *eae*+ (10%) and two were *stx1*+, *stx2* and *eae*- (3.3%). Furthermore, only two isolates detected from fresh cheeses were positive for both *stx1* and *stx2* and negative for *eae.* The virulence factor *eae* has been identified in 6 samples (10%), which were all *stx2*+. The isolates from milk and milk products did not belong to the "top five" serogroups, or to O104:H4 serotype. Only a STEC isolate detected from a deer packed meat sample belonged to the O157 serogroup.

Virulence genes profile	No of isolates (%)
stx1+;eae-	40 (66.7)
stx1+;eae+	0 (0.0)
stx2+;eae-	12 (20.0)
stx2+;eae+	6 (10.0)
stx1+;stx2+;eae-	2 (3.3)
stx1+;stx2+;eae+	0 (0.0)
Total	**60**

Table 3: Distribution of virulence genes in STEC isolates in the Triveneto area, 2019-2021

In the area of Triveneto, 193 out of 1593 samples (12%) were presumptive-positive (Table 3). Among these samples, 188 belonged to milk and milk products, 5 to meat and meat products. As for positive samples, data of virulence factors are available only from 2019 onwards, when 169 samples were classified as presumptive-positive for STEC. The detection of *stx1* was more frequent than *stx2,* as observed in the STEC detected from the positive samples. Many samples (32; 19%). were positive for both *stx1* and *stx2.* The *eae* gene was distributed almost equally, with a slightly larger association with *stx2*, as was observed in the positive samples. Distribution of virulence genes in presumptive-positive samples is shown in Table 4.

Virulence genes profile	No presumptive (%)
stx1+, eae-	63 (37.3)
stx1+, eae+	18 (10.7)
stx2+, eae-	31 (18.3)
stx2+, eae+	25 (14.8)
stx1+, stx2+, eae-	16 (9.5)
stx1+, stx2+, eae+	16 (9.5)
Total	**169**

Table 4:Distribution of virulence genes in presumptive STEC-positive samples in the area of Triveneto, 2019-2021

In the 59 isolates where the *eae* gene was detected, the genes associated with the "top five" serogroups were tested. The most common serogroups were O145 and O103, as shown in Figure 11. Please note that the information for the O104:H4 serotype is not indicative because its detection was not routinely applied but was performed on request only.

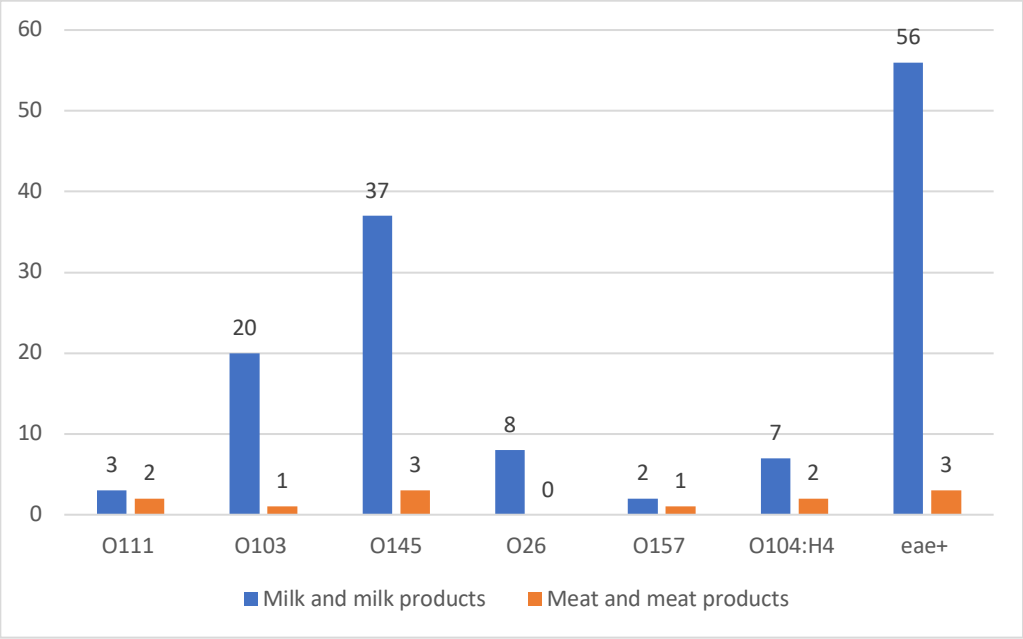

Figure 11: Frequency distribution of serogroups in presumptive samples, 2019-2021

As milk and milk products are the most important food items of the Triveneto, the comparison with data concerning milk and milk products in the EU is of the greatest interest. In 2019, STEC was found in 61 (2.1%) out of 2,981 samples of milk and milk products reported by nine MSs. Concerning raw milk, some MSs reported the results from national monitoring programs. Firstly, eight MSs reported 48 positive samples out of 1,216 (3,9%) raw cow's milk samples. Among these, serogroup information was provided for two isolates only (one STEC O26 and one O157). Secondly, 4 positive samples out of 102 (3,9%) of raw milk (undefined species) was reported by one MS with unspecified serogroup. Lastly, no one positive samples were reported by a monitoring plan made of 27 samples of raw goat's milk. Moreover, four MSs tested 148 RTE-dairy products other than milk and cheese (butter, cream, ice cream, whey, yoghurt and fermented dairy products) obtaining 5 positive samples (3,38%). Only one serogroup was identified, *i.e.* O26. Finally, 25 out of 2,696 (0,9%) cheese samples resulted positive for the presence of STEC (EFSA and ECDC, 2021).

In the EU, characterisation of the *stx* and *eae* gene profiles was performed for a very small number of isolates. In fact, only nine isolates were provided with data on the presence of both *stx* and *eae* genes and included: one *stx1* and *eae* positive; two *eae* and *stx2* positives; two *stx1* positives, *t*wo *stx2* positives, two *stx1* and *stx2* positives. Although only nine isolates were tested at EU level, the important finding is that only three of them were *eae*-positive (EFSA and ECDC, 2021).

6. Occurrence of STEC in foodstuffs

As regarding the occurrence of STEC in foodstuffs in the EU, during 2019 the samples collected in the MSs were almost totally (95%) tested with the ISO 13136:2012 method. Among the main food categories, meat, especially of ruminants, was the most contaminated product (4.1% STEC-positive, out of 12,120 samples), followed by 'milk and dairy products' (2.1% STEC-positive out of 2,981 samples) and 'fruits and vegetables' (0.1% STEC-positive out of 2,171 samples) (ECDC, 2021; EFSA and ECDC, 2021).

Food category	Samples tested for STEC by any method				
		positive (any STEC)		positive for STEC O157	
	n total	n	%	n	%
bovine meat	9,952	320	3.2	19	0.2
ovine and goat meat	923	107	11.6	8	0.9
other ruminants meat	80	11	13.8	1	1.3
pig meat	1,258	59	4.7	5	0.4
other meat	1,281	29	2.3	8	0.6
mixed meat	616	16	2.6	0	0.0
milk and dairy products	3,497	39	1.1	0	0.0
raw milk	1,982	52	2.6	1	0.1
fruit and vegetable	2,658	2	0.1	0	0.0
seeds	994	0	0.0	0	0.0
other food	1,789	5	0.3	0	0.0
Total	**25,030**	**640**	**2.6**	**42**	**0.2**

Table 5: occurrence of STEC in food, 2019.

The most frequently identified serogroup was O157, followed by O26, O145, O103 and O111, as shown in Table 6 (EFSA and ECDC, 2021). Nevertheless, STEC O157 was identified in 0.2% of the samples, while the rate of all the other STEC serogroups reached 2.6 % (Table 5).

Food category	Samples positive for any STEC	O157	O26	O145	O103	O111
	n	n	n	n	n	n
bovine meat	315	14	7	4	4	1
ovine and goat meat	102	3	3	0	2	0
other ruminants meat	10	0	0	0	0	0
pig meat	54	0	0	0	0	0
other meat	21	0	0	0	1	0
mixed meat	16	0	0	0	1	0
milk and dairy products	39	0	3	0	0	0
raw milk	52	1	1	0	0	0
fruit and vegetable	2	0	0	0	0	0
seeds	0	0	0	0	0	0
other food	5	0	0	0	0	0
Total	**616**	**18**	**14**	**4**	**8**	**1**

Table 6: occurrence of serogroups in isolates from different food categories, 2019.

In 2019, the most frequently detected virulotypes among 138 isolates out of 616 (22.4%) were *stx2* +, *eae*- (42) and *stx2*+, *stx1*+, *eae*- (30), followed by *stx1*+, *eae*- (25), *stx1+, eae*+ (25), *stx2+,eae*+ (13) and *stx2+, stx1+, eae*+ (3) (EFSA and ECDC, 2021).

Taking into account that 60% of STEC infections is attributable to food, in Europe dairy products are classified as the second (following beef) major food source of STEC (FAO/WHO, 2018). Furthermore, out of 52 'strong evidence'[2] outbreaks reported in the EU during 2012-2017 (Table 7), 14 (27%) are caused by consumption of milk and milk products and at least seven (50%) of these were of raw milk origin. The food category 'Milk and dairy products' caused 94 cases of infection, 43 hospitalisations and 2 deaths in 2019. One of the outbreaks occurred in Italy, after the ingestion of ricotta cheese, mozzarella cheese and handcraft ice-cream. Overall, bovine meat and meat products ranked first among the strong-evidence outbreaks, causing a greater number of cases (143) compared to milk and dairy products, 76 hospitalizations, but no death.'Vegetables, fruit and products thereof' represent another relevant source of infection; despite the little number of associated outbreaks (7), human cases due to this food category are the most common (575). For this reason, they are considered as one of the most important food vehicles of STEC infection in EU. In addition, the category 'tap water, including well water' ranks third in the source attribution study based on strong evidence outbreaks, but the majority of outbreaks occurred in one MS only and 63% of these was most likely associated with well

[2] The strength of evidence related to an outbreak to be reported to EU level is based on assessment of all available categories of evidence (i.e. descriptive, epidemiological or microbiological evidence) (EFSA,2011,2014)

water, which is not frequently used as source of drinking water in many MSs. Therefore, this finding cannot be extended to the entire EU.

Implicated food vehicle category (number of reported strong evidence outbreaks; number of reporting countries)	**Human cases**	**Hospitalisations**	**Deaths**
Bovine meat and meat products thereof (15;7)	143	76	0
Milk and dairy products (14;8)	94	43	2
Tap water, including well water (8;4)	75	7	0
Vegetables, fruit, and products thereof (7;3)	575	73	2
Pig and meat and products thereof (2;1)	6	2	0
Other or mixed red meat and products thereof (2; 2)	10	0	0
Sheep meat and products thereof (1; 1)	27	9	0
Unspecified meat (1; 1)	2	1	0
Fish and seafood (1; 1)	5	0	0
Herbs and spices (1; 1)	50	3	0
Total	**987**	**214**	**4**

Table 7: Number of human cases, hospitalizations and deaths per implicated food vehicle category reported in strong evidence STEC food-borne outbreaks from 2012 to 2017.

To further understand the risk associated with these products in Europe, Table 8 shows the number of notifications to the Rapid Alert System for Food and Feed (RASFF), the system for reporting food safety issues within the European Union, concerning the presence of STEC in milk products during 2020-2021 (until August 2021). Out of a total of 157 notifications concerning milk and milk products, 12 reported the presence of STEC and most of them were concerned raw milk. The most affected country of origin was France; Italy was the second country involved in STEC notifications to the RASFF with two alert notifications, specifically for a Taleggio cheese made from raw milk and a Fontina cheese PDO (Protected Designation of Origin) (Table 8, Table 9) (RASFF Portal, https://ec.europa.eu/food/safety/rasff-food-and-feed-safety-alerts/rasff-portal_en).

RASFF 2020 report stated that during 2020 there were 29 STEC notifications, with eight notifications (27,6%) for milk and milk products. The major number of notifications are still attributed to *Salmonella*, which is is more than ever the most frequently reported pathogen in food from MSs (537 notifications, up by 45%). It is followed by *Listeria monocytogenes* (123)

and *Norovirus* (50). *Escherichia coli* (mostly STEC) thus ranks fourth among the pathogenic microorganisms reported in food (RASFF, 2020).

Hazard observed in milk and milk products	**Date of notification**	**Notifying country**	**Classification**	**Risk decision**
STEC in goat cheese with raw milk	02/03/20	Switzerland	alert notification	undecided
STEC in taleggio latte crudo	04/03/20	Netherlands	alert notification	serious
Escherichia coli-shigatoxin-producing in organic raw milk goat's cheese	05/03/20	Belgium	alert notification	serious
VTEC in Käse aus den Niederlanden (stx1)	12/03/20	Germany	information notification for attention	serious
Shigatoxin-producing *Escherichia coli* in raw goat milk cheese from France. (Ziegenrohmilchkäse)	14/09/20	Germany	alert notification	undecided
Escherichia coli O157H7 stx2 eae in goat raw milk cheese made in France (PICODON “AOP")	09/10/20	France	alert notification	serious
Shigatoxin-producing *Escherichia coli* in raw milk cheese from France (Käse)	27/10/20	Germany	information notification for attention	serious
E. coli STEC in formaggio Fontina D.o.p.	28/10/20	Italy	alert notification	serious
Shigatoxin-producing *Escherichia coli* in cheese from France (from raw milk)	29/10/20	Germany	alert notification	serious
Shigatoxin-producing *Escherichia coli* (STEC) in soft cheese from France (Weichkäse)	15/03/21	Germany	alert notification	serious
STEC in raw milk soft cheese from france (Camembert)	06/08/21	Germany	alert notification	serious
Escherichia coli shigatoxin-producing in Reblochon chhese from France	20/08/21	France	alert notification	serious

Table 8: Number of STEC notification in milk and milk products done to RASFF during 2020-2021.

Country origin	**Number of notification**
France	7
Italy	2
Switzerland	1
Belgium	1
Germany	1
Total	**12**

Table 9: Number of notifications per country of origin, 2020-2021

7. Conclusion and discussion

The only existing regulatory limit for STEC in foodstuffs concerns sprouts (Regulation CE No 2073/2005). In other food categories, data derive from monitoring activities of MSs following Directive 2003/99/EC, which obliges to the investigation of STEC in food, feed, humans and animals in the UE. Since the directive is not clear about the sampling strategies, MSs investigations are based on non-harmonised samplings. As a consequence, the comparison between MSs monitoring data is not possible, thus precluding the drawing up of spatial and temporal trend in EU.

Food categories other than sprouts play an important role as cause of STEC foodborne disease. As previously mentioned, meat and dairy products represent the major sources of outbreaks in EU during 2010-2017. Especially for milk and milk products, they represent the second cause of outbreaks (Table 7), and, among them, raw milk products are the most notified to RASFF during 2020-2021 (Table 8). The importance of this food category in STEC foodborne disease is clear and also confirmed by the FAO 2018 report which detected milk products as the major source of STEC infection, globally (FAO/WHO, 2018). For these reasons, regulatory measures for these products at EU level are absolutely necessary. The urgency of this regulation is further supported by the fact that more than half of MSs have adopted national sampling plans for the detection of STEC in food, mainly concerning meat, milk and sprouts (EFSA BIOHAZ Panel et al., 2020). Unfortunately, as long as each MS will have its own different sampling strategy and detection method, no real estimation of the presence of STEC at UE level will be feasible.

Another critical element which obstacles the correct interpretation of data on STEC occurrence in foodstuffs, is that for years all studies and tests were directed towards the O157 serogroup. Several studies are observing that O157 is still the main serogroup isolated in food, but non-O157 STEC rates are higher than O157 and in constant rising. Consequently, the real incidence of STEC in foodstuffs has been impossible for many years. Moreover, both data extrapolated by EFSA and ECDC report published in 2021 and collected in the Triveneto area showed that the “top five” serogroups were rarely detected. This gap could be almost totally filled by the implementation of WGS, which can fully type isolates, including O and H antigens. However, it is important to underline that the serogroups are defined as non-pathogenic factor for STEC. This, together with the fact that the same serotype can carry different virulence genes, leads to the conclusion that they are not as useful as the virulence genes in the investigation of outbreaks and in monitoring plans. In fact, even if the reference method ISO 13136:2012

includes the detection of the top-five serogroups associated with severe human illness, several data show that serogroups others than the top-five carrying *stx* genes are constantly growing, and that all STEC are capable to cause severe human illness, regardless of their serogroup.

The ISO 12136: 2012 method also includes the detection of the *eae* gene. However, the *eae* gene has been defined as an aggravating factor for STEC pathogenicity, thus not essential for severe illness, being often replaced by other genes coding for different adhesion mechanisms (EFSA BIOHAZ Panel et al., 2020). In according to this, only a minority of STEC food isolates in the EU and in the Triveneto area were *eae*-positive.

In conclusion, a better understanding of the genes involved in the pathogenesis of STEC is required for the identification and characterization of this complex group of pathogens. This objective could be more easily achieved thanks to the application of WGS to all clinical isolates, in order to identify genes or gene combinations more frequently associated with human illness. For this reason, the detection of virulence factors others than *eae*-gene in samples from food, animals and clinical cases should be performed for a better surveillance/monitoring of STEC in the EU.

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