Emergence of *Escherichia coli* encoding Shiga toxin 2f in human Shiga toxin-producing *E. coli* (STEC) infections in the Netherlands, January 2008 to December 2011

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The Shiga toxins of Shiga toxin-producing *Escherichia coli* (STEC) can be divided into Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2) with several sub-variants. Variant *Stx*$_{2f}$ is one of the latest described, but has been rarely associated with symptomatic human infections. In the enhanced STEC surveillance in the Netherlands, 198 STEC O157 cases and 351 STEC non-O157 cases, including 87 *stx*$_{2f}$ STEC isolates, were reported between 2008 and 2011. Most *stx*$_{2f}$ strains belonged to the serogroups O63:H6 (n=47, 54%), O113:H6 (n=12, 14%) and O125:H6 (n=12, 14%). Of the 87 *stx*$_{2f}$ isolates, 84 (97%) harboured the *E. coli* attaching and effacing (*eae*) gene, but not the enterohaemorrhagic *E. coli* haemolysin (*hly*) gene. *stx*$_{2f}$ STEC infections show milder symptoms and a less severe clinical course than STEC O157 infections. Almost all infections with *stx*$_{2f}$ (n=83, 95%) occurred between June and December, compared to 170/198 (86%) of STEC O157 and 173/264 (66%) of other STEC non-O157. *stx*$_{2f}$ STEC infections in the Netherlands are more common than anticipated, and form a distinct group within STEC with regard to virulence genes and the relatively mild disease.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important pathogen worldwide, associated with human illness, most notably diarrhoea, bloody diarrhoea, haemorrhagic colitis, and haemolytic uraemic syndrome (HUS) [1,2]. Ruminants, especially cattle, are considered the main reservoir for STEC, from where it spreads to humans by contaminated food and/or water. A broad range of virulence factors is associated with the severity of STEC infection [3]. Shiga toxin is an essential factor for the development of severe symptoms like HUS and can be divided into two main types: Shiga toxin 1 (Stx1) and Shiga toxin 2 (Stx2). Within both groups, several variants are distinguished. Variant *stx*$_{2f}$ is one of the latest described in the literature, found in *E. coli* strains from pigeons [4-7]. So far, reports of human illness due to *stx*$_{2f}$ STEC are scarce [5,8,9]. *Stx*$_{2f}$ genes were present in only one of 62 isolates of STEC non-O157 cases in the United Kingdom between 1983 and 2000 [10], but was not found in 530 isolates of STEC non-O157 cases in Germany in the period from 1996 to 2000 [11]. Prager et al. [12] presented data from 32 *stx*$_{2f}$ STEC cases identified between 2004 and 2007 in Germany, suggesting that this might be an emerging pathogen. In Japan, between 1996 and 2006, 24 cases with a rare STEC non-O157 serogroup infection were tested for *stx*$_{2f}$, yielding two cases [9]. Furthermore, two relatives of these cases were found to be asymptomatic *stx*$_{2f}$ STEC carriers. During a multi-centre study in the Netherlands (2005–2006), isolates of 21 STEC cases were tested for *stx*$_{2f}$ of which three (14%) tested positive, which, at that time, was already higher than expected based on the previous international reports [13].

In the Netherlands, STEC isolates are submitted to the National Institute for Public Health and the Environment (RIVM) for confirmation and further typing. Since 2007, submitted strains have been routinely tested for *stx*$_{2f}$. This lead to the observation that *stx*$_{2f}$ STEC was relatively common. The question was raised whether *stx*$_{2f}$ STEC cases had distinct clinical and epidemiological characteristics compared to other STEC cases.

Methods

Since January 1999, an enhanced surveillance of STEC O157 has been implemented in the Netherlands. STEC became notifiable in the same year, effectively being STEC O157. In 2007, STEC non-O157 has been added to the enhanced surveillance, which effectively started running in 2008. The notifications of STEC non-O157 do not cover the whole country, as only a fraction of the

laboratories use molecular methods for the detection of all STEC, although the number of laboratories capable of doing this is rising. All medical microbiological laboratories in the Netherlands have to report a positive result for STEC to the local public health service. In addition, they can voluntarily send up to five isolates per patient to the RIVM for confirmation, free of charge. Putative STEC colonies are tested by polymerase chain reaction (PCR) for the presence of the Shiga toxin 1 (stx_1), Shiga toxin 2 (stx_2), *E. coli* attaching and effacing (*eae*) and enterohaemorrhagic *E. coli* haemolysin (*hly*) genes using primers as described by Paton et al. [14]. The presence of stx_{2f} is tested with the PCR method as described by Schmidt et al. [4]. If *stx*-positive colonies are detected, O- and H-typing are performed [15,16].

The regional public health services gather information about age, sex, symptoms and date of illness onset of each case as part of the notification. In the enhanced surveillance, regional public health services are also asked to complete a more elaborate questionnaire together with the case about the clinical manifestation and possible risk factors, such as food consumption, and outdoor activities in the week before date of onset. Cases with a STEC infection and an isolate confirmed and typed at the RIVM between January 2008 and December 2011 were included in the current analysis. In this period, one national outbreak of STEC O157 (n=19 cases) and the German outbreak of STEC O104 (n=11 cases) were identified [17,18]. Cases linked to these outbreaks were excluded.

Since 2008, a control survey in the general population has been added in the Netherlands; three times a year, a questionnaire intended for all age groups is sent to a sample of the general population, containing similar questions as used for cases with notifiable gastroenteritis pathogens and respiratory infections about health and underlying diseases, food consumption, and outdoor activities. This survey is set up to determine risk factors for these diseases, including trends through the years; the survey can also be helpful in investigations of outbreaks caused by these pathogens and infections, especially when an outbreak is diffuse in space and/or time. Between July 2008 and December 2011, 3,908 control questionnaires were mailed and 1,420 were returned (overall response of 36.3%).

Stx_{2f} STEC was compared with other STEC, divided into STEC O157 and STEC non-O157, regarding O type and presence of other genes, age and symptoms of the cases, and risk factors. Differences were tested using the chi-squared test (with $p<0.05$ considered significant). A similar comparison was done between stx_{2f} STEC cases and controls concerning the risk factors, extended with a logistic regression analysis to calculate adjusted odds ratios.

Results

Between 2008 and 2011, a total of 549 STEC cases were reported for which the STEC could be isolated and typed, resulting in 198 O157 infections and 351 non-O157 infections (Table 1). The steady rise of STEC non-O157 infections over time is most likely due to the increasing number of laboratories using PCR-techniques to identify all STEC infections. A quarter (n=87) of the 351 STEC non-O157 isolates contained the stx_{2f} gene. None of the STEC O157 isolates contained the stx_{2f} variant. Of the 87 stx_{2f} isolates, 84 (97%) harboured the *eae* gene, but not the *hly* gene. The remaining three contained neither *eae* nor *hly*. For the other 264 STEC non-O157 isolates, this was six (2%) with *eae* but not *hly* and 83 (31%) with neither *eae* nor *hly*. All 198 STEC O157 isolates contained *hly* and all but one isolate *eae*.

Table 1

Shiga toxin genes (*stx*) in human STEC non-O157 (n=351) and O157 (n=198) isolates, the Netherlands, 2008–2011

Type	2008	2009	2010	2011	Total
STEC non-O157 total	45	51	81	174	351
Stx_1[a] n(%)	14 (31)	25 (49)	31 (38)	70 (40)	140 (40)
Stx_2[b] n(%)	16 (36)	11 (22)	16 (20)	41 (24)	84 (24)
Stx_1+stx_2 n(%)	5 (11)	4 (8)	12 (15)	19 (11)	40 (11)
Stx_{2f}[c] n(%)	10 (22)	11 (22)	22 (27)	44 (25)	87 (25)
STEC O157 total	45	38	50	65	198
Stx_1[a] n(%)	0 (0)	1 (3)	1 (2)	0 (0)	2 (1)
Stx_2[b] n(%)	18 (40)	17 (45)	17 (34)	19 (29)	71 (36)
Stx_1+stx_2 n(%)	27 (60)	20 (53)	32 (64)	46 (71)	125 (63)
Stx_{2f}[c] n(%)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

STEC: Shiga toxin-producing *Escherichia coli*.

[a] Stx_1 was found but not stx_2 in these isolates.

[b] Stx_2 was found but not stx_1 in these isolates.

[c] In these isolates stx_{2f} was found but not stx_1 or other stx_2.

Besides STEC O-non-typable, 65 different STEC non-O157 O-types were found between 2008 and 2011, of which 11 O-types were also seen with the stx_{2f} gene (Table 2). Especially O63:H6 was related to stx_{2f}, followed by O113:H6 and O125:H6.

Ninety five per cent of the stx_{2f} STEC infections (83/87) occurred between June and December (Figure). STEC O157 infections also show a seasonal trend, although somewhat less pronounced, with 170/198 (86%) between June and December. No clear seasonal trend is seen in the other STEC non-O157 infections (June–December: 173/264; 66%).

The median age of cases with a stx_{2f} STEC infection was 31 years, compared to 28 years for cases with another STEC non-O157 infection and 21 years for cases with an STEC O157 infection (Table 3). The differences in median age and age distribution were not statistically significant, although O157 cases tended to be younger. Cases with an stx_{2f} STEC infection had significantly less frequently blood in the stool than STEC O157 cases ($p<0.0001$) and a smaller proportion had problems with low production of urine during the infection than for cases of STEC O157 ($p<0.0001$) and non-O157 ($p=0.03$) (Table 3). Furthermore, stx_{2f} STEC cases had less frequently stomach ache ($p<0.0001$), were reported with no HUS ($p=0.049$) and were less frequently hospitalised ($p=0.001$) compared to STEC O157 cases. The percentages of stx_{2f} STEC cases with such characteristics were however similar to those in the other non-O157 STEC cases. None of the stx_{2f} STEC cases died due to the infection, compared to one of 224 other STEC non-O157 cases (0.4%) and two of 186 STEC O157 cases (1%) for which the information was known.

Cases with a stx_{2f} STEC infection less frequently reported having had contact with someone with gastrointestinal complaints in the week before illness onset than the other STEC non-O157 ($p=0.030$) and STEC O157 cases ($p=0.046$; Table 4). On the other hand, the stx_{2f} STEC cases more frequently reported having eaten dairy products made of raw milk ($p=0.010$) or bean sprouts ($p=0.018$) than STEC O157 cases. The difference in consumption of bean sprouts between stx_{2f} cases and other STEC non-O157 cases was close to significance (0.055). Bean sprouts were also eaten more often by stx_{2f} cases compared to the controls; the odds ratio for consumption of bean sprouts was 2.3 (95% confidence interval: 1.1–5.1), adjusted for age, sex and urbanisation level. No specific questions about contact with birds were included in the questionnaire, except for owning poultry, but it could be reported in open questions addressing contact with animals. None of the 31 stx_{2f} STEC cases reported contact with birds, compared to six (6%) and nine (7%) of the other 98 STEC non-O157 and the 135 STEC O157 cases for which the information was known, respectively.

Table 2

STEC serotypes (n=11) found to contain the Shiga toxin 2f gene (stx_{2f}), the Netherlands, 2008–2011

Serotype	With stx_{2f}/O-type	Per cent of stx_{2f} (n=87)
O2	2/4	2
O2:H-	1/1	1
O2:H6	1/2	1
O2:H29	0/1	0
O16:H5	1/1	1
O35:H19	1/1	1
O63:H6	47/47	54
O73:H18	1/2	1
O96:H7	1/1	1
O101:H-	1/3	1
O113	15/30	17
O113:H-	2/3	2
O113:H4	0/7	0
O113:H6	12/12	14
O113:H7	1/1	1
O113:H21	0/7	0
O121:H5	1/1	1
O125:H6	12/12	14
O132:H34	4/6	5
ONT:H6	1/2	1
Total	**87/110**	**100**

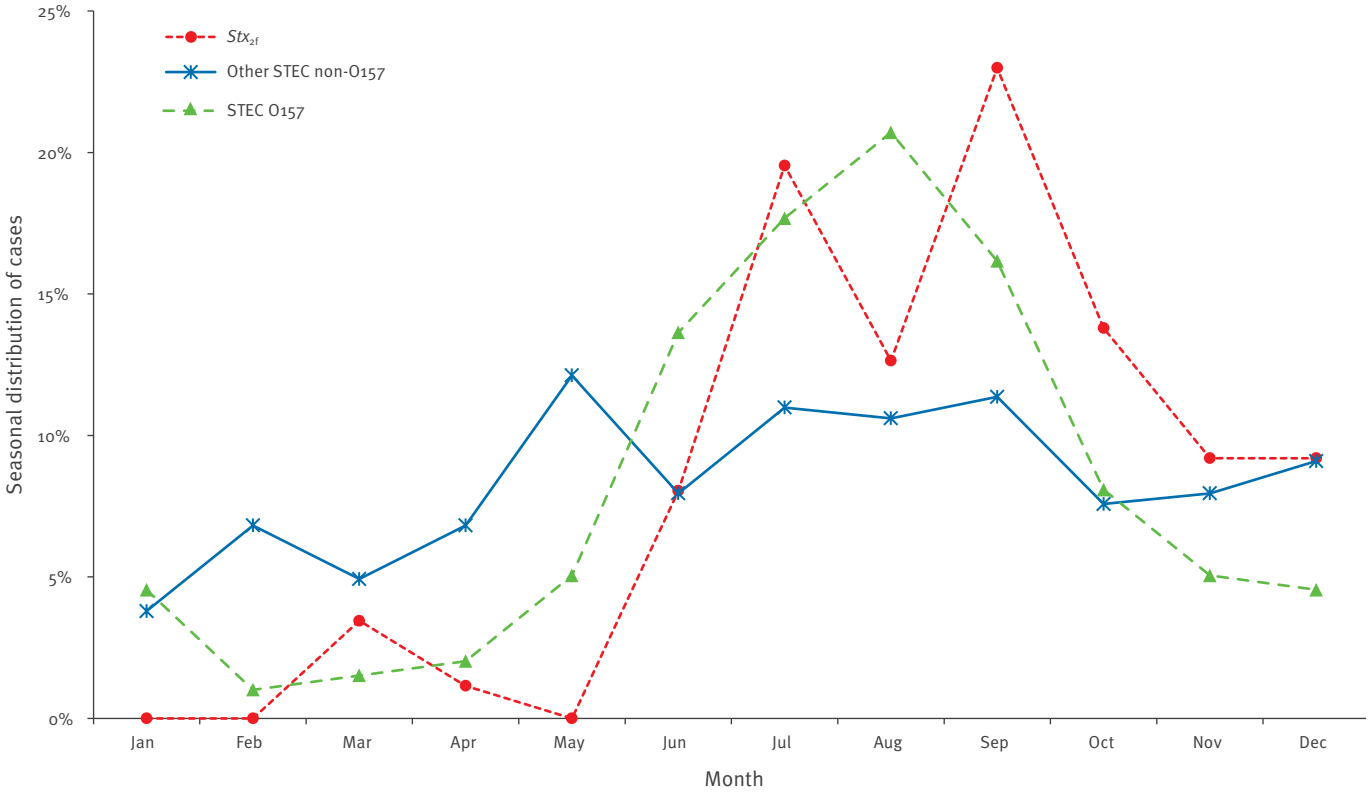
STEC: Shiga toxin-producing *Escherichia coli*.

Discussion

Between 2008 and 2011, stx_{2f} STEC infections comprised 25% of all STEC non-O157 infections in this period and 16% of all STEC isolated. Excluding the laboratories unable to detect all STEC infections, stx_{2f} STEC infections constitute 20% of all STEC infections. This is clearly higher than reported before [9,10,12]. Within a Dutch multi-centre study between 2005 and 2006, three (14%) of 21 STEC cases tested, were positive for stx_{2f}, which was already higher than earlier reports [13]. As these studies all were done before 2008, the relative high percentage in the present study could be a sign that stx_{2f} STEC is emerging. In Belgium, the percentage stx_{2f} STEC was 13% (all STEC) or 17% (STEC non-O157) over the period from 2008 to 2010 [19]. The low frequency of internationally reported human stx_{2f} STEC infections may be due to the mild course of the disease and due to underdiagnosis, as several STEC assays targeting stx (genes) are not capable of detecting the stx_{2f} variant. For example, standard PCR assays and GeneDisc real-time PCR do not detect stx_{2f} [20-22], but requires a specific primer/probe design [21]. Beutin et al. [23] tested two enzyme immunoassays, of which P1-glycoprotein-enzyme immunoassay (EIA) could not and Ridascreen-EIA could detect stx_{2f}. The seemingly mild disease caused by stx_{2f} STEC infections does not stimulate adjusting the commonly used techniques which, in turn, enhances underdiagnosis and underreporting. To be certain however, that stx_{2f} STEC

Figure

Seasonal distribution of *stx*$_{2f}$ STEC (non-O157) cases (n=87), other STEC non-O157 cases (n=264) and STEC O157 cases (n=198), the Netherlands, 2008–2011



STEC: Shiga toxin-producing *Escherichia coli*; *stx*$_{2f}$: Shiga-toxin 2f gene.

Table 3

Reported clinical data of the Shiga toxin-producing *Escherichia coli* (STEC) cases, the Netherlands, 2008–2011 (n=549)

Characteristic	Stx2f STEC non-O157 n/N[a] (%)	Other STEC non-O157 n/N[a] (%)	STEC O157 n/N[a] (%)
Median age in years (minimum–maximum)	31 (0–90)	28 (0–92)	21 (0–85)
Age groups			
0–4 years	17/87 (20)	41/264 (16)	40/198 (20)
5–19 years	15/87 (17)	58/264 (22)	52/198 (26)
20–39 years	17/87 (20)	63/264 (24)	46/198 (23)
40–59 years	20/87 (23)	46/264 (17)	30/198 (15)
>60 years	18/87 (21)	56/264 (21)	30/198 (15)
Sex, % male	41/85 (48)	99/259 (38)	75/198 (38)
Diarrhoea	53/60 (88)	156/179 (87)	164/173 (95)
Stomach ache	36/60 (60)	126/178 (71)	151/172 (88)[b]
Blood in stool	9/60 (15)	47/178 (26)	132/173 (76)[b]
Less urine production	2/59 (3)	24/176 (14)[b]	49/170 (29)[b]
HUS	0/63 (0)	4/191 (2)	11/182 (6)[b]
Hospitalisation	9/58 (16)	29/175 (17)	73/177 (41)[b]
Deceased	0/72 (0)	1/224 (0)	2/186 (1)

HUS: haemolytic uraemic syndrome.
In the Table the denominators of the fractions vary because the information in question was not available from all cases.

[a] Fractions and the resulting percentages are given unless otherwise specified.
[b] Significantly different from *stx*$_{2f}$ cases (p<0.05).

Table 4

Risk factors as reported by the STEC cases (n=417)[a] and community controls (n=1,396)[b], the Netherlands, 2008–2011

Risk factor	Stx_{2f} STEC non-O157 n/N (%)	Other STEC non-O157 n/N (%)	STEC O157 n/N (%)	Controls n/N (%)
Contact with ill person	1/31 (3)	19/98 (19)[c]	24/135 (18)[c]	NA
Contact with livestock	8/31 (26)	28/98 (29)	32/135 (24)	254/1,236 (21)
Dairy products of raw milk consumption	7/31 (23)	16/98 (16)	10/135 (7)[c]	285/1,320 (22)
Raw or undercooked meat consumption	12/31 (39)	52/98 (53)	53/135 (39)	545/1,386 (39)
Raw vegetables/salads consumption	23/29 (79)	71/95 (75)	95/133 (71)	1,080/1,348 (80)
Bean sprouts consumption	11/28 (39)	20/94 (21)	24/129 (19)[c]	301/1,338 (2)[c]
Travel abroad	9/57 (16)	39/182 (21)	32/178 (18)	195/1,364 (14)

NA: not available; STEC: Shiga toxin-producing *Escherichia coli*.

In the Table the denominators of the fractions vary because the information in question was not available from all cases or controls.

[a] The number of STEC cases provided is the number of cases with information on at least one of the risk factors listed in the table available (417 of 549 total STEC cases).

[b] The number of community controls provided is the number of controls with information on at least one of the risk factors listed in the table available (1,396 of 1,420 total controls).

[c] Significantly different from stx_{2f} cases ($p<0.05$).

infections are in fact generally mild, more testing and research is needed.

Almost all stx_{2f} isolates possessed the *eae* gene, which was also reported for the stx_{2f} isolates found in pigeons [7] and in earlier reports of human stx_{2f} STEC infections [9,12]. None of the stx_{2f} isolates contained the *hly* gene, as was also reported by Prager et al. [12] and Seto et al. [9]. The combination of the presence of the *eae* gene but absence of the *hly* gene is rarely seen in the other (non-stx_{2f}) STEC non-O157 infections and not seen at all in STEC O157 infections within the Dutch STEC surveillance. The absence of a finding in this study of a single isolate with a stx_{2f} gene together with a stx_1 gene and the fact that this combination is not reported in the literature so far suggests that stx_{2f} isolates form a distinct group within the STEC infections.

Escherichia albertii has recently been identified as *eae*-positive *Escherichia*, including stx_{2f} strains [24-27]. The stx_{2f} strains were isolated from a patient with diarrhoea and from a healthy crow-like bird [27]. Due to those characteristics, *E. albertii* strains might be misidentified as enterohaemorrhagic *E. coli* (EHEC) or STEC. *E. albertii* and *E. coli* are strongly related and are difficult to discriminate based on 16S sequence (data not shown). Nine isolates in the present study were specifically tested for the inability to ferment lactose, which is a phenotypic trait discriminating *E. albertii* from *E. coli* [27]. Based on this biochemical test all nine tested isolates belonging to the present study appeared to be *E. coli*. However, this does not entirely exclude the possibility that part of the remaining isolates is *E. albertii* instead of *E. coli*.

In the period from 2008 to 2011, the stx_{2f} isolates analysed in this study, belonged to 11 O-types, with four O-types accounting for 87%. O63:H6, appears to be most often associated with stx_{2f}, based on this study and previous reports [9,12,13]. Also, the association with serotype O132:H34 has been reported before [12]. O113:H6, O125:H6 and the more rare O-types of the stx_{2f} isolates have not been related to stx_{2f} before. None of the serogroups found in the current study have been reported in pigeons or other birds [4,7].

Preliminary results from a molecular risk assessment study (data not shown) included five O63 strains and these were all found to harbour relatively low numbers of additional STEC virulence genes. In addition, these O63 strains all belonged to phylogroup B2 while the majority of the other STEC tested belonged to phylogroup B1. There are indications that strains belonging to different phylogroups have different ecological niches and life-history traits. Phylogroup A and B1 strains appear to be generalists, able to occupy a broad range of vertebrate hosts, while B2 and D strains are more commonly isolated from birds and mammals [28]. Phylogroup B2 strains are considered to mainly host adapted *E. coli* with longer persistence in hosts than strains belonging to other phylogroups. In addition, phylogroup B2 generally harbour extra-intestinal virulence traits at higher frequency [29].

No significant difference in age distribution was found between stx_{2f} STEC cases and other STEC cases. Twenty per cent of the Dutch stx_{2f} STEC cases were four years or younger, while 79% of the German stx_{2f} STEC cases reported by Prager et al. [12] were in this age group. The course of a stx_{2f} STEC infection was less severe compared to other STEC infections, especially STEC O157 infections. The reason of this less severe course is unknown, but one could hypothesise that stx_{2f} toxin is less toxic or produced in lower quantities than the other Shiga toxin variants. The putative presence – or

absence – of other virulence genes not determined in this study could also be involved.

This is the first report of possible risk factors and sources for stx_{2f} STEC. The risk factor analysis revealed that person-to-person transmission seems to be less relevant in stx_{2f} STEC infections as compared to other STEC infections. However, the mild course of the infection might mask shedding (contact) persons, which are then not diagnosed nor reported by case patients. stx_{2f} STEC cases reported eating dairy products made of raw milk, and bean sprouts more often than STEC O157 cases.

To confirm whether stx_{2f} STEC is partially foodborne, food products incriminated in our case–control study, or, when occurring, outbreak investigations could be tested for stx_{2f} STEC. When foodborne or other non-human stx_{2f} STEC are found, comparison with human isolates of the same serotype with for example pulsed-field gel electrophoresis (PFGE) or sequence-based typing techniques, could help to further elucidate sources and reservoirs of stx_{2f} STEC and determine the genetic heterogeneity within these serotypes. Beutin et al. [30] tested 219 STEC strains from meat, milk and cheese samples for serotype and genetic variants of Shiga toxins in the period from 2005 to 2006. None of these strains tested positive for stx_{2f}.

Stx_{2f} STEC was first detected in the gastrointestinal tract of apparently healthy pigeons in Italy and Germany [4,31]. With the relative high faecal carriage of stx_{2f} STEC in pigeons, ranging from six to 16%, pigeons were considered as a reservoir [7]. However, the present study did not point in the direction of pigeons or other birds, as none of the serogroups found have been reported in avian species. In addition, none of the stx_{2f} STEC cases reported contact with birds. It should be noted that as the data about risk factors were available for only a part (31/87; 36%) of the stx_{2f} STEC cases, less strong associations might have been missed due to lack of power.

In conclusion, human stx_{2f} STEC infections are more common than anticipated in the Netherlands, with an estimated 20% of all STEC infections constituting the stx_{2f} gene. Stx_{2f} STEC form a clinically and microbiologically distinct group within STEC, mostly harbouring the *eae*, but lacking the *hly* gene and not seen together with other *stx* genes or in STEC O157. Stx_{2f} STEC infections appear to be relatively mild compared to other STEC infections, especially STEC O157. The present study could not confirm exposure to pigeons or other birds as source for infections, but alternatively found, although not very strong, associations with raw dairy products and bean sprout consumption. To further explore such associations, more research would be needed, also using additional diagnostic and typing methods. The trend in stx_{2f} will be further monitored in the coming years and will also allow more powerful case–control analyses.

Conflict of interest

None declared.

Authors' contributions

IF coordinated the collection of data, analysed and interpreted the data and drafted the manuscript. KvdZ confirmed and typed the isolates, and participated in editing the manuscript. TS and MK-S developed/adapted the PCR method for detecting STEC, and participated in editing the manuscript. EF participated in the interpretation of the data and editing the manuscript. YvD previously coordinated the collection of data, and YvD and WvP participated in the interpretation of the data and editing the manuscript. All authors read and approved the final manuscript.

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