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

ANNUAL REPORT 2019

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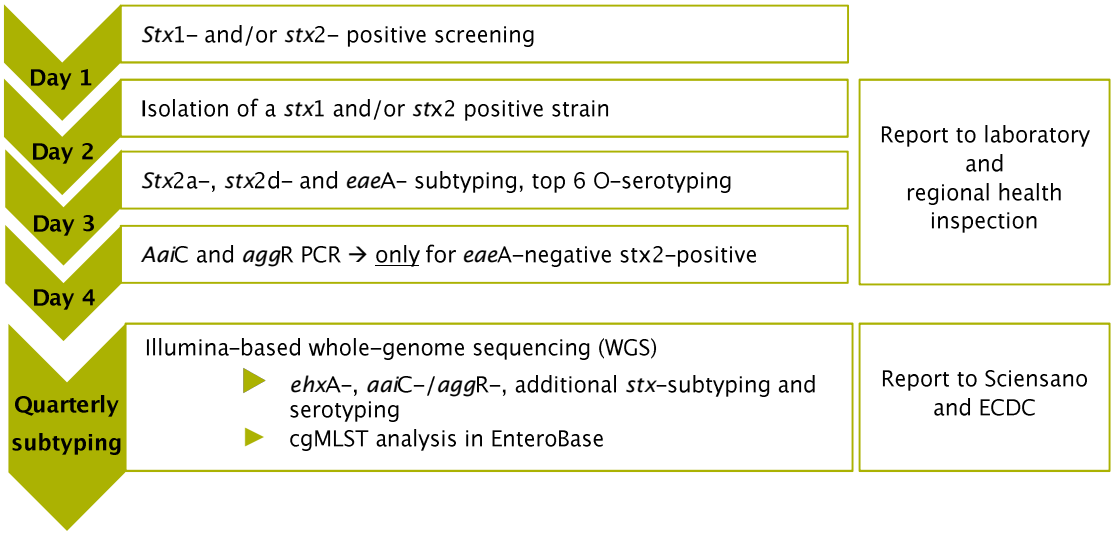
IMPLEMENTATION OF WHOLE-GENOME SEQUENCING FOR CHARACTERIZATION OF STEC AT THE NRC STEC/VTEC

To date traditional typing methods were used at the Belgian National Reference Centre (NRC) for virulence characterisation (Siga toxin [*stx*] subtype, *eae*A, *ehx*A, *aai*C and *agg*R) of Shiga toxin-producing *Escherichia coli* (STEC), which is needed in the context of molecular surveillance. Yet, some reference laboratories are using whole-genome sequencing (WGS) to that end (Dallman et al. 2015; Lindsey et al. 2016). The data obtained by WGS enables full characterisation of STEC isolates, including O:H type, virulence genes and core-genome multilocus sequence typing (cgMLST), which facilitates epidemiologic surveillance and enables data comparison in case of cross-border outbreak. Consequently, WGS was implemented at the NRC in 2019. The method will be performed, from now on, on all STEC strains provided to the NRC, replacing the traditional quarterly subtyping methods (Figure 1). In addition, the sequencing data will be analysed using the *Escherichia/Shigella* cgMLST 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.

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

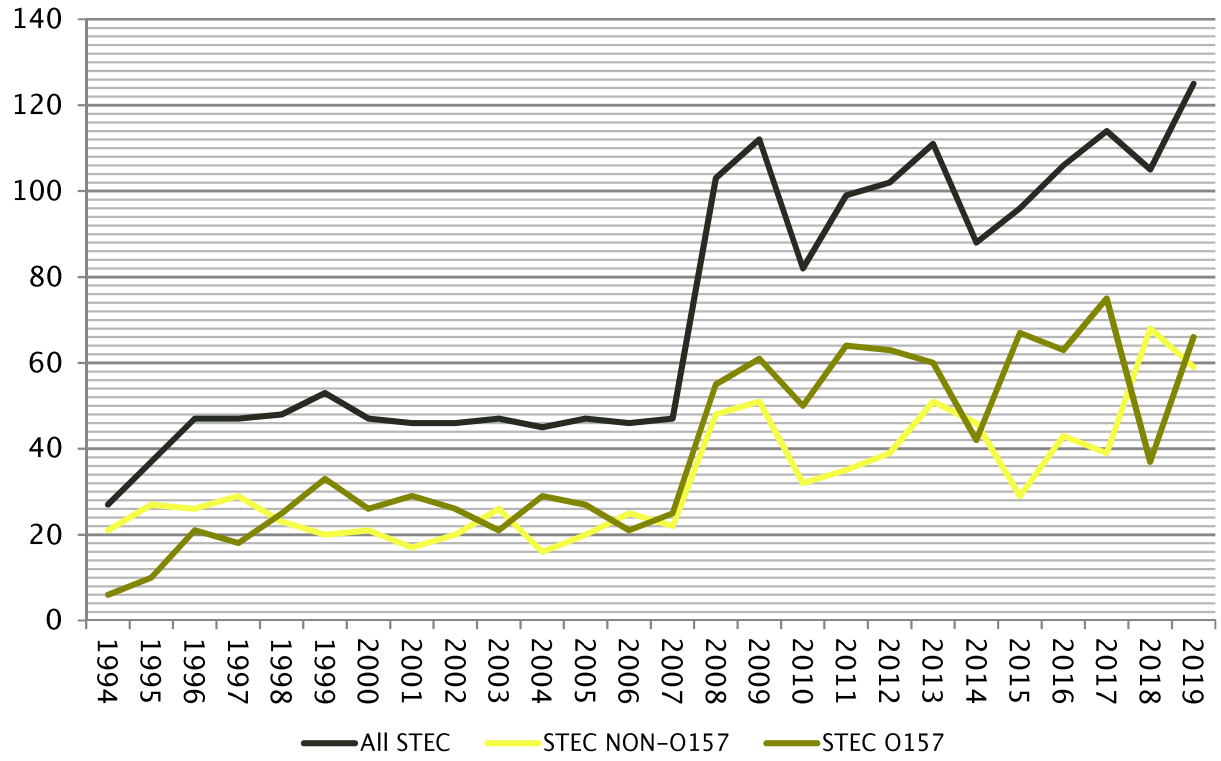


NUMBER OF STEC STRAINS ANNUALLY ISOLATED

Figure 2 gives an overview of the number of STEC strains annually identified at the NRC STEC/VTEC. From 1994 until 2007 an average of 45 (min. 27 (1994) – max. 53 (1999)) strains were identified each year. Since 2008 the yearly number of isolates increased more than twice, to a mean of 100 (min. 82 (2010) – max. 125 (2019)). We believe this increase in STEC isolates since 2008 can be partly explained by external factors. From 2008 until 2010 a research project was performed to study the incidence of STEC infections in the Brussels-Capital Region (Buvens *et al.* 2012). Furthermore in 2008 additional primers were added to our STEC screening PCR assay to target the Shiga toxin (Stx) subtype 2f, which could not be detected before. In 2011 the Belgian National Reference Centers for human microbiology were officially established, leading to more samples for diagnosis being send to us by external laboratories.

In 2019, 125 different STEC strains were isolated from 124 Belgian patients. One hundred and five of them were typical enterohemorrhagic *E. coli* (EHEC) (*eaeA*+, *hlyA*+). Twenty isolates were atypical STEC; lacking one or both of these virulence determinants. None of the strains were found positive for enteroaggregative *E. coli* (EaggEC) virulence genes *aaiC* and *aggR*.

Figure 2: Number of STEC strains annually isolated at the NRC STEC/VTEC (1994–2019)



STEC SEROTYPES

STEC are classified based on their serotype ("O"-antigen:"H"-antigen). Most STEC infections are caused by STEC O157:H7, but non-O157 have also been associated with diarrheal disease worldwide. Figure 2 shows the annual distribution of O157 and non-O157 strains. In the United States the 6 most common non-O157 groups are O26, O45, O103, O111, O121 and O145 (Gould et al. 2009). However, in Belgium O45 is very rare and only one isolate with this serotype was isolated, in 2008. Fifty-four percent of the STEC strains typed at the Belgian NRC STEC between 1994 and 2019 were of the O157:H7:H- serotype (n=1020). The O-serogroup of 735 out of the 853 STEC non-O157 could be determined, classifying them in 82 different O-serogroups. The remaining 118 non-O157 strains could not be serotyped with the methods used until 2018 (NT non-O157) (Figure 3).

Figure 4 gives an overview of the evolution of the 6 most important serogroups, also called the "pathogenic gang of 6": O26 (n_{total}=161), O103 (n_{total}=80), O111 (n_{total}=56), O121 (n_{total}=10), O145 (n_{total}=58) and O157 (n_{total}=1020). Additionally 7 other non-O157 serogroups have been isolated frequently over the years: and O182 (n_{total}=16), O118 (n_{total}=17), O80 (n_{total}=19), O63 (n_{total}=22), O128 (n_{total}=23), O91 (n_{total}=29) and O146 (n_{total}=43). Interestingly, O118 was detected regularly in the early years of screening but has not been found from 2010-2016 and only once in 2017. Serogroups O182, O63 and O80 have only been isolated since 2003 and 2008, respectively. In 2008 primers for the detection of *stx2f* were added to the STEC PCR screening assay and all STEC O63 isolated at our NRC carry the *stx2f* gene, explaining why this serotype was not detected the years before. Ten O80 strains were isolated from 2018 to 2019 while only 9 O80 strains were detected at the NRC from 2008 to 2016 (De Rauw et al. 2019).

In 2019 the majority (66/125; 52.8%) of the STEC strains belonged to serogroup O157 (serotype O157:H7 or O157:H-). Three of the 'top 5' non-O157 serogroups were represented: 16 O26 strains, 6 O103 strains and 6 O145 isolates (Figure 4).

Figure 3: Occurrence of the STEC non-O157-serogroups (1994-2019)

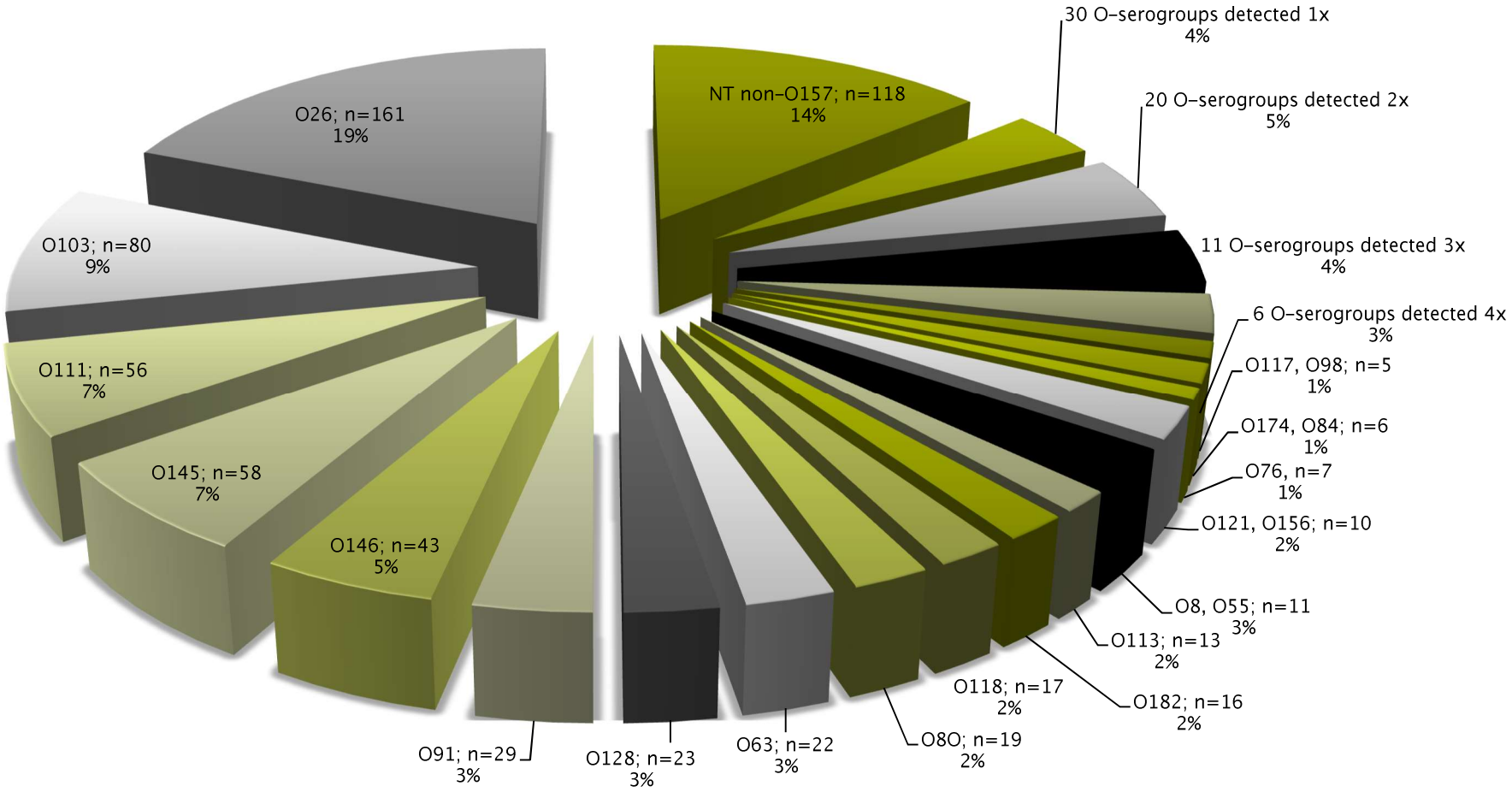
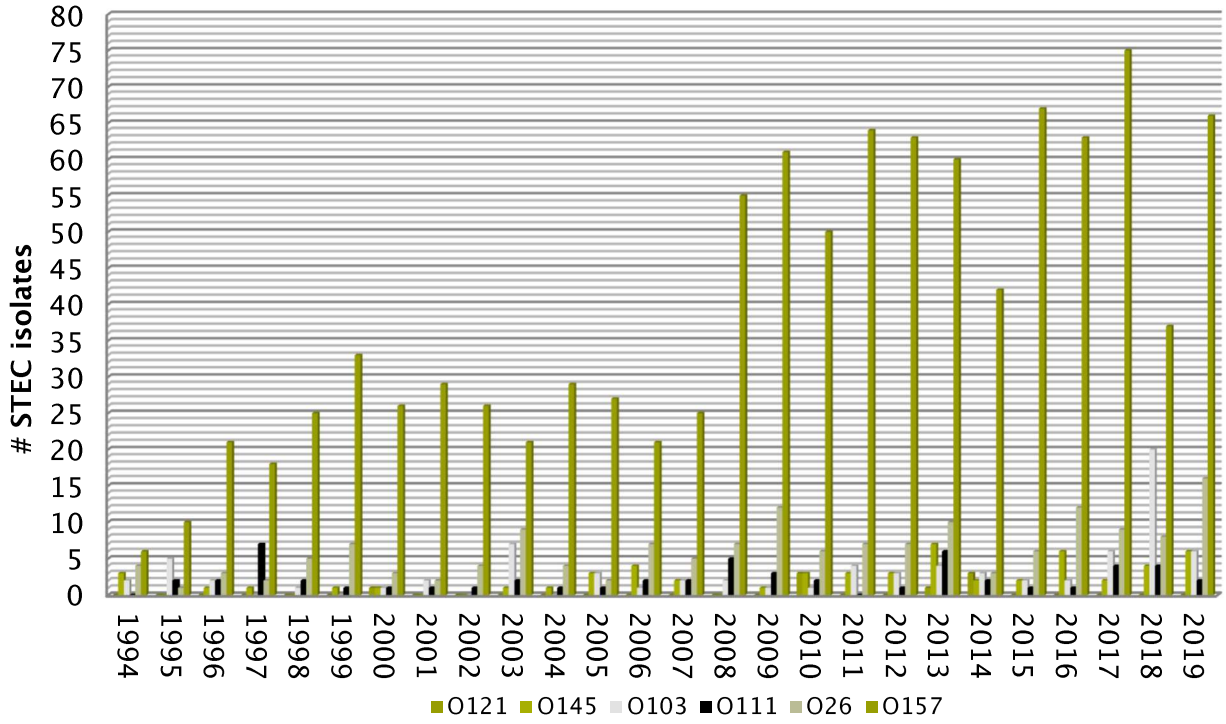


Figure 4: Annual distribution of the 'pathogenic gang of six' (1994–2019)



SHIGA TOXIN SUBTYPES

STEC's main pathogenic mechanism is production of Shiga toxins (Stx) also called verocytotoxins (Vtx), 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. Besides the Stx produced by *Shigella* species, 3 subtypes for Stx1 (Stx1a, stx1c and Stx1d) and 7 subtypes for Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g) have been described in STEC. 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 be easily lost or acquired by horizontal transfer (Scheutz 2014; Scheutz et al. 2012). Determination of the Stx variants is clinically relevant as some Stx2 subtypes -stx2a and stx2d- 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).

In 2019, 40.8 % of the isolates were *stx2* positive (51/125), 16.0 % (20/125) were *stx1* positive and 43.20 % were *stx1* and *stx2* positive (54/125). The predominating *stx* subtypes were: *stx1a* (17), *stx1a+stx2a* (24), *stx1a+stx2c* (26), *stx2a* (24), *stx2a+stx2c* (10) and *stx2c* (7) (Table 1).

Table 1: Shiga toxin subtypes (2016-2019)

stx subtype	2016	2017	2018	2019
stx1a	13	16	21	17
stx1c	6	1	2	3
stx1d	0	1	0	0
stx1a+stx2a	9	25	22	24
stx1a+stx2a+stx2c	1	0	4	0
stx1a+stx2b	3	4	2	1
stx1a+stx2c	17	19	12	26
stx1a+stx2d	1	0	1	1
stx1a+stx2? (gene lost)	1	0	0	0
stx1c+stx2b	0	3	2	2
stx2a	33	21	19	24
stx2a+stx2c	8	6	6	10
Stx2b	0	0	1	3
stx2b+stx2d	1	0	0	0
stx2c	7	16	2	7
stx2d	1	0	7	4
stx2f	5	2	4	3
TOTAL	106	114	105	125

HAEMOLYTIC UREMIC SYNDROME

Shiga toxin- or verocytotoxin-producing *E. coli* (STEC/VTEC) infections are the cause of sporadic and epidemic watery or bloody diarrhoea worldwide. In some individuals it may progress to more severe disease such as the life-threatening haemolytic uremic syndrome (HUS). Table 2 gives an overview of the STEC serotypes detected in Belgian patients suffering from HUS from 2011 until 2019. Every year the majority of HUS-cases were associated with STEC O157. In 2019, STEC strains were isolated from the faecal samples of 21 HUS cases: 9 STEC O157:H-/H7, 4 STEC O26:H11, 1 O145:H28 and 7 NON-O157. Remarkably, three paediatric STEC O80 HUS cases were reported, of which one died as a result of HUS. The *stx* subtypes found in these strains were *stx1a+stx2a* (4), *stx1a+stx2d* (1), *stx2a* (8), *stx2a+stx2c* (4), *stx2c* (1) and *stx2d* (3). Additionally, STEC infection could be confirmed in 3 HUS patients by detection of antibodies against STEC O lipopolysaccharide in their serum samples (O111, O145 and O157) and in 2 HUS patients by detection of *stx2* in their stool samples (Table 2).
It is to be noted that for 18 out of the 125 STEC positive patients, no information was available regarding the development of HUS.

Table 2: Number of HUS-associated STEC serotypes

Characteristic	2011	2012	2013	2014	2015	2016	2017	2018	2019
O157:H-/H7	10	9	6	4	15	9	13	3	9
O157 serology[a]	1	1	3	0	0	7	2	3	1
O26:Hunk	0	1	0	0	1	3	3	0	0
O26:H11								2	4
O26 serology[a]	0	1	2	0	0	2	1	0	0
O45:H2									1
O55:H7	0	1	0	0	0	0	0	0	0
O55:H12								1	0
O61:H-	0	0	1	0	0	0	0	0	0
O63:H6	0	1	0	0	0	0	0	0	0
O74:H42									1
O80:H2	0	0	0	0	1	1	0	0	3
O103:Hunk	0	0	1	0	0	0	1	4	0
O104:H4	0	0	1[e]	0	0	0	0	1[e]	0
O111:H-	0	0	1	0	0	1	0	1	0
O111 serology[a]	0	0	0	0	1[d]	0	0	0	1
O145:Hunk/H-	1	1	4	0	0	2	1	0	1
O145 serology[a]									1
O148:H8	0	1	0	0	0	0	0	0	0
O150:H2									1
O156:Hunk	0	0	0	0	0	0	1	0	0
O174:Hunk	0	0	0	0	0	1	0	0	0
O177:H25									1
O182:H25	0	0	1	0	0	0	0	0	0
Orough:H-[b]	1	0	0	0	0	0	0	0	0
NON-O157[b]	0	0	0	2	1	0	1	0	0
Stx positive[c]	0	1	0	0	0	0	4	5	2
TOTAL	13	17	20	6	18	26	27	20	26

[a] In these HUS patients STEC O LPS was detected with antibodies in the serum sample, but no STEC could be isolated from the stool; [b] Orough: auto-agglutinating; NON-O157: did not agglutinate with O157, O26, O103, O111, O121, O145 and was not typable with molecular gnd-typing; [c] 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.; [d] A STEC O157 was isolated from the stool from the same patient; [e] enteroaggregative-STEC.

OUTBREAKS

STEC infections in Belgium are mainly sporadic, but over the years a few outbreaks have occurred. The outbreaks since 2007 are shortly presented.

STEC O145/STEC O26 – 2007, ANTWERP

In 2007, an outbreak with both STEC O145 ánd STEC O26 occurred among consumers of ice cream produced at a farm in the province of Antwerp. Five children developed HUS and 7 persons contracted severe diarrhoea. In 3 HUS cases STEC O145 infections were laboratory confirmed, one in association with STEC O26 (De Schrijver et al. 2008).

AGG-STEC O104:H4 – 2011, GERMANY

Germany: from May to July 2011, a large outbreak of STEC infections with a serotype that was reported only very rarely before, O104:H4, was observed. This outbreak differed from previous STEC outbreaks by its sheer size, the large percentage of patients with HUS and the high proportion of adults, mainly women. This exceptionally virulent strain combined virulence factors of EAggEC and STEC (Frank et al. 2011). At the same time, a smaller outbreak of Agg-STEC O104:H4 took place in France. Comparison of the back tracing information from the French and German outbreaks led to the conclusion that fenugreek seeds imported from Egypt were the most likely source of infection although the outbreak strain could not be isolated from the seeds (EFSA report 2011). Even though several nationalities were hit by this outbreak no Belgian patients were involved in the outbreak itself, but two sporadic cases of stx2a positive Agg-STEC O104:H4 infection occurred in Belgium in 2012 and 2013 respectively. In both cases travel in a Mediterranean country preceded the infection (De Rauw et al. 2014).

STEC O157 – 2012, LIMBURG

In 2012 an outbreak of STEC O157:H7 in Limburg was successfully traced back to the consumption of raw meat ('filet américain'). The outbreak involved 24 cases, of which 17 were laboratory-confirmed. Five cases developed HUS and fifteen were hospitalized (Braeye et al. 2014).

STEC O157 – 2013, BELGIUM

From May until July 2013 14 stx2a positive typical EHEC O157:H7 strains with undistinguishable or closely related molecular typing profiles (PFGE and IS629-typing) were isolated at NRC STEC. The molecular fingerprints of the strains were identical to those of 5 STEC O157 isolated from meat at the National Reference Laboratory for Foodborne Outbreaks (NRL-FBO) in the same period. Two of the infected patients developed HUS, the others were affected with (bloody) diarrhoea. Eight patients originated from the province East-Flanders, the remaining six patients were from different parts of the country. Despite

the molecular relatedness between the STEC isolates, no clear epidemiological link was found between the patients or the consumption of the meat.

STEC O157 – 2015, Province of Luxembourg

Between the end of August and October 2015 a small outbreak of *stx2a* + *stx2c* positive typical EHEC O157 related to children's day-care took place in Arlon in the province of Luxembourg between August and October. Seven children were involved all between 4 years and 8 months of age, 2 of them suffered from HUS. Some of the patients kept shedding STEC in their feces for several weeks; and for one patient it even took more than 7 weeks to clear the isolate from the stool. Stools of the collaborators of the day-care were also screened for the presence of STEC, but none of them was infected with the outbreak strain. The source of the outbreak was not established.

On 18/9/2015 an urgent inquiry about the outbreak was made on the Epidemic Intelligence Information System for food- and waterborne diseases (EPIS-FWD), a web-based restricted-access communication platform released by the ECDC. But none of the neighbouring countries reported to have detected the outbreak strain.

STEC O157 – 2016, Belgium, several small clusters

In 2016 several small clusters of EHEC O157 infection were detected in Belgium:

May 2016, Flemish Brabant

In May 2016 an outbreak of gastroenteritis occurred in a nursery home in Averbode in the province of Flemish Brabant. The same *stx2a* positive typical STEC O157:H7 could be isolated from the faecal samples of six patients. The patients were between 79 and 91 years old. Two patients had uncomplicated diarrhoea, two patients suffered from bloody diarrhoea, and two patients developed HUS. No source was found.

July 2016, Namur

A two-year-old girl with HUS and her five-year old sister living in the province of Namur were infected with a *stx1a* and *stx2a* positive typical STEC O157:H7 strain. The girls were presumably infected by the consumption of cheese as an identical strain was found in this food item.

September 2016, Liège and Namur

In September 2016 a *stx2c* positive typical STEC O157:H7 was found in the stool samples of two brothers and their nephew living in the province of Liège and Namur, respectively. The boys were only 2 months, 20 months and 21 months of age. The youngest boy had uncomplicated diarrhoea, while the other two were asymptomatic. All had attended a family meeting at a carnival at the end of august. All family members had eaten at a stand selling

French fries and several adult members of the family also suffered from an episode of diarrhoea in the week following the gathering.

September – October 2016, Hainaut and Luxembourg

In October 2016 a three-year-old boy and his twenty-months-old sister who attended a children's day-care centre in Tournai in the province of Hainaut were infected with a *stx2a* positive typical STEC O157:H7. The boy had bloody diarrhoea and the girl uncomplicated diarrhoea. Other children attending the day-care as well as some of the personnel were screened for the presence of STEC, but no other cases were detected. Two weeks earlier the same strain was found in the faecal sample of a girl with diarrhoea in a children's day-care centre located in Marche-en-Famenne in the province of Luxembourg. This specific type is commonly seen in the Belgium STEC O157 isolates. During the screening of the day-care in Marche-of-Famenne a closely related, though not exactly the same, typical *stx2a* positive STEC O157:H7 was found in another asymptomatic girl. No source was found in any of the cases.

October 2016, Brussels-Capital Region

A stx2c positive typical STEC O157:H7 was isolated from an eight-month-old girl who suffered from uncomplicated diarrhoea. The girl attended a children's day-care centre in Anderlecht in the Brussels-Capital Region and stool samples from other children attending the day-care as well as care-takers were screened for the presence of STEC. An identical strain could be isolated from the sample of child-care workers, who was asymptomatic. No other cases were identified.

October 2016, Limburg

Two young patients, a girl and a boy, attending the same day-care centre in Diepenbeek in the province of Limburg were infected with the same *stx1a* and *stx2c* positive typical STEC O157:H- strain. The girl had uncomplicated diarrhoea, the symptoms of the boy were not communicated to the NRC. The parents, other children and personnel working at the day-care centre were screened, but no other STEC infections were identified. The parents were thoroughly questioned, but no source could be identified.

STEC O157 – 2017, LIMBURG

In April/May 2017 12 patients residing in Limburg got infected with the same STEC O157:H- stx1a+ stx2c+ eae+ strain. The same isolate was found in a leftover of 'filet américain' that was consumed by a patient. The relatedness of the human strain and the food isolate was shown by IS629 typing and PFGE. Several patients suffered from bloody diarrhoea, as far as we know none of them developed HUS. The ages of the patients ranged from 2 years to 83 years.

STEC O103 – 2018, Hainaut

A STEC outbreak occurred in June/July 2018 in a family child care home located in the province of Hainaut. Two boys, aged 11 and 18 months, developed HUS. The youngest boy died. The same strain was isolated from eight other children and one family member. The in-home care provider was questioned but no source could be identified. Also, food samples collected from the family child care home were screened and STEC could not be detected.

STEC O157 – 2019, Belgium, several small clusters

In 2019 several small clusters of STEC infection were detected in Belgium:

November 2018, Auvergne-Rhône-Alpes Region, France

One paediatric HUS case, diagnosed in February 2019, could be related to a small outbreak with STEC O26:H11 stx2a+ eae+ in France (EPIS inquiry UI-531). As the source of contamination of this outbreak was known from EPIS inquiry UI-531, trace back investigations performed by the health inspection authorities could confirm that the child consumed the incriminated cheese. The Belgian HUS case was shedding STEC for 8,5 months.

August 2019, Luxembourg

In August 2019, a five-year-old girl with HUS, living in Bastogne in the province of Luxembourg, was infected with a STEC O157:H7 stx1a+ stx2a+ eae+ strain. The same STEC strain was detected in the stool samples of her two sisters, aged one and three-year-old. No source of contamination was found.

August 2019, Antwerp

Two sisters living in Lier, in the province of Antwerp, developed HUS caused by a STEC O157:H7 stx2a+ stx2c+ eae+ strain. A minced-meat hot dog was identified as a probable source of contamination but the food sample could not be tested for confirmation.

November/December 2019, Brussels-Capital Region

A STEC outbreak occurred in November/December 2019 in a day-care center located in Schaarbeek in the Brussels-Capital Region. Two boys, aged 18 and 24 months, developed HUS. The O157:H7 stx2a+ eae+ strain was isolated from eight other children, among which five were asymptomatic. A snackbar, where one of the HUS-cases ate, has been identified as the probable source of contamination. Unfortunately, STEC could not be detected in the food samples collected from the snackbar.

CONCLUSIONS 2019

The number of STEC infections identified at the NRC in 2019 was the highest since 2017. Out of the 125 isolates, 53% belonged to the O157:H7/H- serotype. Though most of the infections were sporadic, one outbreak of STEC O157 in a day-care center was identified in the Brussels Capital Region, including two severe HUS cases.

WGS has been implemented at the NRC in 2019. This method will be performed on all STEC strains from now on, in addition to traditional typing methods.

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