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Rune Micha Pedersen, Marc Trunjer Kusk Nielsen, Sören Möller, Steen Ethelberg, Marianne Nielsine Skov, Hans Jørn Kolmos, Flemming Scheutz, Hanne Marie Holt, Flemming Schønning Rosenvinge

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Title. Shiga toxin-producing *Escherichia coli*: incidence and clinical features in a setting with complete screening of patients with suspected infective diarrhoea

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Running title. Complete STEC screening: incidence and clinical features

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Abstract

Objectives. Shiga toxin-producing Escherichia coli (STEC) causes diarrhoeal disease, bloody diarrhoea and haemolytic uraemic syndrome. The aim of this study was to describe the incidence of STEC and the clinical features of STEC patients from a well-defined Danish population in which all fecal samples of patients with suspected infective gastroenteritis were analysed for STEC.

Methods. In this population-based cohort study, all stool samples referred to two clinical microbiology laboratories, were screened for STEC by culture and/or PCR. Epidemiological (n=170) and clinical (n=209) characteristics were analysed using data from local and national registries.

Results. Overall 75,132 samples from 30,073 patients were screened resulting in 217 unique STEC-isolates. The epidemiological analysis showed an incidence of 10.1 cases per 100,000 person-years, which was more than two-fold higher than the incidence in the rest of Denmark (3.4 cases per 100,000 person-years, p<0.001). Three groups were associated with a higher incidence: age <5 years (n=28, p<0.001), age ≥65 years (n=38, p=0.045) and foreign ethnicity (n=27, p=0.003). In the clinical analysis patients with STEC harboring only the Shiga toxin 1 gene (stx1-only isolates) showed a lower frequency of acute (n=11, p<0.05) and bloody diarrhoea (n=5, p<0.05) and a higher frequency of gastrointestinal symptoms for ≥3 months (n=8, p<0.05) than the other STEC patients.

Conclusions. We report a more than two-fold higher incidence in the project area compared with the rest of Denmark, indicating that patients remain undiagnosed when selective STEC
screening is used. We found an association between patients with $stx_1$-only isolates and long-term gastrointestinal symptoms.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is associated with bloody diarrhoea and haemolytic uraemic syndrome (HUS) [1], and human infections emerge as both spontaneous cases and outbreaks [2,3]. Clinical disease ranges from mild gastroenteritis [4], to bloody diarrhoea, HUS and death [5]. During the 10-year period from 2005 to 2014, the reported incidence of STEC in Denmark increased from 3.1 to 5.0 cases per 100,000 person-years [2,6]. A similar trend has been observed in other parts of Europe and in the United States [6,7]. Knowledge about incidence is needed to assess risk, institute diagnostic strategies and implement public health measures, but generally, STEC incidence data are characterised by a high degree of variation both in time and between countries [6,8]. This is, at least in part explained by variations in diagnostic methods and screening criteria [9]. Although STEC infections have been notifiable in most European Union member states for several years [6], the true STEC incidence among symptomatic patients has yet to be uncovered. These challenges were highlighted in a German study in which the true STEC gastroenteritis incidence in a computed estimate was 32.3-fold higher than the incidence based on notified HUS cases [10]. *E. coli* harboring one or both genes encoding the two Shiga toxins ($stx_1$ and $stx_2$) are defined as STEC [11]. Although the clinical characteristics associated with severe and acute STEC disease have been intensely studied, much less is known about patients with non-severe and long-lasting symptoms. STEC strains harboring only $stx_1$ appear to be associated with non-severe disease [12,13], but detailed data on this group of patients are needed.
Our objective was to describe the STEC incidence, demographic differences and clinical characteristics in a unique setting in which all stool samples from patients with suspected infective diarrhoea were screened for STEC.

**Methods**

Case definition and population

In this population-based cohort study, data on STEC patients were collected from two adjacent laboratories in the Region of Southern Denmark, comprising approximately 13% of Denmark’s population. Laboratory A was the Department of Clinical Microbiology at Odense University Hospital, a tertiary referral hospital, and the data were retrieved from April 2011 through December 2014 (average catchment area: 448,869 inhabitants). Laboratory B was the Department of Clinical Microbiology at Lillebaelt Hospital, Vejle, a secondary referral hospital, and the data were retrieved from November 2013 through December 2014 (average catchment area: 287,199 inhabitants). All stool samples obtained as part of a routine diagnostic work-up for suspected infective diarrhoea were analysed for STEC by culture and/or PCR according to the recommendations on laboratory diagnostics of bacterial gastrointestinal pathogens from the Danish Society of Clinical Microbiology [14]. In addition to STEC, all fecal samples were analysed for other diarrhoeagenic bacteria. Patients with a first-time STEC diagnosis and an isolate confirmed at the National Reference Laboratory (NRL) at Statens Serum Institut (SSI) were included in the study (see Supplementary Material, File S1 for microbiology methods).

The epidemiological analysis was limited to the data from laboratory A, which covered the larger population and had the longer collection period. Patients with residency outside the
catchment area were excluded from the epidemiological analysis. Patients diagnosed at either of the two laboratories were included in the clinical analysis. Patients with more than one distinct STEC type were excluded from this analysis because the contribution of each isolate to the clinical features of the patients could not be determined (Figure 1).

Data collection

Demographic data on the age, gender, residency, income and ethnicity of the STEC patients were collected from the local laboratory information system (LIMS) [15] and the research database of Statistics Denmark [16]. Demographic data from the background population were extracted from StatBank Denmark [16]. Data on STEC isolates, the presence of other diarrhoeagenic bacteria, sample date and requesting physician/hospital were collected from the local LIMS and the National Register of Enteric Pathogens at SSI. Clinical data, which were all available in an electronic health care data system (Cosmic, CGI, Ballerup, Denmark), were extracted by reviewing patient medical charts. Additionally, the laboratory’s clinical charts were reviewed. Laboratory charts contained clinical information on all STEC patients. Since STEC is notifiable in Denmark, the treating physician was routinely interviewed by a physician from the local Department of Clinical Microbiology. Furthermore, all requisition notes contained prompt questions regarding the duration of diarrhoea, blood in stool, travel history and accumulation of cases.

Epidemiology

Patients were compared to the background population based on the following parameters: age, gender, ethnicity, annual equivalised disposable household income and place of residence. Four
age groups were defined: <5 years, 5-14 years, 15-64 years and ≥65 years. Ethnicity was defined according to ancestry, with Danish ancestry defined as having at least one parent who had both Danish citizenship and was born in Denmark. Immigrants and descendants of immigrants were combined to form the other group. The annual equivalised disposable household income was defined according to the modified OECD equivalence scale. Rural residence was defined according to Statistics Denmark [16], in which residential areas less than 200 inhabitants are rural areas.

Clinical characteristics of STEC infection

The following data were extracted: time between the onset of diarrhoea and first positive STEC sample (separated into three groups: <2 weeks, ≥2 weeks to <3 months and ≥3 months), presence of macroscopic blood in stool, travel history, HUS, hospitalisation within one month of first positive STEC test and clinical assessment by a gastroenterologist within 1 year of first positive STEC test. In Denmark, patients must be referred by a physician to be assessed in gastroenterology (GE) clinics. Therefore, only patients with long-term or complicated gastrointestinal symptoms are assessed by gastroenterologists.

Statistics

We applied the Wald test to compare incidence rate ratios, the Wilcoxon rank sum test to compare income groups and Fisher´s exact test to compare the STEC virulence gene combinations with the clinical features of the STEC patients. Associations between virulence genes and clinical characteristics were further explored through multivariate logistic regression. Sensitivity analyses were performed excluding patients aged less than 15 years, the presence of
other diarrhoeagenic bacteria and the presence of the O104:H4 outbreak strain [17]. The
unweighted sum-of-squares test was used to indicate lack of goodness of fit. The analyses were
performed using R version 3.3.1 [18]. The code is available as an R markdown notebook (see
Supplementary Material, File S2).

Ethical considerations

This study was approved by the Danish Data Protection Agency (record nos. 14/26345, 15/5384
and 16/1586) and the Danish Patient Safety Authority (authority no 3-3013-734/1). Approval
from an ethics committee is not required for register-based research in Denmark.

Results

Epidemiology

In the epidemiological analysis, 170 patients were included from the catchment area of
laboratory A (Figure 1). The overall incidence of STEC infection was 10.1 cases per 100,000
person-years (Table 1), whereas the rest of Denmark (n=655) had an incidence of 3.4 cases per
100,000 person-years (p<0.001). The annual incidence during the project period varied from
8.5-10.9 cases per 100,000 person-years. The median age of the cases was 37 (range, 0-92
years) vs. 41 (range, 0-108 years) in the background population. Compared with the incidence
among patients 15-64 years of age, the incidence of STEC infection was higher in children <5
years of age and in the elderly ≥65 years of age (p<0.001 and p=0.045, respectively) (Table 1).
The group of immigrants and descendants consisted of 15 patients (55%) with Middle Eastern
ancestry, 5 patients (19%) with European ancestry and 7 patients (26%) with other foreign
 ancestries. The mean annual equivalised disposable household income of the STEC patients was
29,528 EUR and did not differ from the background population (p=0.07) (see Supplementary Material, Fig. S1). There was a seasonal distribution of STEC diagnosed at laboratory A with a peak in the late summer/early fall (see Supplementary Material, Fig. S2).

Clinical characteristics of STEC infection

Laboratory A tested 67,459 stool samples from 25,555 patients (average: 2.6 samples per patient), and laboratory B tested 7,673 stool samples from 4,518 patients (average: 1.7 samples per patient) for the presence of STEC. A total of 251 patients were diagnosed with STEC by culture and/or PCR. The NRL confirmed 217 STEC isolates from 213 patients, and 209 of these patients were included (Figure 1). STEC isolates representing 83 different serotypes were grouped according to the presence of \textit{stx}_1, \textit{stx}_2 and the \textit{E. coli} attaching and effacing gene (\textit{eae}). Differences were observed in the time gap between the onset of diarrhoea and first positive STEC sample (p<0.001), assessment by a gastroenterologist (p=0.002) and bloody stools (p=0.016) (Table 2). Time until positive STEC sample, bloody stools and assessment by a gastroenterologist were analysed by multivariate logistic regression models adjusting for age group, \textit{stx} profile and the presence of \textit{eae}. Using \textit{stx}_1-only as a reference, time until positive STEC sample of less than two weeks was associated with the presence of \textit{eae} (OR 4.23, 95% CI: 2.03-9.31) and \textit{stx}_2 (OR 2.08, 95% CI: 1.05-4.22). In contrast, time until positive STEC sample ≥3 months was inversely associated with \textit{eae} (OR 0.29, 95% CI: 0.07-0.91) and \textit{stx}_2 (OR 0.39, 95% CI: 0.15-0.99). The outcome bloody stools was associated with \textit{eae} (OR 3.27, 95% CI: 1.61-6.84) and \textit{stx}_2 (OR 2.56, 95% CI: 1.27-5.43) (Table 3). An association between \textit{stx}_1-only and assessment by a gastroenterologist was found when the reference was set to \textit{stx}_2 (OR 3.54, 95% CI: 1.70-7.64). This association remained significant in the sensitivity analysis (see
Supplementary Material, Table S1). The values indicating goodness of fit were not critical (p=0.28-0.90) (see Supplementary Material, File S2).

Discussion

The incidence reported in this study is more than twice as high as the Danish national STEC incidence reported to ECDC/EFSA [6], which indicates that more cases will be found if all patients with suspected infective diarrhoea are screened for STEC. This indication is supported by the 88% increase in the number of STEC cases diagnosed at laboratory A upon the introduction of non-selective STEC screening [19]. The incidence of STEC infections was highest in children <5 years of age and in adults ≥65 years of age. This finding was also observed when using selective screening [20,21] and raises the question of whether these age-associated incidence peaks are related to increased exposure, susceptibility or diagnostic efforts.

The epidemiological analyses showed an accumulation of cases among immigrants and descendants, as also noted previously [22]. We speculate that this could be due to different food habits (e.g., consumption of imported food) and travel activity (destinations, frequency and types of accommodation). The country of origin or socioeconomic status were not included in the analysis, due to the small number of patients in the foreign ethnicity group.

Consistent with other studies, this report shows that the clinical symptoms of STEC infections are related to the stx type and the presence of eae [23–25]. STEC isolates harboring eae have been associated with HUS in Scandinavia [26]. In our study, eae was associated with bloody diarrhoea and acute presentation of illness, both of which are indicators of serious STEC disease. We did not find an association between eae/stx2 and HUS in this study, probably
because of the low number of patients with HUS. Furthermore, in this STEC cohort, infections with \( stx_1 \)-only isolates were associated with long-term gastrointestinal symptoms and assessment by a gastroenterologist, implying the presence of persistent and unclarified gastrointestinal symptoms. Other studies have described \( stx_1 \)-only isolates in relation to persistent diarrhoea and asymptomatic colonisation [27,28].

This work has a number of limitations. Differences in the thoroughness of the diagnostic workup within various groups of patients can skew the comparison of the incidence rates. Another limitation of this study is the differences in the diagnostic approach between the two laboratories. Laboratory A used a primary culture-based approach, whereas laboratory B used a primary culture-independent approach. Nearly four times as many patients had to be excluded from laboratory B than from laboratory A due to the lack of cultured isolates. This likely reflects inter-laboratory differences in the diagnostic methods and in the number of diagnostic samples per patient with diarrhoea. The size of the STEC cohort did not allow for a clinical comparison at the \( stx \) subtype level.

In summary, compared with national data, we found a high STEC incidence in the project area, reflecting different diagnostic strategies: unselective vs. selective STEC screening of stool samples. This indicates that our current clinical strategies and health measures are based on incomplete and biased data. STEC infection appears to be associated with the extremes of age and foreign ethnicity. We also found an association between STEC virulence genes and clinical manifestations: \( stx_2 \) and particularly \( eae \) were related to acute and bloody diarrhoea, whereas \( stx_1 \)-only was associated with persistent gastrointestinal illness. This finding indicates that STEC infection is a heterogeneous disease entity. Thus, the likely associations between STEC toxin
subtypes and clinical symptoms should be addressed in future research. Gaining this knowledge is essential for improving patient management and implementing cost-effective screening strategies.

**Funding**

No external funding was received for this study.

**Transparency declaration**

The authors declare no conflicts of interest related to this work.

**References**


Table captions

Table 1. Demographic characteristics of the STEC patients compared with the background population in the catchment area of laboratory A from April 2011 through December 2014

Table 2. Association between virulence genes and clinical features/exposures in a univariate model

Table 3. Associations of three different clinical features with age groups and STEC virulence genes within a univariate and multivariate model

Figure captions

Fig. 1. Study profile. Patient distribution flow chart. Abbreviations: STEC, Shiga toxin-producing *Escherichia coli*. 
<table>
<thead>
<tr>
<th>Demographic group</th>
<th>STEC Cases</th>
<th>Population</th>
<th>Incidence rate per 100,000 person-years</th>
<th>Incidence rate ratios</th>
<th>95% CI</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
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<td>Age &lt;5 Y</td>
<td>28</td>
<td>23,190</td>
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<td>4.08</td>
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<td>Age 5-14 Y</td>
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<td>51,470</td>
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<td>Danish</td>
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<td>Immigrants and descendants</td>
<td>27</td>
<td>41,897</td>
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<td><strong>Total</strong></td>
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<td>448,869</td>
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</table>

Abbreviations: CI, confidence interval; NA, not applicable; Ref, reference group; STEC, Shiga toxin-producing *Escherichia coli*; Y, years old.

<sup>a</sup>The Wald test was used to compare the incidence rates. p<0.05 is considered statistically significant and marked in bold.

<sup>b</sup>Data on ethnicity were missing in two STEC patients.
### Combinations of virulence genes

<table>
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<tr>
<th>Clinical features/exposures</th>
<th>stx₁-only</th>
<th>stx₂-only</th>
<th>stx₁ + stx₂</th>
<th>stx₁ + eae</th>
<th>stx₂ + eae</th>
<th>stx₁ + stx₂ + eae</th>
<th>p-valueᵃ</th>
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<tr>
<td>Time between onset of diarrhoea and positive STEC sampleᵇ</td>
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<tr>
<td>&lt;2 weeks (%)</td>
<td>11 (37)</td>
<td>35 (59)</td>
<td>13 (43)</td>
<td>29 (78)</td>
<td>19 (79)</td>
<td>22 (96)</td>
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<td>≥2 weeks - &lt;3 months (%)</td>
<td>8 (26)</td>
<td>16 (27)</td>
<td>5 (17)</td>
<td>4 (11)</td>
<td>3 (13)</td>
<td>1 (4)</td>
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<td>≥3 months (%)</td>
<td>8 (26)</td>
<td>6 (10)</td>
<td>6 (20)</td>
<td>3 (8)</td>
<td>1 (4)</td>
<td>0 (0)</td>
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<tr>
<td>No diarrhea (%)</td>
<td>3 (10)</td>
<td>2 (3)</td>
<td>6 (20)</td>
<td>2 (5)</td>
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<td>Hospitalization (%)</td>
<td>12 (39)</td>
<td>18 (30)</td>
<td>7 (23)</td>
<td>13 (33)</td>
<td>4 (17)</td>
<td>7 (30)</td>
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<tr>
<td>Gastroenterologyᶜ (%)</td>
<td>17 (55)</td>
<td>19 (31)</td>
<td>9 (30)</td>
<td>12 (31)</td>
<td>4 (17)</td>
<td>1 (5)</td>
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<td>Bloody stools (%)</td>
<td>5 (16)</td>
<td>19 (31)</td>
<td>5 (17)</td>
<td>10 (25)</td>
<td>11 (46)</td>
<td>12 (52)</td>
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<td>HUS (%)</td>
<td>1 (3)</td>
<td>5 (8)</td>
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<tr>
<td>International travelᵈ (%)</td>
<td>12 (41)</td>
<td>18 (31)</td>
<td>6 (21)</td>
<td>10 (27)</td>
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<tr>
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<td>61</td>
<td>30</td>
<td>40</td>
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Abbreviations: eae, *E. coli* attaching and effacing gene; HUS, haemolytic uraemic syndrome; STEC, Shiga toxin-producing *Escherichia coli*; stx₁, Shiga toxin 1 gene; stx₂, Shiga toxin 2 gene.

ᵃFisher’s exact test was used to compare the clinical features between groups of STEC patients with strains of different genetic virulence profiles. P<0.05 is considered statistically significant and marked in bold.

ᵇData on time from the onset of diarrhoea to positive STEC sample were missing in 6 STEC patients.

ᶜData on assessment by a gastroenterologist were missing in 2 STEC patients.

ᵈData on international travel were missing in 9 STEC patients.
### Time until first positive STEC sample <2 weeks

<table>
<thead>
<tr>
<th>Association group</th>
<th>Univariate OR (95% CI)</th>
<th>Multivariate OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 0-5 Y</td>
<td>4.52 (1.63-16.11)</td>
<td>2.36 (0.76-8.93)</td>
<td>.16</td>
</tr>
<tr>
<td>Age 5-14 Y</td>
<td>1.13 (0.45-3.04)</td>
<td>0.81 (0.29-2.31)</td>
<td>.68</td>
</tr>
<tr>
<td>Age ≥65 Y</td>
<td>0.48 (0.23-0.98)</td>
<td>0.53 (0.25-1.12)</td>
<td>.10</td>
</tr>
<tr>
<td>eae</td>
<td>4.68 (2.46-9.34)</td>
<td>4.23 (2.03-9.31)</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>stx₁-only</td>
<td>Ref</td>
<td>Ref</td>
<td>NA</td>
</tr>
<tr>
<td>stx₂-only</td>
<td>1.36 (0.74-2.48)</td>
<td>2.08 (1.05-4.22)</td>
<td>.04</td>
</tr>
<tr>
<td>Number of observations</td>
<td>203</td>
<td>203</td>
<td></td>
</tr>
</tbody>
</table>

### Bloody stool

<table>
<thead>
<tr>
<th>Association group</th>
<th>Univariate OR (95% CI)</th>
<th>Multivariate OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 0-5 Y</td>
<td>0.64 (0.25-1.50)</td>
<td>0.35 (0.12-0.92)</td>
<td>.04</td>
</tr>
<tr>
<td>Age 5-14 Y</td>
<td>1.15 (0.43-2.92)</td>
<td>0.88 (0.30-2.42)</td>
<td>.81</td>
</tr>
<tr>
<td>Age ≥65 Y</td>
<td>0.82 (0.38-1.74)</td>
<td>0.91 (0.40-2.00)</td>
<td>.81</td>
</tr>
<tr>
<td>eae</td>
<td>1.96 (1.08-3.59)</td>
<td>3.36 (1.64-7.10)</td>
<td>.001</td>
</tr>
<tr>
<td>stx₁-only</td>
<td>Ref</td>
<td>Ref</td>
<td>NA</td>
</tr>
<tr>
<td>stx₂-only</td>
<td>1.93 (1.00-3.86)</td>
<td>2.56 (1.27-5.43)</td>
<td>.01</td>
</tr>
<tr>
<td>Number of observations</td>
<td>209</td>
<td>209</td>
<td></td>
</tr>
</tbody>
</table>

### Assessment by a gastroenterologist

<table>
<thead>
<tr>
<th>Association group</th>
<th>Univariate OR (95% CI)</th>
<th>Multivariate OR (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age 0-5 Y</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Age 5-14 Y</td>
<td>0.15 (0.02-0.54)</td>
<td>0.15 (0.02-.58)</td>
<td>.02</td>
</tr>
<tr>
<td>Age ≥65 Y</td>
<td>1.15 (0.57-2.31)</td>
<td>1.08 (0.51-2.26)</td>
<td>.84</td>
</tr>
<tr>
<td>eae</td>
<td>0.43 (0.22-0.80)</td>
<td>0.64 (0.29-1.37)</td>
<td>.26</td>
</tr>
<tr>
<td>stx₁-only</td>
<td>Ref</td>
<td>Ref</td>
<td>NA</td>
</tr>
<tr>
<td>stx₂-only</td>
<td>0.45 (0.24-0.83)</td>
<td>0.32 (0.15-0.65)</td>
<td>.002</td>
</tr>
<tr>
<td>Number of observations</td>
<td>207</td>
<td>207</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; eae, E. coli attaching and effacing gene; NA, not applicable; OR, odds ratio; Ref, reference group; STEC, Shiga toxin-producing Escherichia coli; stx₁, Shiga toxin 1 gene; stx₂, Shiga toxin 2 gene; Y, years old.

* p<0.05 is considered statistically significant and marked in bold.

* Multivariate analyses were explored through multiple logistic regression.

* p-values refer to the multivariate analysis.
203 patients with a first-time STEC diagnosis

195 STEC patients

8 patients excluded: no isolate

25 patients excluded: residency outside catchment area

170 STEC patients

Epidemiological analysis

Laboratory A (Odense)
Primary culture-dependent diagnostic test
67,459 stool samples from 25,555 patients

Laboratory B (Vejle)
Primary culture-independent diagnostic test
7,673 stool samples from 4,518 patients

48 patients with a first-time STEC diagnosis

18 STEC patients

30 patients excluded: no isolate

213 STEC patients

4 patients excluded: >1 STEC type

209 STEC patients

Clinical analysis