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Real-time genomic investigation underlying the public health response to a Shiga toxin-producing *Escherichia coli* O26:H11 outbreak in a nursery

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SUMMARY

Shiga toxin-producing *Escherichia coli* (STEC) is a significant cause of gastrointestinal infection and the haemolytic-uremic syndrome (HUS). STEC outbreaks are commonly associated with food but animal contact is increasingly being implicated in its transmission. We report an outbreak of STEC affecting young infants at a nursery in a rural community (three HUS cases, one definite case, one probable case, three possible cases and five carriers, based on the combination of clinical, epidemiological and laboratory data) identified using culture-based and molecular techniques. The investigation identified repeated animal contact (animal farming and petting) as a likely source of STEC introduction followed by horizontal transmission. Whole genome sequencing (WGS) was used for real-time investigation of the incident and revealed a unique strain of STEC O26:H11 carrying *stx2a* and intimin. Following a public health intervention, no additional cases have occurred. This is the first STEC outbreak reported from Israel. WGS proved as a useful tool for rapid laboratory characterization and typing of the outbreak strain and informed the public health response at an early stage of this unusual outbreak.

Key words: *Escherichia coli*, haemolytic-uremic syndrome, investigation, outbreak, paediatric, Shigatoxin, whole genome sequencing.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a significant cause of gastrointestinal infection, ranging from mild diarrhoea to severe bloody diarrhoea. Of STEC infections (O157:H7), 5–10% develop the haemolytic-uremic syndrome (HUS), which is a

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leading cause of paediatric renal failure [1]. Progression to HUS occurs most commonly in younger children [2]. Human STEC infection is commonly associated with exposure to contaminated food but the role of contact with infected animal excretions is increasingly being recognized [3].

STEC strains carry the *stx1* and/or *stx2* genes that encode for the respective Shiga toxin genes. There are three variants for *stx1* and seven variants for *stx2* [4], which contribute to the varying severity of STEC infection, together with other virulence determinants such as the *eae* gene encoding the intimin protein of the locus of enterocyte effacement [5]. Culture-based screening for STEC using media such as SMAC or chromogenic agars is much less sensitive for non-O157 STECs, and therefore molecular detection assays for *stx1* and *stx2* in faecal samples have been widely implemented [6].

STEC infections should prompt an epidemiological investigation aimed at identifying and controlling the source of infection. Recently, whole genome sequencing (WGS) has been shown to be very useful for the typing of STEC [7] with increased discriminatory power as compared with standard techniques such as pulsed-field gel electrophoresis (PFGE) and multilocus variable number tandem repeats analysis [8]. Moreover, WGS also has the potential for replacing several frontline and reference microbiology assays as a 'one-stop-shop' for rapid pathogen characterization.

On 25–27 April 2016, admission to the regional hospital of two young infants with HUS (index cases) residing in the same rural community (Kibbutz) in Southern Israel and attending the same nursery at the local day-care centre, prompted an immediate epidemiological investigation by the Southern District Health Office. The aim of this paper is to describe the epidemiological and genomic investigation performed in real time during an unusual STEC outbreak, which is also the first being reported from Israel.

METHODS

Epidemiological investigation

Questioning, aiming at identifying plausible risk factors, involved parents of the two HUS cases as well as parents of other infants attending the nursery and the caregivers. Case finding in the community was carried out by alerting parents and staff members at the community as well as health personnel at local and regional primary care clinics and also the regional hospital. Possible risk factors and exposures were assessed using standard questionnaires, addressing close contacts, travel, food and water consumption and animal contact.

A case definition was subsequently constructed and classified into four groups: (1) a confirmed case was defined as any individual living in the community with a compatible gastrointestinal illness and/or HUS and laboratory evidence for STEC infection (PCR and/or culture) over the last month; (2) a probable case was defined as any individual with current or recent (up to 1 month) compatible and evident gastrointestinal illness but no laboratory evidence for STEC infection; (3) a possible case was defined as any individual with a reported history of gastrointestinal illness in the recent month; (4) laboratory-confirmed excretion of STEC in the absence of any reported illness was defined as carriage.

Laboratory investigation

Fresh stool samples obtained from patients and contacts (nursery, adjacent day-care centres and close contacts) were transported within 24 h to the Central Laboratories of the Ministry of Health (national reference laboratory for STEC) for a total of 120 samples. Samples were tested for the presence of STEC by means of standard culture on selective and non-selective agar plates (Sorbitol MacConkey, CHROMagar STEC, CHROMagar Orientation (CHROMagar Company, Paris, France)), colony picking and molecular screening of up to 15 suspected colonies using PCR for *stx1* and *stx2* and intimin (*eae*) [9–12]. PFGE was performed per the PulseNet protocol using *Xba*I. In parallel, direct molecular testing of stool was performed using the same PCR assay following DNA extraction using the easyMAG platform (bioMerieux, France) and with the addition of an internal inhibition control (*gfp*). Faecal samples obtained from hospitalized patients linked to the outbreak and suspected to be positive for STEC were referred for confirmatory testing at the national laboratory.

Environmental testing was performed on freshly voided animal faeces obtained from potential sources using the same approach, for a total of 111 samples. Sampling of the petting zoo involved freshly voided animal faeces and covered the entire area of the zoo premises as well as the different animal species present in the zoo. Sampling of cowsheds involved collection of fresh cow manure from all farms and cowsheds in

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the local area, including but not restricted to those identified through questionnaires. Water sampling was performed by obtaining drinking water from the main water line and testing for the presence of coliforms using standard water quality methods.

WGS analysis

Starting in the second week of the outbreak (10 May 2016), all STEC isolates were analysed by rapid WGS. DNA extraction was carried out with a manual kit (Geneaid, Taiwan). DNA libraries were prepared at the University Medical Centre Groningen, The Netherlands, using Nextera XT according to manufacturer's protocols (Illumina, San Diego, California, USA) and then run on a Miseq (Illumina) for generating paired-end 250-bp reads aiming at a coverage of at least 50-fold. Data analysis was performed as previously described [13]. Raw reads were quality checked and trimmed, followed by *de novo* assembly using CLC Genomic Workbench v. 9.5.2 (CLC bio, Denmark) and gene annotation using RAST v.2. O and H serogrouping, determination of *eae* and *stx* variants and additional virulence genes, and calling of *in silico* multi-locus sequence type (MLST) [14] was performed using online tools (SerotypeFinder 1.1, VirulenceFinder 1.5 and MLST Finder 1.8) at the Center for Genomic Epidemiology server (http://www.genomicepidemiology.org/). For the CGE server, the threshold of ID was set to 85% and the percentage of minimum overlapping gene length to 60%.

Fifteen genomes were included in the phylogenetic analysis, consisting of 11 isolates sequenced in this study and four additional STEC O26:H11 genomes downloaded from NCBI which represent different geographical regions, isolation dates, genotype and pathotype (Table 1). Phylogenetic analysis performed using the gene-by-gene approach was carried out in SeqSphere^{+} v3.0 (Ridom GmbH, Münster, Germany). For this, an *ad hoc* core genome MLST (cgMLST) scheme was used as described previously [13]. Briefly, the genome of *E. coli* O26:H11 strain (NC_013361·1) was taken as a reference genome and 12 additional *E. coli* genomes were used as query genomes to extract open reading frames (ORFs) using the MLST+ Target Definer 2.1.0 of SeqSphere^{+}. The list of genomes appears in a Supplementary File. Only the ORFs without premature stop codon and ambiguous nucleotides from contigs of assembled genomes were included. The genes shared by the genomes of all isolates analysed in this study, as listed in Table 1 and shown in Figure 2, were defined as the core genome for phylogenetic analysis. According to the sequence identity of the genes, a numerical allele type was assigned by SeqSphere and the allelic profiles generated by the combination of all alleles in each genome were considered for constructing the minimum-spanning tree and measure allelic differences between the isolates. Genomic sequences have been deposited (accession numbers appear in Table 1).

An additional analysis of single nucleotide polymorphisms (SNPs) was performed for the six isolates belonging to the outbreak strain and using the O26:H11 NC_013361 reference genome. The analysis was carried out by CSI Phylogeny with the following settings: a minimum depth of 10 at SNP positions, a minimum relative depth of 10% at SNP positions, a minimum distance of 10 bp between SNPs (prune), a minimum quality of 30, a minimum SNP quality of 25 and a minimum Z-score of 1·96. The percentage of the reference genome covered by all isolates was 88·2% (5 022 775 bp).

RESULTS

Investigation of the outbreak setting identified 14 young infants at age range of 6–18 months, which were regularly attending the nursery. In total, there were four definite cases (three of whom had HUS), one probable case, two possible cases and five carriers (three in the nursery and two in adjacent classes). Faecal samples obtained from the two index HUS cases were PCR-positive for *stx2*, one of whom was also culture-positive (the other patient had received empirical antibiotics). Out of the 14 infants, two had a recent history of gastroenteritis which resolved 1–2 weeks earlier and were thus classified as possible cases. An additional three infants had symptomatic gastroenteritis and were subsequently admitted to the hospital for further diagnosis and treatment. Two symptomatic infants were STEC *stx2*-positive, one of whom later developed HUS during the hospital course and the third was a probable case. A sixth infant had a very brief gastrointestinal episode reported but was STEC-negative (possible case). The timeline of the outbreak is depicted in Figure 1 and the clinical features of hospitalized cases are summarized in Table 2. Notably, all three HUS cases resolved with supportive therapy without sequelae.

The public health response to the STEC outbreak was mounted within 2 days following the reporting of HUS cases to local health authorities over the

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Table 1. *Escherichia coli isolates analysed by whole genome sequencing in this study*

Isolate no.	Country/year	Source	Clinical illness	Serotype	*stx* subtype	Sequence type	No. of contigs	No. of reads	Coverage	Accession no.
1	Israel/2016	Human	HUS	O26:H11	*stx*2a	New	235	1 675 537	64	MRVR00000000
2	Israel/2016	Human	HUS	O26:H11	*stx*2a	New	183	1 579 103	55	MRVS00000000
4	Israel/2016	Human	Diarrhoea	O26:H11	*stx*2a	New	226	4 170 353	92	MRVT00000000
6	Israel/2016	Human	Asymptomatic	O26:H11	*stx*2	New	224	3 938 003	89	MRVU00000000
7	Israel/2016	Human	Asymptomatic	O26:H11	*stx*2a	New	219	5 088 810	113	MRVV00000000
15	Israel/2016	Human	Asymptomatic	O26:H11	*stx*2a	New	245	1 846 971	69	MRVW00000000
16	Israel/2016	Human	Asymptomatic	O157:H7	*stx*1a + *stx*2c	ST11	212	1 595 325	62	MRVX00000000
440	Israel/2015	Human	HUS	O71:H8	*stx*1a + *stx*2c	ST6	205	5 659 470	126	MRVY00000000
514	Israel/2016	Calf	NA	O174:H21	*stx*2c	ST677	222	1 320 151	49	MRVZ00000000
573	Israel/2016	Cow	NA	O171:H29	*stx*2d	ST515	128	1 502 546	61	MRWA00000000
587	Israel/2016	Cow	NA	O177:H25	*stx*2a + *stx*2c	ST659	249	1 646 472	67	MRWB00000000
11368	Japan/2001	Human	Diarrhoea (outbreak)	O26:H11	*stx*1a	ST21	NA	NA	NA	AP010953
2011C-3506	USA/unknown	Human	Unknown	O26:H11	*stx*1a	ST21	NA	NA	NA	JHLS00000000
36708	France/2013	Human	HUS	O26:H11	*stx*2a	ST29	NA	NA	NA	NZ_LDXG00000000
CMV9942	1983	Cow	NA	O26:H11	*stx*1a	ST21	NA	NA	NA	AJVW00000000

HUS, haemolytic-uremic syndrome; ST, sequence type; cgMLST, core genome multi-locus sequence typing; NA, not applicable.

weekend. It included immediate closure of all local day-care centres, active case finding and microbiological surveillance. Upon questioning, possible risk factors included extensive contact with various animals at a local petting zoo, inhabited by different animal species, and frequent contact with animal husbandry, particularly calves and young cattle in dairy farms (several hundred heads of cattle) situated in proximity with residential premises. This appeared to have occurred directly through farm visits that took place at least once per week and in which all nursery infants participated. Visits involved direct animal contact at cowsheds. Family members of some infants were also working in the local animal farms. No suspected foodborne exposure was evident, especially since infant nutrition largely involved baby formula. Tests of drinking water revealed no faecal contamination.

Stool samples obtained from the six asymptomatic infants in the nursery revealed three STEC-positive infants (carriers). Eight women working as caregivers in the nursery were asymptomatic and tested negative, as were 10 sampled adults, including four with inconsistent gastrointestinal symptoms and six relatives of the nursery infants. Microbiological surveillance also included 39 children aged 2–5 years attending the adjacent day-care centres, of whom two were STEC-positive. One of those was a sibling of a carrier from the nursery. Of 25 representative samples obtained from the petting zoo, covering most of its area and resident animals, one sample (related to a calf) was STEC-positive. Of 86 samples obtained from three local cowsheds, two samples obtained from two different farm locations were STEC-positive.

An educational intervention was performed, starting 1 May 2016, focusing on proper personal and hand hygiene, elimination of direct animal contact through temporary prohibition of farm and zoo visits and reiteration of hygiene practices among the farm workers. After thorough cleansing, the day-care system was re-opened. STEC-positive children were followed up clinically and microbiologically at least twice weekly and were excluded from the nursery until two consecutive stool samples taken at least 48 h apart were PCR-negative for STEC. The duration of asymptomatic STEC shedding in those individuals ranged between 2 and 6 weeks (Fig. 1).

A total of 11 non-duplicate isolates were subject to rapid NGS and are summarized in Table 1. The mean coverage, N50 value and number of contigs were 76

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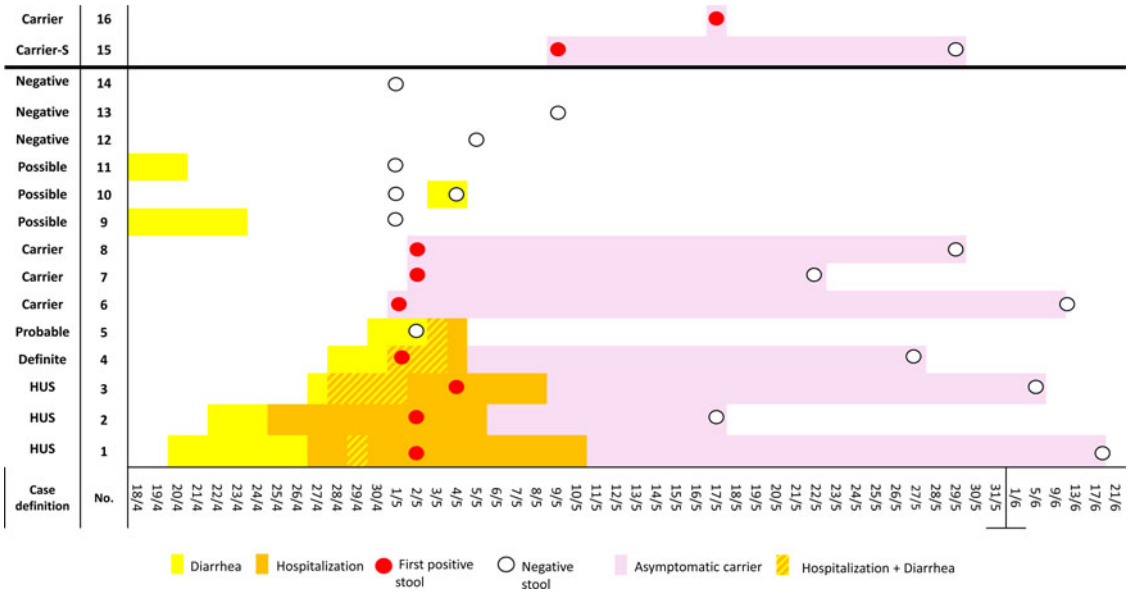

Fig. 1. Description of the outbreak. The illustration includes 14 infants attending the affected nursery and additional two children attending other day-care centres found to be carriers, including three cases of haemolytic-uremic syndrome, one definite, one probable and three possible cases with symptomatic diarrhoea, five carriers (one of whom was a sibling – carrier-S) and three negative individuals. Gastrointestinal symptoms (yellow), hospital stay (amber) and faecal excretion of the outbreak strain (purple) are shown over time. Red and white circles correspond with first positive and first negative stool samples, respectively.

(49–126), 92 297 (range 52 061–126 505) and 213 (range 128–249), respectively. WGS revealed the outbreak strain was an O26:11 STEC strain, harbouring the *stx2a* toxin variant encoding gene and the intimin gene (*eae*). A list of additional virulence genes appears in the Supplementary Table S1. MLST performed *in silico* revealed that the outbreak strain was a new sequence type (allelic profile: X, 4, 12 ,16, 9, 7, 7), being a single-locus variant of the ST21 international clone. The combination of O26/*stx2a*/ST21-like has not been previously reported.

Analysis of isolates by cgMLST is presented in Figure 2. All isolates belonging to the outbreak strain (all stool isolates from symptomatic and asymptomatic individuals) tightly clustered together, showing up to one allele difference. These isolates also belonged to the same pulsotype per PFGE. A historical isolate from a case of HUS reported a year before the outbreak from an adjacent village was found to be unrelated (an O71:H8, ST6). The three bovine isolates recovered through environmental sampling were also unrelated to the outbreak strain (O17:H21/ST677; O171:H29/ST515 and O177:H25/ST659). Interestingly, the STEC recovered from one of two carriers found among older children was an O157:H7, ST11 strain. During 6 months of follow-up, no additional STEC cases were reported in the area.

Apart from the gene-by-gene approach, a SNP analysis on the core genome of the six outbreak isolates was performed. Within the shared genome (5 022 775 nt), 7–35 SNPs were found between the outbreak isolates, indicating that they were almost identical given the extremely dynamic nature of STEC.

DISCUSSION

We report an investigation of an outbreak of STEC in a nursery at a rural community, involving a high attack rate of HUS among symptomatic individuals. HUS related to STEC infection is very uncommon in Israel with only a handful of cases reported annually [15]. Notably, this is the first outbreak of STEC documented in Israel. An epidemiological investigation implicated contact with animal husbandry as a likely source of introduction of STEC into the nursery and outbreak initiation and the dynamics of the outbreak suggest an important role for secondary person-to-person transmission in propagation of the outbreak.

STEC O26:H11 is one of the 'big six' STEC serotypes and the most common non-O157 STEC causing infection in Europe [16]. It is a highly diverse group containing inter-mixed clusters of isolates from human and animal origin [17]. Over recent decades,

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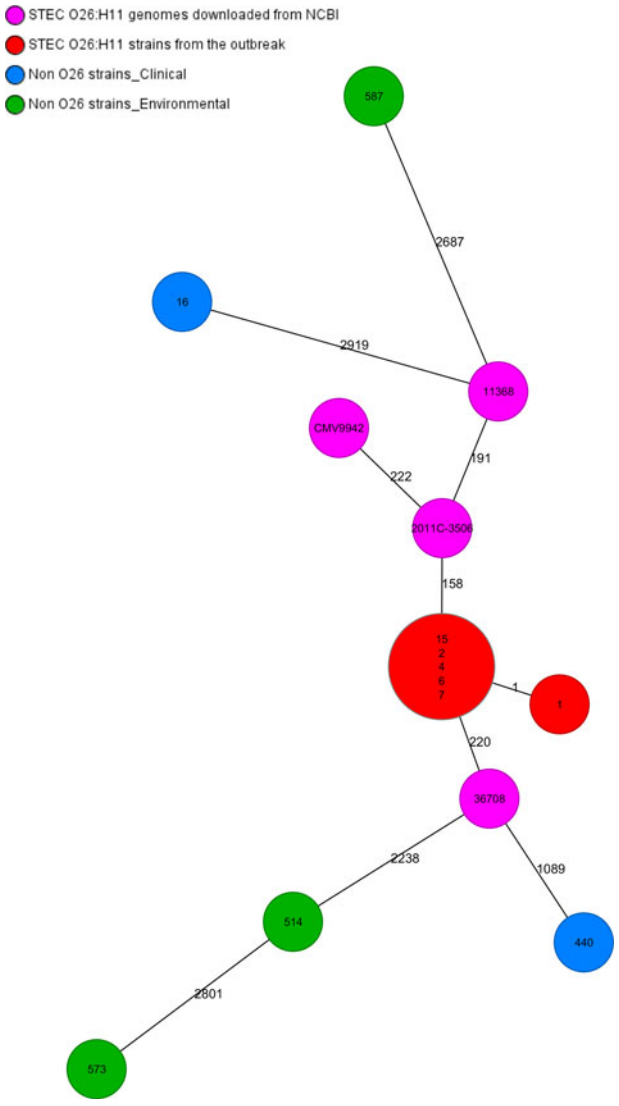

Fig. 2. Phylogenetic analysis using a gene-by-gene approach. A minimum spanning tree of 15 STEC isolates subject to WGS, generated using an *ad hoc* core genome of 2962 genes. The analysis included clinical and environmental isolates (green) recovered during the outbreak, an isolate from a historic HUS case in the region (blue, #440) and international O26:H11 reference strains (pink). All O26 isolates belonging to the outbreak strain are tightly clustered together (red). Isolates found during environmental sampling and comprising three bovine isolates are indicated in green.

a shift between *stx1*-positive and *stx2*-positive STEC O26 strains has been noted, with *stx2*-positive STEC O26 being considered highly virulent [18] and showing a predilection for causing diarrhoea and HUS, especially in young children [16, 19]. Moreover, strains harbouring the *stx2a* variant in combination with intimin are more likely associated with HUS [20].

STEC outbreaks involving nurseries or child day-care centres have been reported with varying rates of HUS. There are several reports of STEC O26:H11 infection in day-care centres, including the USA, UK, Germany and Japan [21–24]. Clusters of paediatric STEC occurring in day-care settings emphasize the role of secondary transmission in those centres as well as within families [10, 25, 26]. Such STEC outbreaks have been reported to involve prolonged shedding of STEC among clinical cases [27] but less is known about the duration of shedding in asymptomatic carriers. In the outbreak reported herein, shedding among asymptomatic carriers lasted 3–5 weeks and exclusion of excreting carriers from the nursery was associated with significant parental distress, as reflected through communications to the District health office directly by parents and indirectly by staff of local authorities, but was probably instrumental in curbing the outbreak and preventing further infections.

WGS is increasingly being used as a highly discriminative tool for investigating STEC outbreaks [28], mainly as a retrospective tool either corroborating standard typing techniques or offering improved typing resolution [24, 29, 30]. WGS utilized via rapid protocols may be performed in comparable or even faster turnaround times such as standard techniques (e.g. PFGE or MLST) but is advantageous, not only due to its improved resolution but also as it allows to extract additional important information from the genomes. This may prove helpful for both clinical and reference settings. The current investigation utilized rapid WGS performed in real time at a very early stage of the outbreak in a non-reference microbiology setting. The use of WGS generated timely information including calling of the serotype and ST *in silico*, typing of the *stx* toxin, assessment of over 20 virulence genes as well as phylogenetic analysis for genomic epidemiology using cgMLST and SNP methods. Such information is usually produced at later stages of outbreak investigation, only at reference laboratory level and requires multiple laborious tests to be performed over a longer timeframe. In the current outbreak, rapidly refuting the association between the nursery cases and a case that have occurred in that area a year before was important for risk communication purposes and obviated the need for performing additional surveillance at the adjacent village. While other methods (e.g. PFGE) may be used to rule out epidemiological links, performing WGS is advantageous as in cases of identical

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Table 2. *Characteristics of symptomatic infants hospitalized during the outbreak*

No.	Age (months)	Underlying conditions	Nutrition	Presenting symptom	Antibiotic therapy	Fever	HUS	ICU	Stool culture	*stx2* PCR	Therapy	LOS	Outcome
1	10	Preterm	Formula and food	Watery diarrhoea	Azithro	Yes	Yes	Yes	Neg	Pos	AH, FFP, PC, IV, D	11	Cure
2	7	None	Formula	Bloody diarrhoea	None	No	Yes	Yes	Pos	Pos	AH, FFP, PC, IV, D	14	Cure
3	4	None	Formula	Bloody diarrhoea	None	No	Yes	No	Pos	Pos	PC, IV, D	11	Cure
4	8	None	Lactation	Watery diarrhoea	None	Yes	No	No	Pos	Pos	IV	4	Cure
5	11	None	Formula and food	Watery diarrhoea	None	No	No	No	Neg	Neg	IV	2	Cure

HUS, haemolytic-uremic syndrome; ICU, intensive care unit; LOS, length of hospital stay; AH, anti-hypertensive therapy; FFP, fresh frozen plasma; PC, packed cells; IV, intravenous fluids; D, diuretics.

PFGE, it provides an immediate higher resolution that could inform public health measures.

Contact with farm animals, such as calves and lambs, has repeatedly been implicated in STEC outbreaks [28, 30, 31]. STEC outbreaks involving direct animal contact have also been reported to be associated with increased rates of HUS [1] but the persistence of STEC strains, particularly O26 in cow manure and slurries [32] may contribute to transmission. Using WGS we were able to demonstrate co-circulation of several unrelated clones of STEC in that rural community, suggesting multiple transmission opportunities repeatedly occurred in this ecological niche. This is in agreement with previous reports of multi-source STEC infections [33]. The occurrence of an HUS case in a similar adjacent community a year earlier suggests circulation of STEC in that setting is not new. That one particular virulent clone may become successful in establishing ongoing transmission and illness has been demonstrated in similar settings [30]. This phenomenon may be related to virulence and pathogenicity features yet to be discovered, the inoculum, number of introduction/transmission opportunities, the susceptibility to infection of patient groups such as young infants and/or nursery hygiene practices (contributing to secondary transmission). In our case, the very close proximity between premises and farm animals, continuous movement of vehicles and people to and from the farms and inadequate environmental control measures were possible contributing factors to the outbreak.

WGS is transforming public health microbiology with its unprecedented capability for epidemiological typing and has been successfully used for investigating the genomic epidemiology of STEC. Nevertheless, WGS remains to be validated and standardized before it could be globally implemented for routine use in public health [34]. Several pioneering examples already exist for using WGS routinely for surveillance and these should pave the way for integration of WGS even in frontline settings. The use of WGS to characterize STEC outbreak strains, by combining rapid epi- and patho-typing, has a great potential for shortening laboratory turnaround and decision-making times in outbreak settings and favours its application in real time during investigations.

SUPPLEMENTARY MATERIAL

The supplementary material for this article can be found at https://doi.org/10.1017/S0950268817001923.

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DECLARATION OF INTEREST

None.

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