NATIONAL REFERENCE CENTRE FOR SHIGA TOXIN/VEROTOXIN-PRODUCING *ESCHERICHIA COLI* (NRC STEC/VTEC)

ANNUAL REPORT 2023

Universitair Ziekenhuis Brussel

UZ BRUSSEL

Department of Microbiology and Infection Control

NRC STEC/VTEC

Florence Crombé PhD

Denis Piérard MD, PhD

✉ lmiknrc@uzbrussel.be ☎ +32 2 474 93 83

Universitair Ziekenhuis Brussel

TABLE OF CONTENTS

MAIN POINTS

- In 2023, 335 cases of Shiga toxin-producing *Escherichia coli* (STEC) were submitted to the National Reference Centre (NRC) STEC. These included 331 culture-confirmed cases of STEC.

- Twenty-nine patients developed the hemolytic uremic syndrome (HUS): two with O157, 8 with O26, 15 with non-O157 and 4 without STEC-positive culture (i.e. toxin production genes directly detected in faeces or *E. coli* lipopolysaccharide [LPS] positive serology).

- Four cases, including two cases of HUS (3- and 88-year-old), died as direct or indirect consequence of STEC infection.

- The most common STEC serogroup was O157 (n=73 out of 332, 22.0%).

- The most commonly isolated non-O157 STEC serogroup was O63 (n=43 out of 332, 13.0%), which is in contrast to previous years where O26 was most common.

- The new Stx2i subtype was identified for the first time in an *eae*-negative strain of serotype O30:H25.

- Twenty-six molecular clusters of two to seven cases were identified by core genome multilocus sequence typing (cgMLST) analysis in 2023.

BACKGROUND

Shiga toxin-producing *Escherichia coli* (STEC) infections, also known as vero(cyto)toxin-producing *Escherichia coli* (VTEC) infections, are the cause of sporadic and epidemic watery or bloody diarrhoea worldwide. A small proportion of patients, mainly children, develop hemolytic uremic syndrome (HUS) (Launders et al. 2016).

The main reservoir for STEC is cattle but other ruminants such as sheep, goats and deer can also be carriers of STEC. Transmission can occur through direct or indirect contact with animals or their environments, consumption of contaminated food or water, and person-to-person contact (Kintz et al. 2017).

STEC strains are classified based on their *E. coli* O:H-serotype (Orskov and Orskov 1984). STEC O157:H7/H- causes most of the STEC infections but other serotypes (termed non-O157) have also been associated with diarrheal disease worldwide. In the European Union/European Economic Area (EU/EEA), the 5 most common non-O157 serogroups are O26, O91, O103, O145 and O146 (ECDC, STEC infection, 2022).

STEC's main pathogenic mechanism is the production of Shiga toxins (Stx), which cause cell death by blocking the protein synthesis. The Stx family can be divided in two major antigenically distinct types, Stx1 and Stx2, which show distinct immunogenic and genetic properties. Stx variants are presently organized into a taxonomic system of three Stx1 (a, c and d) and seven Stx2 (a, b, c, d, e, f, and g) subtypes. Yet, novel Stx1e and Stx2 (h, I, j, k, l, m, n and o) subtypes have recently been described (Bai et al. 2021; Yang et al. 2020; Hughes et al. 2019). STEC strains can produce either only one Stx or a combination of different Stx subtypes. The Stx-coding genes (*stx*) are carried by bacteriophages that can easily be lost or acquired by horizontal transfer (Scheutz 2014; Scheutz et al. 2012). Determination of the Stx variants is clinically relevant as some Stx2 subtypes – *stx*2a and *stx*2d - seem to be more often associated with severe human illness, especially when they are present in combination with the *eae* virulence gene coding for intimin (Brandal et al. 2015; Werber and Scheutz 2019). The NRC STEC uses a self-designed virulence typing algorithm to classify the risk of HUS development (De Rauw et al. 2018).

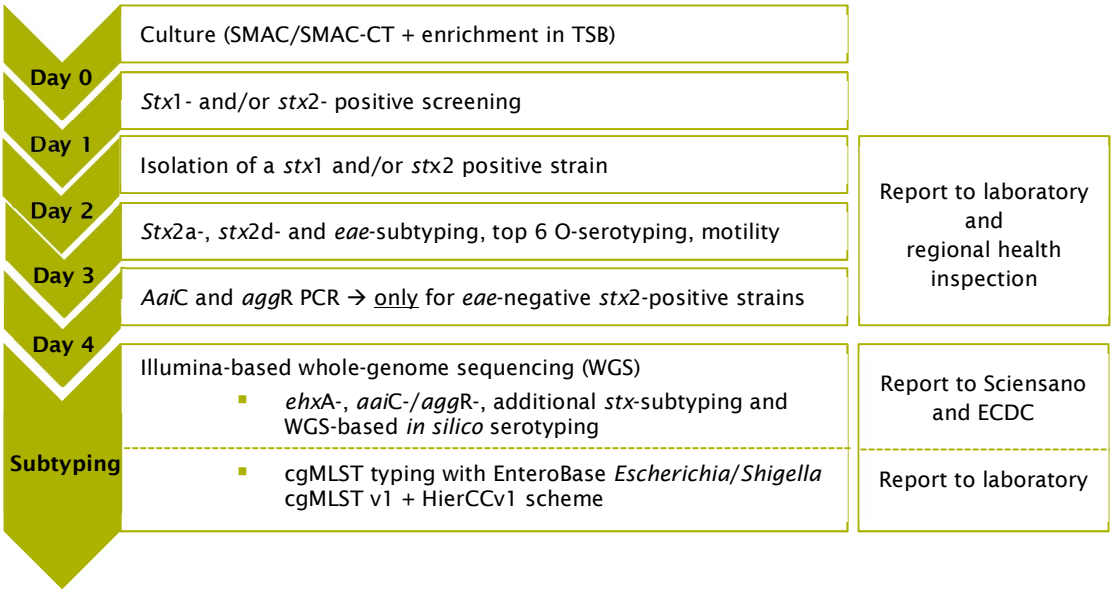
Since the outbreak of STEC/enteroaggregative *E. coli* (EAEC) O104:H4 in Germany in 2011 (Buchholz et al. 2011), the so-called cross-pathotype or hybrid strains are emerging. Besides STEC/EAEC, other hybrid STEC isolates have been reported, such as STEC/extraintestinal pathogenic *E. coli* (ExPEC) (Cointe et al. 2021), STEC/enterotoxinogenic *E. coli* (ETEC) (Bai et al. 2019) and STEC/uropathogenic *E. coli* (UPEC) (Gati et al. 2019).

This report summarises the typing data of human STEC strains reported to the Belgian NRC in 2023 and compares it to previous years. It is to be noted that the NRC STEC only reports cases of STEC infection from whom STEC was isolated (see page 4), toxin production genes were directly detected in faeces (HUS cases only) or *E. coli* serogroup-specific (LPS) antibody response was observed (HUS cases only) (see page 13). The NRC STEC does not report non-HUS cases from whom toxin production genes were directly detected in faeces without STEC isolation as per EU decision 2018/945.

WHOLE-GENOME SEQUENCING FOR CHARACTERIZATION OF STEC AT THE NRC STEC

WGS has been implemented at the NRC in 2019. The method is performed on all STEC strains provided to the NRC, replacing the traditional quarterly subtyping methods (Figure 1). In addition, the sequencing data is analysed using the *Escherichia/Shigella* cgMLST v1 + HierCCv1 typing scheme in EnteroBase (Zhou et al. 2020). This analysis enables confirmation of epidemiologically linked cases on the one hand and clustering of cases that were not identified based on traditional typing data complemented with epidemiological data on the other hand. The cluster threshold is set at hierarchical clustering level HC5, i.e. all strains in this cluster have links no more than 5 alleles apart. The sequences are available in EnteroBase[1]. To that end, a search can be performed by clicking the **Field** dropdown and selecting *Lab Contact*; and typing in *Laboratory of Microbiology, UZ Brussel* in the **Value** field. The isolate genomes from 2023 are also available on NCBI (Bio-Project PRJEB61522).

Figure 1: Screening and virulence typing algorithm used at the Belgian NRC STEC from 2019 onwards



[1] https://enterobase.warwick.ac.uk/

NUMBER OF STEC STRAINS ANNUALLY ISOLATED

Figure 2 gives an overview of the number of STEC strains annually identified at the NRC STEC from 2008 to 2023. On average 122 strains were identified each year (min. 81 [2020] – max. 332 [2023]).

In 2020, as for other gastrointestinal pathogens, a decrease in number of STEC strains was recorded probably due to the COVID-19 pandemic. In 2022, on the other hand, a substantial increase in number of STEC strains was observed as a probable consequence of the implementation of gastrointestinal molecular panels by a number of clinical laboratories. This number of STEC strains, non-O157 in particular, continued to increase drastically in 2023. Indeed, in 2023, 332 different STEC strains were isolated from 331 Belgian patients (Table 1). Two strains with identical cgMLST profile were isolated from the stool of a same patient: O157:H7, *stx*1a, *eae*-positive and O157:H7, *stx*1a *stx*2c, *eae*-positive.

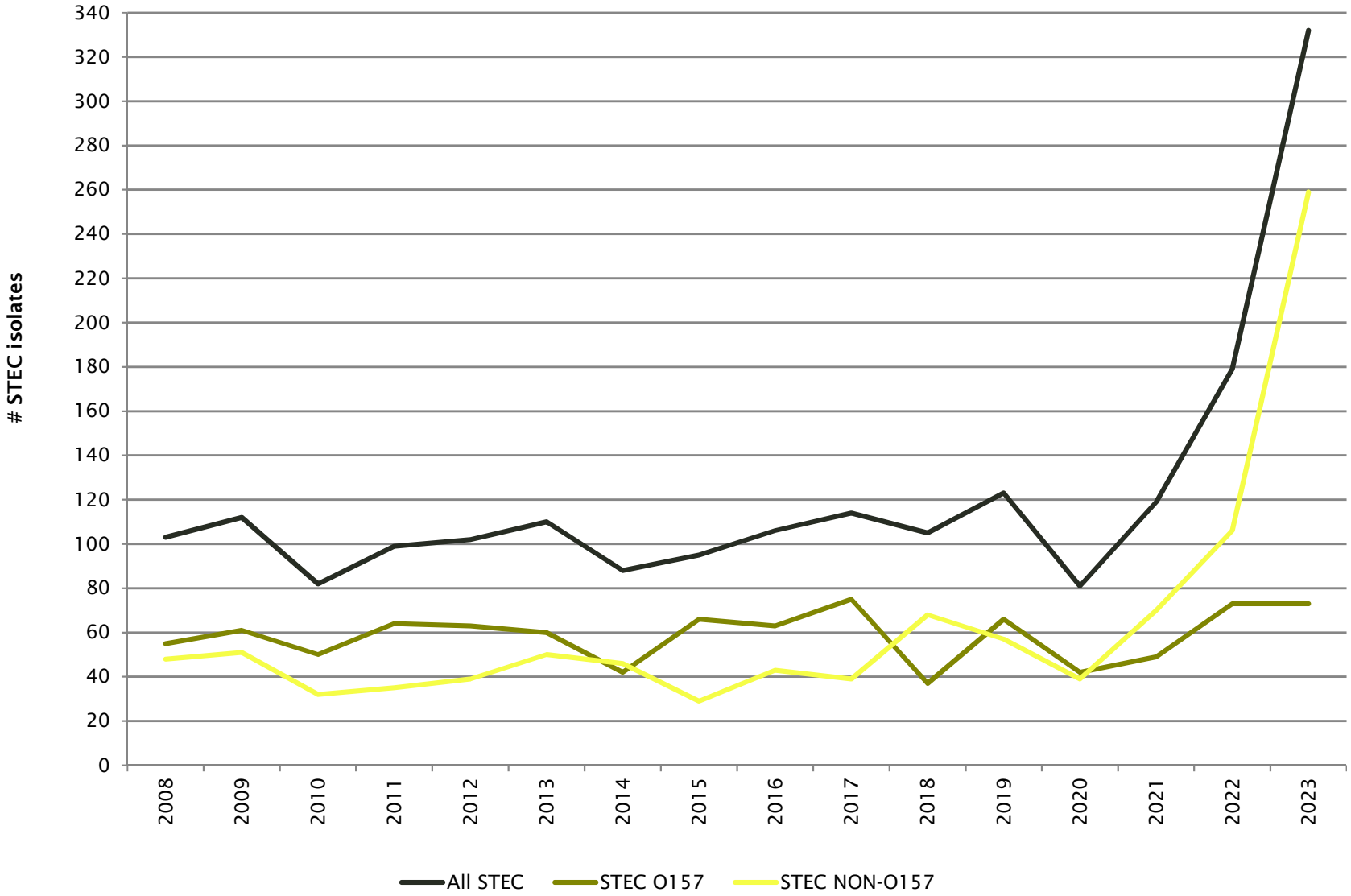
Four cases, including two cases of HUS, died in 2023. The serotypes of the isolated STEC strains were O27:H30 (86-year-old case, no HUS but unknown clinical symptoms), O45:H2 (88-year-old HUS case), O145:H28 (3-year-old HUS case) and O177:H11 (76-year-old asymptomatic cancer case).

One hundred eighty-one of them were typical STEC (*eae*+, *hlyA*+). One hundred fifty-one isolates were atypical STEC; lacking one or both of these virulence determinants. One strain with serotype O136:H20 (*stx*1c, *eae*-negative) was found positive for EAEC virulence gene *aaiC* and negative for *aggR*. One strain with serotype O181:H4 (*stx*2a, *eae*-negative) was found positive for both EAEC virulence gene *aaiC* and *aggR*.

Table1: Number of STEC strains annually identified at the NRC STEC in relation to the number of cases with STEC infection (2017-2023)

Total numbers	2017	2018	2019	2020	2021	2022	2023
STEC strains	114	105	123	81	119	179	332
Culture-positive cases of STEC infection	112	104	122	78	119	177	331

Figure 2: Number of STEC strains annually isolated at the NRC STEC (2008-2023)



STEC SEROTYPES

Figure 2 shows the annual distribution of O157 and non-O157 strains. Forty-nine percent of the STEC strains typed at the Belgian NRC STEC between 1994 and 2022 were of the O157:H7/H- serotype (n=1256).

O-serogroups of 1249 out of the 1324 STEC non-O157 could be determined, classifying them in 108 different O-serogroups and 5 additional O-genotypes (OgN1, OgN9, OgN10, OgN15 and OgN-RKI4)[2] (Figure 3). The remaining 75 non-O157 strains could not be serotyped with the methods used at the moment of analysis[3] (NT non-O157). It is to be noted that three strains have been counted in as NT non-O157 as the *stx* gene was not detected anymore after sub-culture for WGS: 1 *stx*2f in 2021, 1 *stx*1 and 1 *stx*2 other than *stx*2a, *stx*2d and *stx*2f in 2023.

Figure 4 gives an overview of the evolution of the six most common serogroups in the EU/EEA in Belgium: O26 (n_{total}=247), O91 (n_{total}=49), O103 (n_{total}=121), O145 (n_{total}=104), O146 (n_{total}=75) and O157 (n_{total}=1256). Additionally seven other non-O157 serogroups have been isolated frequently over the years: O63 (n_{total}=74), O80 (n_{total}=36), O111 (n_{total}=71), O113 (n_{total}=25), O118 (n_{total}=17), O128 (n_{total}=31) and O182 (n_{total}=22).

Interestingly, O118 was detected regularly in the early years of screening but has not been found from 2010 to 2016 and only once in 2017. Serogroups O63, O80 and O182 have only been isolated since 2008 and 2003, respectively.

In 2008, primers for the detection of *stx*2f were added to the STEC PCR screening assay. All STEC O63 isolated at our NRC carry the *stx*2f gene, explaining why this serogroup was not detected the years before. Surprisingly, in 2023, 43 STEC O63 have been recorded while on average two strains were identified each year between 2008 and 2022. It is to be noted that 17 out of these 43 STEC O63 isolates were belonging to six molecular clusters (see page 18). Although pigeons are considered as a natural reservoir for *stx*2f-carrying *E. coli*, it seems that pigeon and human isolates differ regarding serotypes and phylogenetic clusters based on comparative genomics (van Hoek et al. 2019). The reservoir of human STEC *stx*2f is still unknown. Humans could be considered as plausible reservoir for STEC O63-infections (van Hoek et al. 2019).

Twenty-six STEC O80 strains were isolated from 2018 to 2023 while only ten were detected at the NRC between 2008 and 2017 (De Rauw et al. 2019). The identification of STEC O80:H2 is of importance as this hybrid pathotype has recently emerged in France and in other European countries and has been associated with HUS as well as HUS associated with bacteraemia (Ingelbeen et al. 2018; Nuesch-Inderbinen et al. 2018; Rodwell et al. 2021; Cointe et al. 2021).

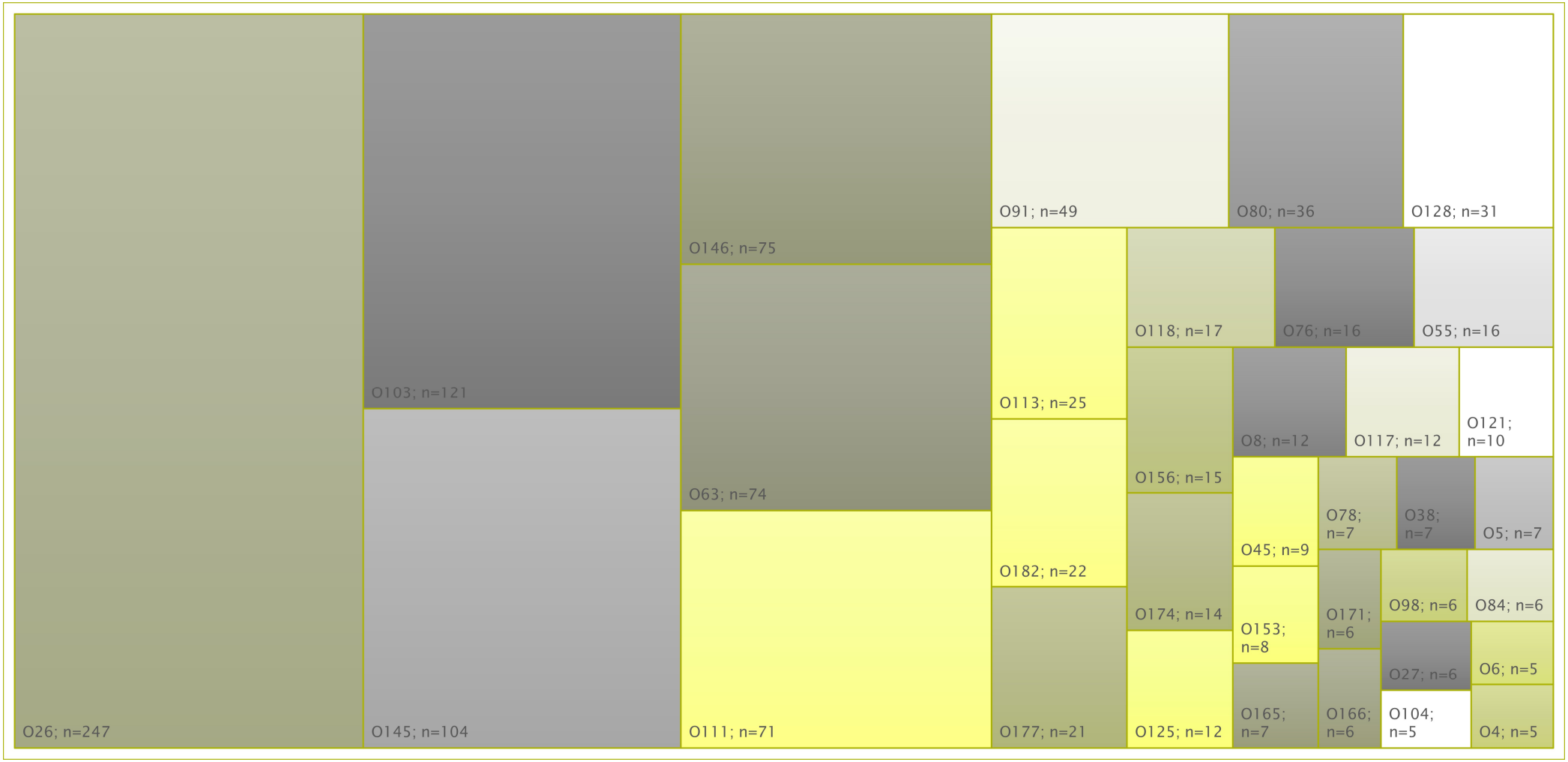
In 2023, the most common STEC serogroup was O157 (serotype O157:H7/H-) (73/332; 22.0 %). All of the 'top 5' non-O157 serogroups were represented: 34 O26 serogroup, 13 O91 serogroup, 20 O103 serogroup, 21 O145 serogroup and 18 O146 serogroup (Figure 4).

---

[2]O-genotyping was performed by Dr. Lang and Dr. Fruth at the Robert Koch Institute as described in Lang et al. (2019).

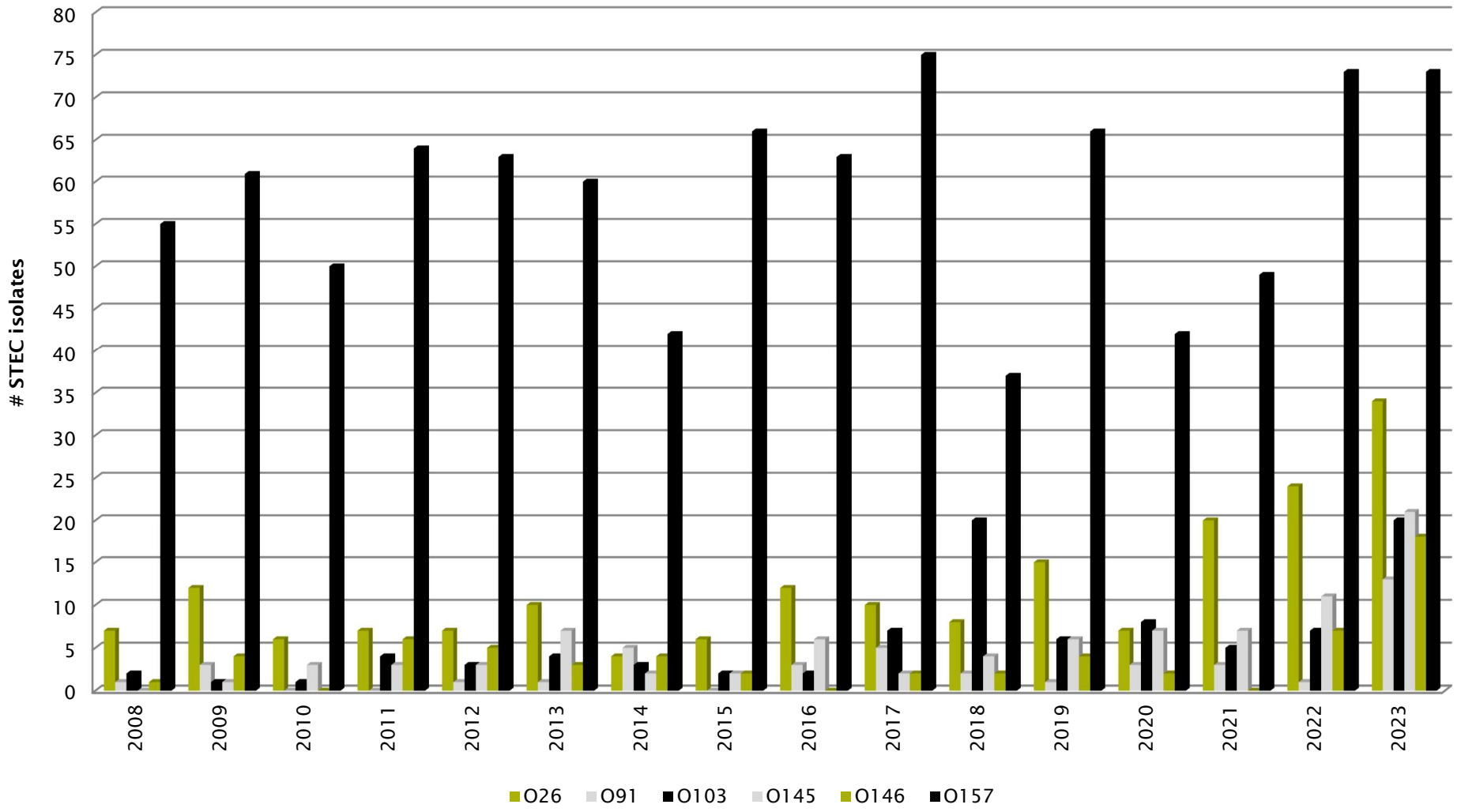
[3]*Gnd*-sequencing for O-typing from 2015 to 2018 (Gilmour et al. 2007); WGS-based O:H-typing from 2019 to present (*E.coli* functional genotyping plugin in BioNumerics v.8.1.1. (Applied Maths, BioMérieux, France)). In 2022, WGS has been performed retrospectively on all NT non-O157 strains isolated between 2011 and 2018 (n=48). Therefore, the numbers of NT non-O157 differ from previous annual reports.

Figure 3: Occurrence of the STEC non-O157 serogroups (1994-2023)[4]



[4] Serogroups detected ≤ 4 times, including NT non-O157, are not included in Figure 3.

Figure 4: Annual distribution of the six most common STEC serogroups reported in the EU/EEA (2008-2023)



SHIGA TOXIN SUBTYPES

In 2023, 19.0 % (63/332) were *stx*1 positive, 53.9 % of the isolates were *stx*2 positive (179/332), and 27.1 % were *stx*1 and *stx*2 positive (90/332). The majority of the STEC strains were at medium (50.0 %) or high (31.3 %) risk for developing HUS (Table 2) (De Rauw et al. 2018). The predominating Stx subtype profiles were: stx1a (51), stx1a stx2a (23), stx1a stx2c (33), stx1c stx2b (20), stx2a (56), stx2c (23) and stx2f (58) (Table 3). Surprisingly, stx2f, generally associated with mild symptoms, was the most common Stx subtype profile in 2023. The second most common Stx subtype profile, stx2a alone, was harboured by fourteen different serotypes, with O26:H11 predominating (n=23/56; 41.1 %) (Table 4). The new Stx2 subtype, Stx2i (O30:H25, *eae*-negative), was identified for the first time in 2023.

Table 2: Risk classification for the development of HUS among STEC isolates (2017-2023)

Risk for HUS development	2017	2018	2019	2020	2021	2022	2023
High	52	59	62	30	49	70	104
Medium	44	23	42	37	51	74	166
Low	18	23	19	14	19	35	62
TOTAL	114	105	123	81	119	179	332

Table 3: Shiga toxin subtype profiles detected among STEC isolates (2017-2023)

Stx subtype	2017	2018	2019	2020	2021	2022	2023
stx1a	16	21	17	13	16	30	51
stx1c	1	2	2	1	3	5	10
stx1d	1	0	0	0	0	0	1
stx1a stx2a	25	22	23	7	12	19	23
stx1a stx2a stx2c	0	4	0	0	0	0	5
stx1a stx2b	4	2	1	2	2	1	9
stx1a stx2c	19	12	26	24	31	27	33
stx1a stx2d	0	1	1	0	0	0	0
stx1c stx2b	3	2	2	2	1	11	20
stx2a	21	19	24	16	33	38	56
stx2a stx2c	6	6	10	4	2	7	5
stx2a stx2d	0	0	0	0	0	2	1
stx2b	0	1	3	2	2	3	18
stx2b stx2c	0	0	0	0	0	1	2
stx2b stx2d	0	0	0	1	0	1	1
stx2c	16	2	7	6	6	19	23
stx2c stx2d	0	0	0	0	1	0	1
stx2d	0	7	4	2	1	3	11
stx2e	0	0	0	0	0	2	1
stx2f	2	4	3	1	9	10	58
stx2i	0	0	0	0	0	0	1
No Stx subtype	0	0	0	0	0	0	2[5]
TOTAL	114	105	123	81	119	179	332

[5]The Stx subtype of two isolates could not be determined, as the *stx* gene was lost after subculture for WGS: 1 *stx*1 and 1 *stx*2 other than *stx*2a, *stx*2d and *stx*2f.

Table 4: Number of Stx subtype profiles reported amongst the top 10 STEC serogroups in Belgium, 2021-2023

Serogroup	Stx subtype	2021	2022	2023
O26	stx1a	4	10	10[6]
	stx2a	16	10	23[7]
	stx1a stx2a	0	2	1
O63	stx2f	3	5	43[8]
O80	stx1a	0	1	0
	stx2a	1	0	0
	stx2d	1	2	9
O91	stx1a	1	0	2
	stx1c	0	0	1
	stx1a stx2b	2	1	9
O103	stx1a	4	6	16[9]
	stx1a stx2a	1	1	3[10]
	stx2a	0	0	1
O111	stx1a	0	5	1
	stx1c	1	0	0
	stx1a stx2a	1	4	2
O128	stx1c stx2b	0	1	1
	stx2b	1	2	1
	stx2f	0	0	1
O145	stx2a	7	8	15[11]
	stx2a stx2c	0	1	0
	stx2a stx2d	0	1	0
	stx2f	0	1	6[12]
O146	stx1c	0	0	1
	stx1c stx2b	0	7	9
	stx2b	0	0	8[13]
O157	stx1a	0	0	1
	stx1a stx2a	8	11	10[14]
	stx1a stx2c	31	26	33[15]
	stx1a stx2a stx2c	0	0	5[16]
	stx2a	4	15	5[17]
	stx2a stx2c	2	4	4
	stx2c	4	17	15[18]

[6] Two isolate genomes within HC5_185264 (2023-FWD-00029).
[7] Two isolate genomes within HC5_204886 (familial cluster); two isolate genomes within HC5_259824 (familial cluster); two isolate genomes within HC5_268107; two isolate genomes within HC5_270508 (familial cluster).
[8] Three isolate genomes within HC5_257986; two isolate genomes within HC5_257987; two isolate genomes within HC5_257989; three isolate genomes within HC5_257991; five isolate genomes within HC5_258004; two isolate genomes within HC5_258007.
[9] One isolate genome within HC5_205179; two isolate genomes, 11 months apart, within HC5_231733.
[10] Three isolate genomes within HC5_205179.
[11] Four isolate genomes within HC5_249005 (familial cluster).
[12] Three isolate genomes within HC5_250266.
[13] Seven isolate genomes within HC5_250262 (2023-FWD-00080).
[14] Three isolate genomes within HC5_177415 (familial cluster); six genomes within HC5_259826 (2023-FWD-00078).
[15] Three isolate genomes within HC5_150164; five isolate genomes, including one genome with 5 AD compared to the cluster representative, within HC5_212693.
[16] Five isolate genomes, including one with 9 months apart, within HC5_74816.
[17] Three isolate genomes within HC5_197305 (familial cluster).
[18] Two isolate genomes within HC5_234951.

PATIENTS

Among the 335 cases with STEC infection, 43.6 % were males and 56.4 % were females, with a male-to-female ratio of 0.8:1 (Figure 5). The highest number of cases was observed in the age group 0-4 years. This age group accounted for 26.9% of the cases (n=90).

The incidence of STEC infection decreased with age and was lowest in the 25-44 age group (Figure 6).

Figure 5: Distribution of cases of STEC infection by age and gender, 2023

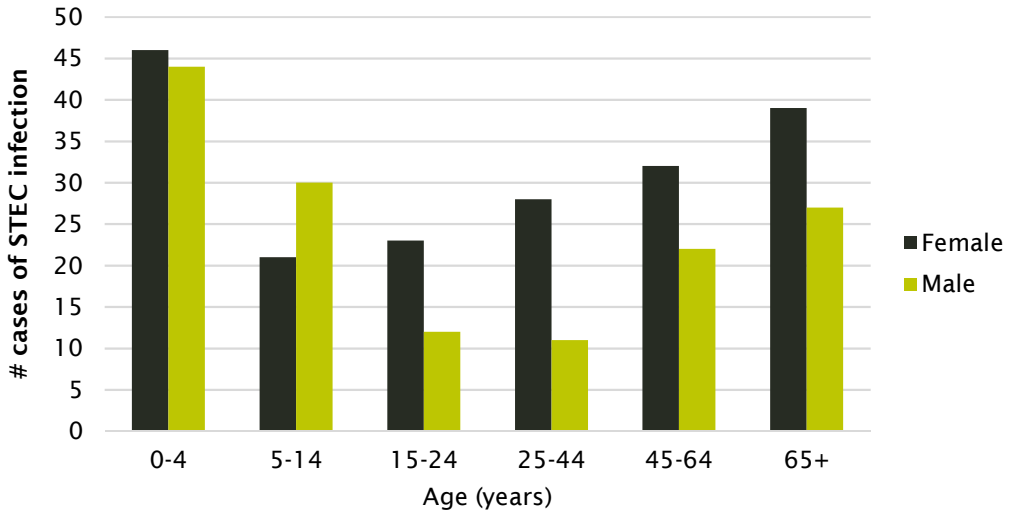
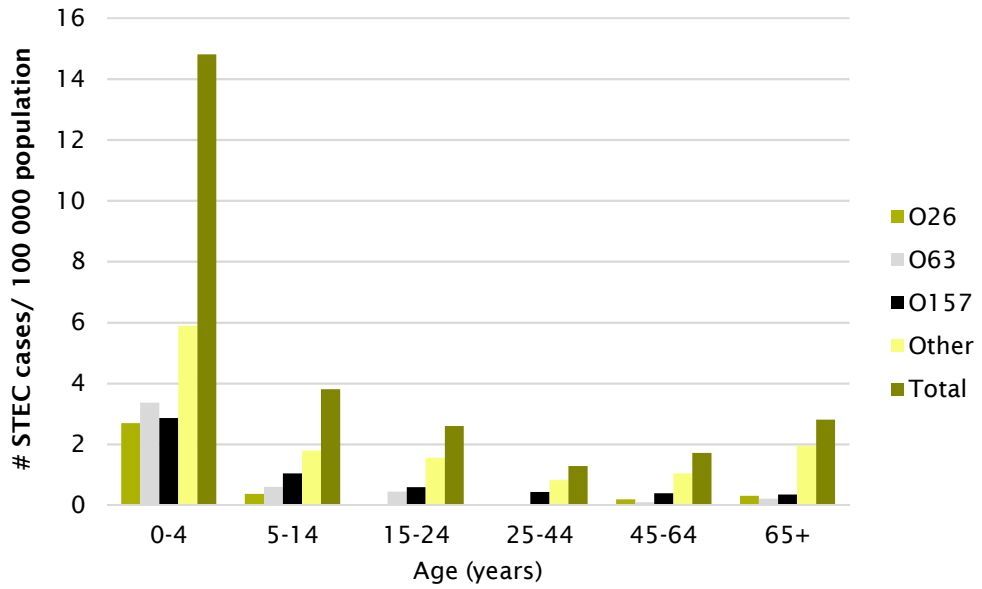


Figure 6: Incidence of STEC infection by age and serogroup, 2023[19]



[19] The cases of STEC infection for which an isolate is available are presented (n=331).

HEMOLYTIC UREMIC SYNDROME

The annual total numbers of HUS cases reported to the NRC STEC from 2017 to 2023 are presented in Table 6.

In 2023, the number of HUS cases dropped back to normal after an unexpected increase in 2022 (41 cases in 2022 vs. an average of 23.6 cases over the years 2017-2021).

Table 6: Number of cases in relation to HUS (2017-2023)[20]

Total numbers	2017	2018	2019	2020	2021	2022	2023
Cases of STEC infection	119	112	127	81	127	187	335
HUS cases	27	19	26	19	27	41	29
Culture-positive HUS cases	20	11	21	16	19	31	25
Percentage of HUS cases	22.7	17.0	20.5	23.5	21.3	21.9	8.7
UNK clinical status	36	14	18	17	18	9	9

As expected, the majority (72,0 %) of the STEC strains isolated from HUS cases were at high risk for developing HUS (Table 7).

Table 7: Risk classification for the development of HUS among STEC isolates of HUS cases (2017-2023)

Risk for HUS development	2017	2018	2019	2020	2021	2022	2023
High	16[a]	9[a]	20	13[c,d]	17	27[e]	18
Medium	2	0	1	3	2	3	7
Low	3[a]	3[b]	0	2[c,d]	1	2[e]	0
TOTAL	21	12	21	18	19	32	25

[a]Co-infection with two STEC strains (low risk O145 *stx*1a and high risk O157 *stx*2a); [b]Co-infection with two STEC strains (low risk O103 *stx*1a and high risk O111 *stx*1a *stx*2a); [c]Co-infection with two STEC strains (low risk O103 *stx*1a and high risk O103 *stx*1a *stx*2a); [d]Co-infection with two STEC strains (low risk O98 *stx*1a and high risk O157 *stx*2a); [e]Co-infection with two STEC strains (low risk O26 *stx*1a and high risk O145 *stx*2a).

Table 8 gives an overview of the STEC serotypes detected in Belgian patients suffering from HUS from 2017 until 2023. Every year the majority of HUS cases was associated with STEC O157, except in 2021 and 2023. At that time, the majority of the HUS cases was associated with O26:H11 (2021: n=12 out of the 19, 63.2%; 2023: n=8 out of the 25, 32.0%).

In 2023, 25 STEC strains were isolated from the faecal samples of 25 HUS cases: 8 STEC O26:H11, 2 O45:H2, 3 O145:H28, 2 STEC O157:H7/H-, 4 O177:H25 and 6 other different serotypes. The Stx subtype profiles found in these 25 strains were stx1a stx2a (2), stx2a (15), stx2a stx2d (1), stx2b (1), stx2b stx2c (1), stx2c (4) and stx2e (1). The subtype profile Stx2a alone was detected in four serotypes including O26:H11 (n=8), O45:H2 (n=2), O103:H25 (n=1), O145:H28 (n=3) and O157:H7 (n=1).

[20] Clinical laboratories are systematically sending samples from all HUS cases to the NRC over the years in order to exclude STEC associated HUS. This is of importance for the reimbursement of eculizumab (Soliris®) for patients with non-STEC related HUS.

Additionally, STEC infection could be confirmed in one HUS patient by detection of antibodies against *E. coli* O LPS (O26 and O145) in the serum sample only and in three HUS patients by detection of *stx*2 alone in stool samples without isolation of the STEC strain.

Table 8: Number of HUS-associated STEC serotypes (2017-2023)

Characteristic	2017	2018	2019	2020	2021	2022	2023
O2/O50:H6[a]						1	0
O26:Hunk	3	0	0	0	0	0	0
O26:H11		2	4	4	12	8	8
O26:H11 + O145:H28[b]						1	0
O26:H11 + O111 serology[c]						1	0
O26 serology[d]	1	0	0	0	0	0	0
O26 and O145 serology[d]							1
O45:H2			1	0	0	1	2
O55:H7	1	0	0	0	0	0	0
O55:H12		1	0	0	0	0	0
O74:H42			1	0	0	0	0
O80:H2	0	0	2	0	0	1	0
O80:H2 + O26 serology[c]			1	0	0	0	0
O93:H28							1
O100:H30							1
O103:Hunk	1	3	0	0	0	0	0
O103:H2				1[g]	0	1	0
O103:H25							1
O103:Hunk + O111:H-[b]		1	0	0	0	0	0
O103 serology[d]					1	0	0
O104:H4[e]	0	1	0	0	1	0	0
O111:H8						1	0
O111 serology[d]	0	0	1	0	0	0	0
O113:H4				1	0	1[h]	0
O145:H-	1	0	0	0	0	0	0
O145:H28			1	5	1	3	3
O145 serology[d]			1	1	0	1	0
O146:H28							1
O150:H2			1	0	0	0	0
O156:H25	1	0	0	0	0	0	1
O157:H7	12	3	9	4	3	11	2
O157:H7 + O98:H21[a]				1	0	0	0
O157:H7 + O145:H-[a]	1	0	0	0	0	0	0
O157 serology[c]	2	3	1	0	1	0	0
O171:H2							1
O177:H25			1	0	1	1	4
OgN9:H2					1	0	0
Stx positive[f]	4	5	2	2	6	9	3
TOTAL	27	19	26	19	27	41	29

[a]O2/O50 are genetically nearly identical *wzx*/*wzy*-genes and *wzm*/*wzt*-genes; [b]Co-infection with two STEC strains; [c]In these HUS patients STEC could be isolated from stool but the serogroup does not correspond to the *E. coli* O LPS detected in the serum sample; [d]In these HUS patients *E. coli* O LPS was detected with antibodies in the serum sample, but no STEC could be isolated from stool; [e]EAEC-STEC; [f]The sample was found positive for *stx* genes, but it was not possible to isolate a STEC strain. *Stx* PCR on non-cultivable faecal samples from HUS patients was routinely introduced in 2017; [g]Two STEC O103 (*stx*1a and *stx*1a *stx*2a; cgMLST 150264) were isolated from the stool of a same patient; [h]Vanesse et al. (2023) reported a severe clinical case of HUS with neurological involvement associated with STEC O113:H4 (Vanesse et al. 2023).

MOLECULAR CLUSTERS

Twenty-six molecular clusters of two to seven cases were identified by cgMLST analysis in 2023 (Table 9, Figure 7). Six out of these were familial: HC5_177415 (3), HC5_197305 (3), HC5_204886 (2), HC5_249005 (4), HC5_259824 (2) and HC5_270508 (2) (Table 10).

Table 9: Number of molecular clusters of STEC by serogroup (2019-2023)

Serogroup	2019			2020			2021			2022			2023		
	Total #	Isolate #	Range	Total #	Isolate #	Range	Total #	Isolate #	Range	Total #	Isolate #	Range	Total #	Isolate #	Range
O26	1	4	4	1	2	2	2	12	5-7	4	8	2	5	10	2
O157	8	26	2-10	4	13	2-4	4	25	2-17	12	36	2-6	7	25	2-6
Other	2	6	3	2	9	4-5	1	2	2	5	12	2-3	14	43	2-7

Certain of these clusters are briefly presented below.

STEC O26 – April 2023, West Flanders and France

Two cases of STEC **O26:H11 *stx*1a *eae*-positive** could be related to a multi-country outbreak based on cgMLST analysis (cgMLST **HC5|185264**) (2023-FWD-00029). France reported five confirmed cases in April 2023. Fermented raw milk was identified as vehicle of infection by the French health inspectors. The milk was imported from Belgium (RASFF notification 2023.2928). Based on this information, the cases were questioned again by the health inspectors. The adult case confirmed consumption of fermented raw milk, which was not the case for the paediatric patient.

STEC O63 – 2023, Belgium, several clusters

Six molecular clusters of STEC **O63:H6 *stx*2f *eae*-positive** were identified by the NRC in 2023: HC5|257986 (3 children; August-October), HC5|257987 (1 child and 1 adult; August), HC5|257989 (1 child and 1 adult; July-October), HC5|257991 (1 child and 2 adults; July - August), HC5|258004 (2 children and 3 adults; August-November) and HC5|258007 (2 children; August - September). None of the cases developed HUS. Despite the efforts of the health inspection authorities, no epidemiological link could be found between the cases within each molecular cluster.

STEC O146 – July–November 2023, Belgium, Denmark and Sweden

Seven cases of STEC **O146:H28 *stx*2b *eae*-negative** were reported to the NRC between July and December 2023: 3 children (age range: 2 to 16 years old) and 4 adults (27 to 68 years old). The cases were originating from Antwerp, Flemish Brabant, East and West Flanders. One adult case developed HUS. No epidemiological link could be found between the cases. Yet, based on cgMLST analysis, all strains showed high similarity and belonged to cgMLST **HC5|250262**. Inquiry 2023-FWD-00080 was created in EpiPulse. Two countries, Denmark (2) and Sweden (16), responded positively to the inquiry. Yet, the source of contamination of this multi-country cluster could not be identified.

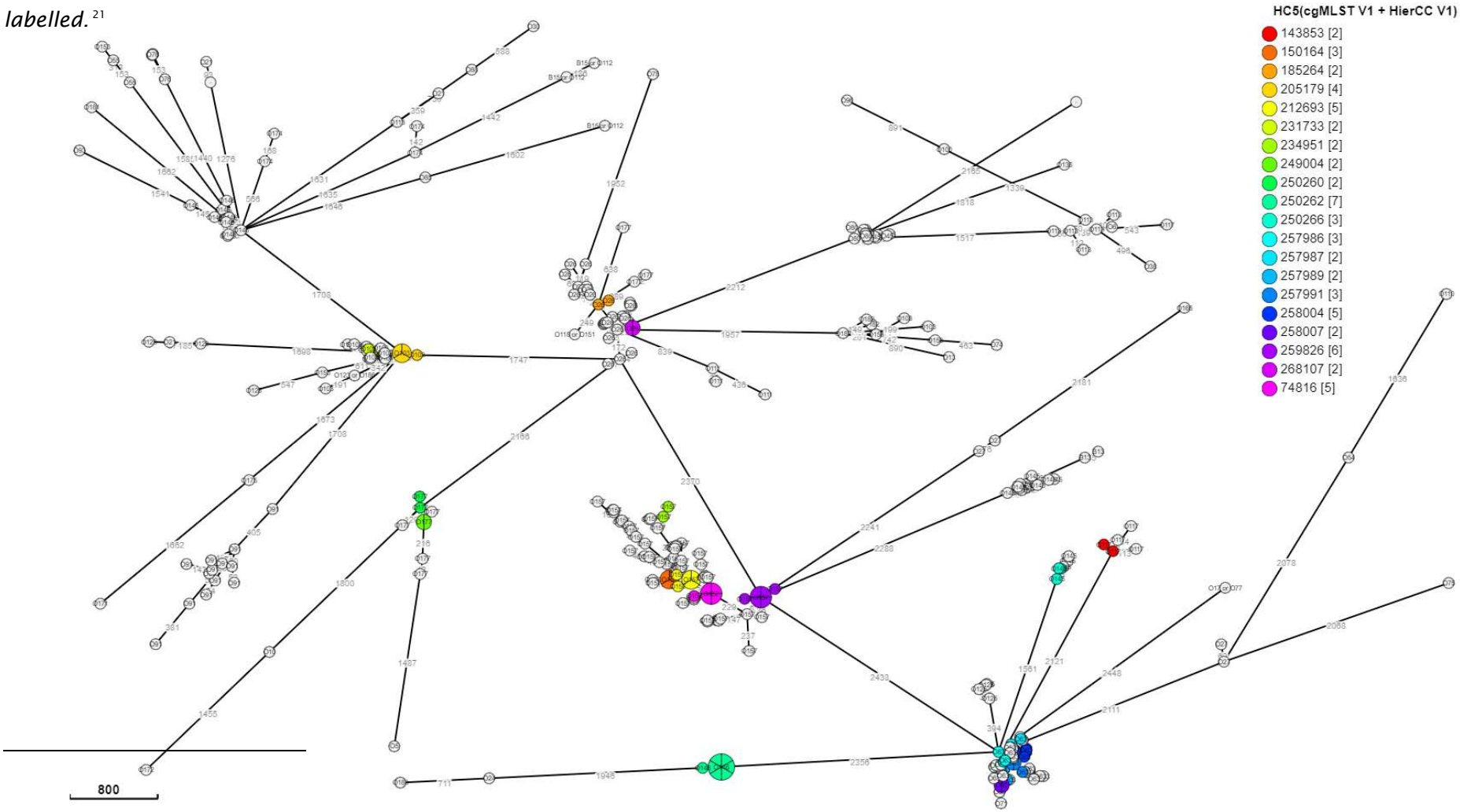
STEC O157 – September 2023, East Flanders and Liège

Six cases of STEC **O157:H7 *stx*1a *stx*2a *eae*-positive** were reported to the NRC in September 2023. One seven-year-old case developed HUS while the other cases had bloody diarrhoea. All strains had identical IS629-profile. All strains showed high similarity based on cgMLST analysis and belonged to cgMLST **HC5|259826** (EpiPulse inquiry 2023-FWD-00078). No epidemiological link was reported between the cases.

STEC O157 – October 2023, East Flanders

Four adult cases of STEC **O157:H7 *stx*1a *stx*2a *stx*2c *eae*-positive** were reported to the NRC in October 2023. Three cases had bloody diarrhoea and one case had gastrointestinal complaints other than diarrhoea. For two cases, consumption of meat was identified as probable source of contamination. All four isolate genomes had identical cgMLST profiles and belonged to cgMLST **HC5|74816**. Interestingly, a molecular cluster of 17 isolates belonging to the same HC5-cluster was already identified end 2021 by the NRC (EpiPulse inquiry 2021-FWD-00081). No source of contamination could be identified at that time.

Figure 7: Minimum spanning tree of cgMLST data from 319 Belgian STEC genomes analysed in 2023. *Clusters of at least two STEC cases are highlighted based on Hierarchical Clustering of cgMLST data. The analysis was carried out in EnteroBase, using the 'Create MLST GrapeTree' tool and selecting the Escherichia/Shigella cgMLST v1 + HierCC v1 scheme and the MSTree V2 algorithm. Nodes are colour-coded by HC5 cluster as labelled.* [21]



[21] Only one isolate genome has been included per familial cluster: HC5_177415 (3), HC5_197305 (3), HC5_204886 (2), HC5_249005 (4), HC5_259824 (2) and HC5_270508 (2); only one isolate genome has been included per patient.

Table 10: Results obtained after traditional typing and Hierarchical Clustering of cgMLST (HierCC) of 319 STEC genomes analysed in 2023[22]

Serotype	#HUS cases/ # cases	HierCC HC5	IS629-type	Stx subtype profile	Epidemiological link	Suspected source	Region
O157:H7[23]	0/4	74816[24]	AW/BZ	stx1a stx2a stx2c	No link	Meat (2/4)	East Flanders
O117:H7	0/2	143853		stx1a	No link	Not identified	Antwerp, East Flanders
O157:H7[20]	0/3	150164	AW	stx1a stx2c	No link	Not identified	West Flanders
O157:H7	0/3	177415	BU	stx1a stx2a	Familial link	Not identified	East Flanders
O26:H11	0/2	185264		stx1a	No link (2023-FWD-00029)	Fermented raw milk (1/2)	West Flanders
O157:H7	1/3	197305	B2	stx2a	Familial link	Not identified	Liège
O26:H11	0/2	204886		stx2a	Familial link	Not identified	East Flanders
O103:H2	0/4	205179		stx1a stx2a (3), stx1a (1)	No link	Not identified	Antwerp, East Flanders
O157:H7[20]	0/4	212693[25]	BZ	stx1a stx2c	No link	Not identified	Antwerp, West Flanders
O103:H2	0/2	231733[26]		stx1a	No link	Not identified	East Flanders, West Flanders
O157:H7[20]	0/2	234951	BK	stx2c	No link	Not identified	Antwerp
O177:H25	1/2	249004		stx2c	No link	Not identified	Antwerp, West Flanders
O145:H28	0/4	249005		stx2a	Familial link	Not identified	West Flanders
O177:H25	1/2	250260		stx2c	No link	Not identified	Antwerp, West Flanders
O146:H28	1/7	250262		stx2b	No link (2023-FWD-00080)	Not identified	Antwerp, Flemish Brabant, East and West Flanders
O145:H34	0/3	250266		stx2f	No link	Not identified	Brussels Capital Region, Liège, West Flanders
O63:H6	0/3	257986		stx2f	No link	Not identified	Brussels Capital Region, East Flanders, Flemish Brabant
O63:H6	0/2	257987		stx2f	No link	Not identified	Flemish Brabant, Liège
O63:H6	0/2	257989		stx2f	No link	Not identified	East Flanders
O63:H6	0/3	257991		stx2f	No link	Not identified	East Flanders

[22] Only one isolate genome has been included per familial cluster: HC5_177415 (3), HC5_197305 (3), HC5_204886 (2), HC5_249005 (4), HC5_259824 (2) and HC5_270508 (2).
[23] WGS-based O:H-type is O157:H7. Yet, these strains are non-motile.
[24] One additional isolate genome (January 2023), with 9 AD compared to the representative cluster isolate (October 2023), belonged to HC5_74816.
[25] One additional isolate genome (October 2023), with 5 AD compared to the representative cluster isolate (June 2023), belonged to HC5_212693.
[26] HC5_231733 includes one isolate from January and one from December 2023.

Serotype	#HUS cases/ # cases	HierCC HC5	IS629-type	Stx subtype profile	Epidemiological link	Suspected source	Region
O63:H6	0/5	258004		stx2f	No link	Not identified	West Flanders
O63:H6	0/2	258007		stx2f	No link	Not identified	Liège
O26:H11	0/2	259824		stx2a	Familial link	Not identified	Luxembourg
O157:H7	1/6	259826	BF	stx1a stx2a	No link (2023-FWD-00078)	Not identified	East Flanders, Liège
O26:H11	1/2	268107		stx2a	No link	Not identified	Flemish Brabant
O26:H11	1/2	270508		stx2a	Familial link	Not identified	East Flanders

HierCC HC5: hierarchical cluster level HC5, which includes all strains with links no more than 5 alleles apart – other than missing data; HUS: hemolytic uremic syndrome.

CONCLUSION

A significant increase in number of STEC strains, non-O157 in particular, was observed in 2023 compared to the other years. Yet, this increase was already observed in 2022 as a probable consequence of the implementation of gastrointestinal molecular panels by a number of clinical laboratories.

Serogroup O157 was the most frequently isolated STEC serogroup (73/332; 22.0%). Surprisingly, serogroup O63 was the second most common (43/332; 13.0%) serogroup in 2023. All non-O157 serogroups of the 'top 5' most common serogroups in the EU/EEA were represented: 34 O26 serogroup, 13 O91 serogroup, 20 O103 serogroup, 21 O145 serogroup and 18 O146 serogroup.

After an increase in total number of HUS cases observed in 2022, this number was comparable with those of the previous years in 2023.

Though most of the infections were sporadic, multiple small molecular clusters of STEC, mainly with serogroup O63 and O157, have been observed.

ACKNOWLEDGEMENTS

We would like to thank all clinical laboratories for voluntarily sending faecal samples of patients with symptoms of STEC infections to the Belgian NRC STEC. Also, we would like to thank the health inspectors for their great collaboration and communication with us over the past year. We would also like to acknowledge Dr Lang and Dr Fruth of the Robert Koch Institute for their support in O-genotyping.

REFERENCES

Bai, X. N., F. Scheutz, H. M. Dahlgren, I. Hedenstrom, and C. Jernberg. 2021. 'Characterization of Clinical Escherichia coli Strains Producing a Novel Shiga Toxin 2 Subtype in Sweden and Denmark', *Microorganisms*, 9.

Bai, X., J. Zhang, A. Ambikan, C. Jernberg, R. Ehricht, F. Scheutz, Y. Xiong, and A. Matussek. 2019. 'Molecular Characterization and Comparative Genomics of Clinical Hybrid Shiga Toxin-Producing and Enterotoxigenic Escherichia coli (STEC/ETEC) Strains in Sweden', *Sci Rep*, 9: 5619.

Brandal, L. T., A. L. Wester, H. Lange, I. Lobersli, B. A. Lindstedt, L. Vold, and G. Kapperud. 2015. 'Shiga toxin-producing escherichia coli infections in Norway, 1992-2012: characterization of isolates and identification of risk factors for haemolytic uremic syndrome', *BMC Infect Dis*, 15: 324.

Buchholz, U., H. Bernard, D. Werber, M. M. Bohmer, C. Remschmidt, H. Wilking, Y. Delere, M. an der Heiden, C. Adlhoch, J. Dreesman, J. Ehlers, S. Ethelberg, M. Faber, C. Frank, G. Fricke, M. Greiner, M. Hohle, S. Ivarsson, U. Jark, M. Kirchner, J. Koch, G. Krause, P. Luber, B. Rosner, K. Stark, and M. Kuhne. 2011. 'German outbreak of Escherichia coli O104:H4 associated with sprouts', *N Engl J Med*, 365: 1763-70.

Cointe, A., E. Bizot, S. Delannoy, P. Fach, P. Bidet, A. Birgy, F. X. Weill, S. Lefevre, P. Mariani-Kurkdjian, and S. Bonacorsi. 2021. 'Emergence of New ST301 Shiga Toxin-Producing Escherichia coli Clones Harboring Extra-Intestinal Virulence Traits in Europe', *Toxins (Basel)*, 13.

De Rauw, K., R. Buyl, S. Jacquinet, and D. Pierard. 2018. 'Risk determinants for the development of typical haemolytic uremic syndrome in Belgium and proposition of a new virulence typing algorithm for Shiga toxin-producing Escherichia coli', *Epidemiol Infect*: 1-5.

De Rauw, K., D. Thiry, B. Caljon, M. Saulmont, J. Mainil, and D. Pierard. 2019. 'Characteristics of Shiga toxin producing- and enteropathogenic Escherichia coli of the emerging serotype O80:H2 isolated from humans and diarrhoeic calves in Belgium', *Clin Microbiol Infect*, 25: 111 e5-11 e8.

Gati, N. S., B. Middendorf-Bauchart, S. Bletz, U. Dobrindt, and A. Mellmann. 2019. 'Origin and Evolution of Hybrid Shiga Toxin-Producing and Uropathogenic Escherichia coli Strains of Sequence Type 141', *J Clin Microbiol*, 58.

Gilmour, M. W., A. B. Olson, A. K. Andrysiak, L. K. Ng, and L. Chui. 2007. 'Sequence-based typing of genetic targets encoded outside of the O-antigen gene cluster is indicative of Shiga toxin-producing Escherichia coli serogroup lineages', *J Med Microbiol*, 56: 620-28.

Hughes, A. C., Y. Zhang, X. Bai, Y. Xiong, Y. Wang, X. Yang, Q. Xu, and X. He. 2019. 'Structural and Functional Characterization of Stx2k, a New Subtype of Shiga Toxin 2', *Microorganisms*, 8.

Ingelbeen, B., M. Bruyand, P. Mariani-Kurkjian, S. Le Hello, K. Danis, C. Sommen, S. Bonacorsi, and H. de Valk. 2018. 'Emerging Shiga-toxin-producing Escherichia coli serogroup O80 associated hemolytic and uremic syndrome in France, 2013-2016: Differences with other serogroups', *PLoS One*, 13: e0207492.

Kintz, E., J. Brainard, L. Hooper, and P. Hunter. 2017. 'Transmission pathways for sporadic Shiga-toxin producing E. coli infections: A systematic review and meta-analysis', *International Journal of Hygiene and Environmental Health*, 220: 57-67.

Launders, N., L. Byrne, C. Jenkins, K. Harker, A. Charlett, and G. K. Adak. 2016. 'Disease severity of Shiga toxin-producing E. coli O157 and factors influencing the development of typical haemolytic uraemic syndrome: a retrospective cohort study, 2009-2012', *BMJ Open*, 6: e009933.

Nuesch-Inderbinen, M., N. Cernela, D. Wuthrich, A. Egli, and R. Stephan. 2018. 'Genetic characterization of Shiga toxin producing Escherichia coli belonging to the emerging hybrid pathotype O80:H2 isolated from humans 2010-2017 in Switzerland', *International Journal of Medical Microbiology*, 308: 534-38.

Orskov, F., and I. Orskov. 1984. 'Serotyping of Escherichia-Coli', *Methods in Microbiology*, 14: 43-112.

Rodwell, E. V., B. Vishram, R. Smith, L. Browning, A. Smith-Palmer, L. Allison, A. Holmes, G. Godbole, N. McCarthy, T. J. Dallman, and C. Jenkins. 2021. 'Epidemiology and genomic

analysis of Shiga toxin-producing Escherichia coli clonal complex 165 in the UK', *J Med Microbiol*, 70.

Scheutz, F. 2014. 'Taxonomy Meets Public Health: The Case of Shiga Toxin-Producing Escherichia coli', *Microbiol Spectr*, 2.

Scheutz, F., L. D. Teel, L. Beutin, D. Pierard, G. Buvens, H. Karch, A. Mellmann, A. Caprioli, R. Tozzoli, S. Morabito, N. A. Strockbine, A. R. Melton-Celsa, M. Sanchez, S. Persson, and A. D. O'Brien. 2012. 'Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature', *J Clin Microbiol*, 50: 2951-63.

van Hoek, Aham, J. N. J. van Veldhuizen, I. Friesema, C. Coipan, J. W. A. Rossen, I. L. Bergval, and E. Franz. 2019. 'Comparative genomics reveals a lack of evidence for pigeons as a main source of stx(2f)-carrying Escherichia coli causing disease in humans and the common existence of hybrid Shiga toxin-producing and enteropathogenic E. coli pathotypes', *BMC Genomics*, 20: 271.

Vanesse, P., H. Georgery, T. Duprez, L. Gerard, C. Collienne, A. Verroken, F. Crombe, J. Morelle, and P. Hantson. 2023. 'Severe Neurological Involvement in an Adult with Shiga Toxin-Producing Escherichia coli-Hemolytic Uremic Syndrome Treated with Eculizumab', *Case Rep Nephrol Dial*, 13: 7-14.

Werber, D., and F. Scheutz. 2019. 'The importance of integrating genetic strain information for managing cases of Shiga toxin-producing E. coli infection', *Epidemiol Infect*, 147: e264.

Yang, X., X. N. Bai, J. Zhang, H. Sun, S. S. Fu, R. Y. Fan, X. H. He, F. Scheutz, A. Matussek, and Y. W. Xiong. 2020. 'Escherichia coli strains producing a novel Shiga toxin 2 subtype circulate in China', *International Journal of Medical Microbiology*, 310.

Zhou, Z., N. F. Alikhan, K. Mohamed, Y. Fan, Group Agama Study, and M. Achtman. 2020. 'The EnteroBase user's guide, with case studies on Salmonella transmissions, Yersinia pestis phylogeny, and Escherichia core genomic diversity', *Genome Res*, 30: 138-52.