A case of haemolytic uraemic syndrome (HUS) revealed an outbreak of Shiga toxin-2-producing Escherichia coli O26:H11 infection in a nursery, with long-lasting shedders and person-to-person transmission, Italy 2015

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Abstract

Purpose. Shiga toxin-producing Escherichia coli (STEC) represents a major issue for public health because of the severity of the associated illnesses, including haemolytic uraemic syndrome (HUS). In 2015, investigation of a case of HUS revealed an outbreak of Shiga toxin-2-producing E. coli O26:H11 infection in a nursery in Italy. The investigation showed that the infection was transmitted to cases’ contacts via person to person.

Methods. The case finding was performed by testing for STEC stool samples of the HUS case’s contacts within the family and the nursery. STEC O26 isolates were characterized by whole genome sequencing. Confirmed cases were repeatedly tested to monitor the duration of STEC shedding.

Results. Eleven STEC O26 cases were identified, including adults and asymptomatic patients. Clinical illness was only observed in children. Strain characterization revealed that a single clone harbouring the stx2a and eae genes and the complete array of STEC-associated virulence genes, belonging to ST(21), was implicated in the outbreak. To reduce bacterial shedding, patients were treated with cefixime following clinical recovery. This antibiotic was well tolerated and did not induce any apparent consequences on patients’ health.

Conclusions. This study confirms that Stx2-producing E. coli O26 represents an emerging public health problem. The occurrence of outbreaks of infection by Stx2-producing E. coli O26 in nurseries is of particular concern, given the high probability of infection progressing in severity and resulting in secondary cases.

INTRODUCTION

Haemolytic uraemic syndrome (HUS) is a life-threatening condition characterized by microangiopathic haemolysis, platelet consumption and multi-organ (mainly the kidneys) damage [1]. It typically occurs after a prodromal diarrhoea, due to Shiga toxin (Stx)-producing Escherichia coli (STEC) infection. STEC infection progresses to overt HUS only in a limited proportion of cases, most often in children. Long-term sequelae are frequently described and, together with acute mortality, account for most of the burden of STEC infection.

To date, supportive care represents the most effective option to treat STEC infection [2]. The use of antibiotics still represents a challenge given the dearth of conclusive data to support generalizable evidence in favour or against their use in STEC infection [3].

HUS occurrence is considered an important sentinel event revealing the circulation of less severe cases of STEC infection in a population. In Italy, surveillance of HUS is carried out by the National Registry of HUS in cooperation with the National Reference Laboratory (NRL) for E. coli [4], and this revealed that STEC belonging to non-O157 serogroups,
in particular O26, has been the most frequently detected STEC among HUS cases in the paediatric population since the late 1990s [5].

In May 2015, a 15-month-old girl presenting with HUS and who tested positive for STEC O26 (stx2+, eae+) was admitted to the Pediatric Hospital Bambino Gesù in Rome (Italy). Epidemiological and microbiological investigations, extended to the case’s family and the nursery attended by the HUS case, revealed an underlying outbreak of STEC infection. This report describes the epidemiological, clinical and microbiological features of the outbreak.

METHODS

Epidemic settings and sample collection

The HUS patient’s family home and nursery were both located in a densely populated urban area of central Italy, which was considered at lower risk for HUS (mean annual incidence rate in 0–15-year-old population: 0.19 cases per 100 000), compared to other Italian provinces [6].

The HUS case’s parents were interviewed on 21 May 2015 using the standard questionnaire adopted by the National Registry for HUS. Because contacts with other children suffering from diarrhoeal illness in both the family and at the nursery attended by the patient emerged as the main risk factor in the previous two weeks, an active search for cases, based on stool sample collection and testing for the presence of STEC, was carried out among the HUS case’s family, the nursery and the general population, in order to evaluate the extent of the outbreak.

Information on the occurrence of gastroenteric symptoms and exposure to potential risk factors for STEC infection, from 7 May 2015, was obtained from the HUS case’s family members (two adults and two children) and the attendees of the nursery. Stool samples from all the family contacts were repeatedly collected and tested for STEC.

At the nursery, the investigation was initiated soon after reporting of the HUS case and revealed that children (aged 13–44 months) were grouped into three different classrooms, according to age. Each group had dedicated nursery staff. All children, in particular those presenting with diarrhoea from 7 May 2015, were invited to submit stool samples for STEC examination. Stool specimens from all nursery staff members, even in the absence of symptoms, were also tested for STEC. Household contacts of STEC-positive children were also invited to submit stool samples.

A local enhanced surveillance in the general population was carried out with the support of the paediatricians practising in the same area. Patients with a clinical illness compatible with STEC infection were invited to submit stool samples for STEC microbiological testing.

Laboratory investigations

All stool samples were examined for the presence of STEC at the NRL for E. coli, as previously described [4]. Briefly, stool samples were inoculated in tryptic soy broth (TSB) and incubated at 37°C for 18 h. DNA was extracted from the culture using the Instagene DNA matrix (Biorad Laboratories, Carlsbad, CA, USA) and tested by real-time PCR assay to detect the presence of stx and eae genes and top-5 STEC serogroup (O157, O26, O111, O145, O103)-associated genes. Positive samples were streaked onto MacConkey agar plates. Single colonies resembling E. coli were tested for the presence of stx and eae genes by PCR. The STEC strains isolated were tested with O antisera against the main STEC serogroups (Statens Serum Institut, Copenhagen, Denmark) by slide agglutination. Antibiotic susceptibility testing was performed using the disk diffusion method with antimicrobial discs (Becton Dickinson, MD, USA). Antibiotic concentrations (µg) were as follows: nalidixic acid (30), perfluorocacin (5), ampicillin (10), cefixime (5), amoxicillin/clavulanic acid 2 : 1 (30), meropenem (10), chloramphenicol (30), gentamicin (10), kanamycin (30), streptomycin (10), sulfonamides (0.25), tetracycline (30), trimethoprim (5) and trimethoprim-sulfamethoxazole (1.25/23.75). The reference strain E. coli ATCC 25922 was used as a control for each experiment. Data were interpreted using the EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/v.5.0). Stool specimens were also examined for the presence of free faecal Stx by Vero cell assay [4].

Food samples were tested for STEC by ISO/TS 13136:2012.

Whole genome sequencing (WGS) of STEC strains and bioinformatics analysis

Total DNA was extracted using the E. Z. N. A. Bacterial DNA kit (Omega Bio-tek, Norcross, GA, USA) from 2 ml overnight cultures of STEC O26 strains grown in TSB at 37°C. The genome sequence of seven strains was obtained using an Ion Torrent Personal Genome Machine (Life Technologies, Carlsbad, USA) with the specific kits for library preparation through enzymatic shearing, followed by HiQ kits for One-Touch2 emulsion PCR, enrichment and sequencing with 400 bp protocols on Ion 316 and 318 chips. Whole genome sequencing of three isolates (ED1020, ED1021 and ED1022) was outsourced and performed on an Illumina MiSeq sequencer with a 250 bp paired end protocol. The targeted coverage was 30× for all the sequencing experiments performed. The sequences were uploaded to the European Nucleotide Archive [Study Accession Number: PRJEB21570; whole genome sequences: ERS1803097- ERS1803103 (ED1012-ED1018) and ERS1803086- ERS1803088 (ED1020-ED1022)].

All bioinformatics tools used are available on the Galaxy public server ARIES (https://w3.iss.it/site/aries/). The raw sequences were trimmed, both to remove the adaptors and to accept a minimum Phred value of 25. The detection of virulence genes in the genomes was performed by mapping the trimmed reads on the E. coli virulence genes database [7] using the Bowtie2 tool [8]. In silico multi-locus sequence typing was performed using the MLST.UCC database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli/) and the SRST2 software [9]. The trimmed reads were subjected to de novo
assembly using the tool SPADES [10] and the A5 pipeline [11], respectively.

The serotype was determined in silico using the NCBI BLAST+ blastn tool by aligning the assembled contigs with the database containing the sequences of serotype-associated genes of *E. coli* [12]. Genes responsible for antimicrobial resistance were investigated on the whole genome sequences with the tool ResFinder, available at the Center for Genomic Epidemiology (http://genomicepidemiology.org/index.html).

Phylogenetic analyses were conducted with the reference-free kSNP3 tool [13] to identify the total SNPs in the set of sequences analysed, as previously described [14]. The optimum value for the kmer size (21 nucleotides) was selected as that producing the highest number of unique kmers of median length in all genomes of the dataset, and was calculated using the kchooser tool included in the kSNP3 pipeline [13]. The phylogenetic tree was estimated using the maximum likelihood clustering algorithm and Figtree software (http://tree.bio.ed.ac.uk/software/figtree/) to edit the image of the dendrogram. The whole genome sequences of an additional 12 STEC strains belonging to serotype O26: H11, isolated in Italy in the period 1989–2010 [15], were included in the analysis for comparison, together with the genomes of STEC O26 strains 11368, 36708, CVM9942 and 2011C-3506 recovered from NCBI databases and used as background data (accession numbers AP010953, NZ_LDXYG00000000, AJVW00000000 and JHLS00000000, respectively).

**Case definition**
A confirmed case was defined as an individual who had attended or worked at the nursery since 7 May 2015 or was a family contact of ill nursery attendees, and whose stool tested positive for STEC O26, by culture or real-time PCR or Vero-cell assay. Cases were repeatedly tested for STEC, approximately on a weekly basis, and tested by real-time PCR and Vero-cell assay.

Duration of shedding was calculated as the number of days between the onset of diarrhoea and the date of the last sample that tested positive. Regarding asymptomatic cases, the date of the first positive sample was used rather than the day of onset. Clearance time was calculated from the same starting points until the specimen collection date of the first sample that tested negative. A specimen tested by real-time PCR was considered positive if the genes *stx2, eae* and *wzyO26* were detected.

**RESULTS**

**Investigation of nursery, family contacts and general population**
At the nursery, 11 children reported a history of diarrhoea in the previous 10 days. Seven were from classroom A (the youngest age group), which included 13 toddlers at the time of investigation. Four children were from classroom B (25 children). No children with gastrointestinal symptoms were observed among the oldest children (group C – 25 children). Hospitalization was necessary for two children from group A, due to the severity of the clinical illness. One developed HUS while the other severe bloody diarrhoea (BD). BD was also reported by another child in group A who was admitted to an emergency unit but was soon discharged after rehydration. The median duration of illness in the 11 symptomatic children was 2 days (range 2; >10 days). A diarrhoeal illness was also reported from two out of the seven staff employees. Stool samples were obtained from all 11 children reporting diarrhoea and two asymptomatic children (one each from group A and group B). Isolation of STEC O26: H11 positive for the *stx2* and *eae* genes was obtained for six children, all symptomatic, from group A. In group B, none of the four children reporting diarrhoea tested positive for STEC. One asymptomatic child from group B also had stool tested for STEC, yielding isolation of STEC O26 (*stx2+, eae+*). No children were sampled in group C. Stool samples collected from the seven nursery staff employees were all negative for STEC, except one. STEC O26 (*stx2+, eae+) was isolated from a nursery assistant from group A, who denied having had gastrointestinal symptoms in the previous four weeks. Based on microbiological results (Table 1), eight out of 20 persons in the nursery who had stool sample tested met the outbreak case definition: seven children (six symptomatic and one asymptomatic) and one asymptomatic adult. All confirmed cases and children with diarrhoea were excluded from the nursery until complete clinical recovery and at least one negative test with real-time PCR and Vero-cell assay for the presence of genes *stx, eae* and *wzyO26* and free Stx in stools, respectively (Fig. 1). The nursery was closed from 26–29 May 2015, to allow a thorough environmental cleaning and sanitisation of the equipment and facilities.

Investigation of the HUS case’s four family contacts allowed identification of three further confirmed outbreak cases. A STEC O26 (*stx2+, eae+) strain was isolated from the 5-year-old sister who had onset of illness five days before the onset of diarrhoea of the HUS case, and who had recovered at the time of the first specimen collection (21 May 2015). The case’s 3-year-old sibling and father also tested positive for STEC, two weeks later, in the absence of symptoms. STEC O26 (*stx2+, eae+) was isolated from the child while genes *stx2, eae* and *wzyO26* were detected in the father’s faeces by real-time PCR. Six other contacts of confirmed cases or symptomatic children attending the nursery were also tested for STEC. All yielded negative results, except the 3-year-old asymptomatic sibling of a confirmed case of group A, who also attended the nursery in group B. Seven other children reporting gastrointestinal symptoms and not linked to the nursery were tested for STEC, as part of the enhanced surveillance, all with negative results.

Overall, eleven confirmed cases among the nursery attendees and the family contacts were identified in this outbreak (Fig. 1). Seven of these, all children <6 years, developed
clinical illness including HUS (1 case), BD (2 cases) and watery diarrhoea (4 cases). The remaining cases, two children and two adults, were asymptomatic. No cases reported a history of recent travel, exposure to ruminants and/or environment potentially contaminated by animal manure. All cases were subjected to close clinical monitoring of general health status and a daily urine dipstick analysis for the detection of blood and proteins, until a negative stool sample test for STEC was obtained.

**Environmental and food investigation**

Inspection at the nursery revealed good general conditions of the facilities and satisfactory compliance with hygiene requirements and standard practices for food administration and childcare (e.g. use of gloves and other protective equipment; diapering; routine handwashing). Food consumed by children was provided pre-cooked by an external private service, excepting snacks and fresh fruit, which were internally prepared. STEC testing of food served in the nursery could not be performed because left-overs were no longer available. Left-over corn flour used for recreational activities was sampled and tested negative for STEC. No other sources of potential exposure to STEC could be identified.

**Microbiological monitoring of STEC shedding and treatment**

Periodic monitoring of STEC O26 faecal shedding was carried out, revealing that all confirmed cases had at least one further positive test (Fig. 1). The HUS case and the three positive family contacts tested negative for STEC after receiving oral treatment with cefixime (8 mg/kg/day for children and 400 mg/day for adults), for a seven-day period, in the absence of diarrhoea (Fig. 1). Cefixime was chosen based on the results of antibiotic susceptibility testing and because it is generally well tolerated in paediatric patients and well absorbed from the gastrointestinal tract. Antibiotic susceptibility testing showed that the STEC O26 implicated in this outbreak was sensitive to the entire panel tested. Cefixime treatment was administrated according to parental

Table 1. Number of subjects attending the nursery and family contacts with stool samples examined for Shiga toxin-producing E. coli (STEC) O26, by test results, age and presence of clinical symptoms (Italy) May–June 2015

<table>
<thead>
<tr>
<th>Age</th>
<th>Subjects tested for STEC O26 (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Symptomatic</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
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<tr>
<td></td>
<td>Negative</td>
</tr>
<tr>
<td>Nursery</td>
<td></td>
</tr>
<tr>
<td>Children, group A</td>
<td>1</td>
</tr>
<tr>
<td>Children, group B</td>
<td>4</td>
</tr>
<tr>
<td>Adults (staff members)</td>
<td>2</td>
</tr>
<tr>
<td>Cases’s family contacts</td>
<td></td>
</tr>
<tr>
<td>Children</td>
<td>2</td>
</tr>
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<td>Adults</td>
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</table>

*Includes the three-year-old sibling of a confirmed case also included among the nursery cases (group B).
agreement, in the absence of clinical symptoms, to primarily reduce the risk of transmission of STEC infection to the HUS case’s mother, who was pregnant at the time of the outbreak. Detailed information on benefits and risks connected with the antibiotic treatment of a Stx2-producing E. coli infection was provided, in particular on the risk of increasing the likelihood of events leading to the development of HUS. The same treatment was also administered, according to informed parental consent, to six other confirmed cases who continued shedding STEC O26 after clinical recovery. All cases were treated after clinical recovery, under daily clinical monitoring. The antibiotic was well tolerated by all cases and did not induce any apparent consequence on patient health. On average, children started cefixime treatment 23 days after disease onset (range 12–40). After treatment, all but one case yielded STEC O26-negative stool samples. In one case, treatment was repeated two weeks apart and STEC-negative faeces was eventually obtained. The STEC O26-positive asymptomatic employee was the only case with spontaneous clearance.

The estimated median duration of STEC O26 shedding, based on cases with available information (n=8) was 14.5 days (range 4–40). The median clearance time, based on all cases, was 22 days (range: 13–54). Due to STEC shedding, cases were subjected to a median of 22 days of exclusion from the nursery (range: 19–54). Overall a total of 253 days of exclusion was counted, corresponding to 186 working days potentially lost by nursery employees and the cases’ parents, to care for children at home. Moreover, due to nursery closure, a further 228 working days were potentially lost by the children’s families.

Whole genome sequencing of STEC O26 strains isolated from cases

All STEC O26 strains (n=10) isolated from cases were of sequence type (ST) 21. All strains harboured eae and stx2, additionally identified as the stx2a subtype, and the accessory virulence genes cif, efa1, espA, espB, espF, espJ, espP, iha, lpfA, nleA, nleB, nleC and tir and the genes ehxA, katP and toxB, typically located on the STEC large-virulence plasmids. No determinants for antimicrobial resistance were identified.

Whole genome SNPs analysis showed that all strains isolated from the outbreak cases clustered together (Fig. 2) with a total of 240 SNP alleles, uniquely present in the group and absent in the other unrelated STEC O26 strains included in the analysis. The number of SNP alleles specific to the internal nodes in the outbreak comprised between 0 and 11.

DISCUSSION

Although many outbreaks of STEC O26 infection in nurseries have been described worldwide to date [16–19], few were associated with Stx2-producing strains. Stx2-producing E. coli O26 is considered a hyper-virulent emerging clone in Europe [20]. In Italy, STEC O26 has been the most frequently reported STEC serogroup in HUS patients since the late 1990s [5]. Surveillance data from the European Union indicate that in 2016, for the first time, the number of HUS cases resulting from STEC O26 outnumbered those from STEC O157 [21]. Recently, two severe large community-wide foodborne outbreaks caused by STEC O26 (stx2+, eae+) with high proportions of children developing HUS, neurological sequelae and deaths, were reported from Italy [4] and Romania [14].

In the episode described here, the timing of disease onset and the high genetic similarity of the strains isolated from patients, suggest that person-to-person (PTP) transmission of the infection occurred in both the nursery and the families. We cannot exclude, however, that the first cases in the younger age group A had a point-source exposure to a primary source of STEC, which remains unknown. This finding confirms the premise that risk factors such as crowding, young age and attending a nursery while infected are key contributors to the inter-human transmission of STEC O26. Secondary cases via PTP transmission are frequently reported in STEC outbreaks [22], and recently PTP was identified as the most common route of dissemination of STEC infection in Irish childcare [23]. Inter-human transmission of Stx2-producing E. coli O26 in a nursery setting is of particular concern, given the severity of the associated clinical illness.

In this episode, almost half of the symptomatic cases (3/7) developed severe clinical illness such as HUS and BD, and required hospitalization. These findings are fully compatible with the virulence genes asset of the implicated STEC O26 strain, characterized by the presence of both stx2 (subtype stx2a) and eae genes. STEC strains with a similar virulence asset are considered to be of the highest potential risk for public health [24]. Characterization of STEC strains by WGS showed that the isolate possessed the complete panel of virulence genes usually associated with O26 STEC strains, including nle genes for non-LEE-encoded effectors of the type III secretion system, the efa1 gene, marker of the pathogenicity island (OI-122) and the virulence genes typically harboured on STEC virulence plasmids [25]. It also allowed assigning the strains to ST21, the most represented ST of STEC O26 worldwide [20]. Additionally, genetic comparative study of STEC O26 isolates and whole genome SNP analysis showed that all strains were grouped into the same cluster, confirming that a single clone was implicated in the outbreak.

The outbreak was identified thanks to the prompt investigation of a single case of HUS, confirming that this event is a sensitive sentinel of a possible broader circulation of STEC in the community. Our findings underline the importance of actively screening contacts of HUS case patients, given the likelihood of secondary cases. This may dramatically improve the identification of STEC outbreaks in the community [26]. The detection of cases of STEC infection in the early course of the disease is also critical to reducing the individual burden of STEC infection, by increasing the options for supportive care.
Long-lasting STEC O26 shedders were identified in our study, with symptomatic and younger cases shedding for longer. As in other nursery outbreaks [19, 27–31], shedders were also identified among contacts actively screened for STEC in the absence of symptoms. This finding challenges the effectiveness of those control policies based on exclusion from nurseries of attendees positive for STEC [32], when asymptomatic children and adults are not simultaneously tested for STEC. Estimates from the literature indicate that the length of time involved in shedding of STEC may be great, with important socio-economic consequences for the families involved, who may experience difficulties in complying with exclusion policies [30, 32, 33]. In our study, although the number of cases was relatively small and the median duration of STEC O26 shedding and the clearance time were shorter than in other nursery outbreaks, the potential societal costs to cover for the working days lost (414 days overall) were not negligible. In Ireland, a median STEC clearance time of 39 days (maximum 283 days) in children <6 years was reported, with asymptomatic cases clearing faster [34]. In England, a median of 31 days of STEC shedding was estimated in children <5 years attending childcare facilities, with 24% shedding for more than six weeks [30]. Similar estimates were reported in childcare outbreaks involving STEC O26 in Argentina, Ireland and the USA [19, 28, 33, 35, 36]. Shorter times (6/13 days) were observed only in Japan [29]. One limitation of our study, however, was that asymptomatic carriers might have been overlooked, as not all children in the nursery were tested for STEC. Moreover, a possible occurrence of intermittent STEC shedding could not be properly evaluated, as no repetition of STEC testing after a negative result was possible.

In this outbreak, patients were treated with cefixime after remission of symptoms to reduce the duration of STEC shedding, aiming at limiting the spread of a potentially life-

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**Fig. 2.** Phylogenetic analysis based on whole genome SNP comparison. The tree was obtained using the kSNP3 tool to identify the total SNPs in the set of sequences analysed. The phylogenetic tree was estimated based upon those SNPs using the maximum likelihood clustering algorithm. The different groups of O26:H11 STEC strains are labelled according to the following colour legend: blue for sequences of strains isolated outside Italy downloaded from NCBI; green for strains isolated from Italy in the period 1989–2010; and red for strains included in the outbreak described in the present study. The tips labels include the names of the strains, the corresponding case number (only for strains from outbreak patients) and the strain-specific allele count, separated by underscores. The scale indicates the legend for branch length, expressed in terms of changes per number of SNPs.
threatening infection. Antibiotic treatment of STEC infections is highly controversial, and concerns exist about the possible increase of Stx release triggering the pathogenetic cascade of HUS [37]. A recent meta-analysis conducted by Fredman et al. [37], however, failed to find significant association between early illness antibiotic administration and HUS, leaning towards the hypothesis that such treatment may increase the risk of HUS. However, ciprofloxacin administration in patients with STEC O104:H4 shedding was not associated with an increased risk of HUS [38], and fosfomycin treatment, in a non-randomized prospective study carried out in children with STEC O157 infection, was reported to lower the risk of developing HUS [39]. Evidence is available in the literature that the administration of antibiotic at a late stage of the clinical course may not result in recurring or late HUS. In particular, azithromycin was found to successfully reduce the duration of STEC O104:H4 shedding [40]. In our study, we observed in all patients that the shedding of STEC O26 ceased following the administration of cefixime. However, this finding should be interpreted with caution, given that only a single negative laboratory test for STEC could be performed to follow up the end of shedding. Moreover, no practical conclusion on the effectiveness of cefixime treatment can be drawn from our observations, which could not conclusively prove that microbiological clearance was attributable to the treatment itself, given the lack of untreated control STEC shedders and the randomization of treatment. Cefixime administration was well tolerated by all patients, with no apparent effect on their health status. It is possible that STEC bacterial load in the intestine at this late stage of the infection may be insufficient to trigger events leading to HUS, or that the host immune response may be by that stage effective in preventing further complications.

In conclusion, infections by Stx2-producing E. coli O26 represent an emerging public health issue and the occurrence of outbreak in nurseries is of particular concern, given the high probability of infection progressing to severity and resultant secondary cases. When sporadic HUS is reported in small children, the potential of an underlying outbreak should always be considered, and active screening for other STEC cases should include both symptomatic and asymptomatic subjects and be extended to the case’s household. This study further underlines the importance of routinely implementing preventive policies in nurseries based on high-level standards of hygiene, nursery staff training and exclusion of persons with confirmed STEC infection.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
Patients were treated with antibiotic upon patients’ (or patient’s parents) informed consent on the benefits and risks connected to the antibiotic treatment of a STEC infection.

References

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