SHORT REPORT
Cluster investigation of mixed O76:H19 Shiga toxin-producing Escherichia coli and atypical enteropathogenic E. coli infection in a Spanish household

S. SÁNCHEZ1*, M. GARCÍA CENOZ2, C. MARTÍN3, X. BERISTAIN3, M. T. LLORENTE1 AND S. HERRERA-LEÓN1
1 Centro Nacional de Microbiología, Instituto de Salud Carlos III, Majadahonda, Spain
2 Instituto de Salud Pública de Navarra, Pamplona, Spain
3 Complejo Hospitalario de Navarra, Pamplona, Spain

Received 1 April 2013; Final revision 29 May 2013; Accepted 9 July 2013; first published online 2 August 2013

SUMMARY
A Spanish household was identified through a Public Health follow up on a Shiga toxin-producing Escherichia coli (STEC)-positive 14-month-old girl reporting bloody diarrhoea, with the four household members experiencing either symptomatic or asymptomatic STEC and/or atypical enteropathogenic E. coli (aEPEC) shedding. In total, two different O76:H19 STEC strains and six aEPEC strains belonging to multiple serotypes were isolated and characterized in the household during a 5-month period. Prolonged asymptomatic shedding of O76:H19 STEC and O51:H49 aEPEC was detected in two family members. Although there was no conclusive evidence, consumption of vegetables fertilized with sheep manure was the suspected source of infection. This study highlights the risk of cross-infections posed by prolonged asymptomatic carriage and close household contact between family members, and illustrates the importance of molecular epidemiology in understanding disease clusters.

Key words: Atypical enteropathogenic E. coli (aEPEC), household transmission, prolonged shedding, sheep manure, Shiga toxin-producing Escherichia coli (STEC).

Shiga toxin (Stx)-producing Escherichia coli (STEC) can cause a broad spectrum of clinical symptoms in humans, ranging from haemolytic uraemic syndrome (HUS) to mild non-bloody diarrhoea or even asymptomatic carriage [1]. In particular, non-O157 STEC are considered emerging pathogens, despite being currently underrecognized because methods for their detection and isolation are not widely implemented. STEC infection is commonly acquired through the consumption of faecally contaminated food or water, through direct or indirect contact with animal carriers, mainly ruminants, or via secondary person-to-person transmission [1]. Enteropathogenic E. coli (EPEC) are one of the most common causes of infantile diarrhoea worldwide and are further divided into two subtypes, typical and atypical EPEC, depending on the presence or absence of the bundle-forming pilus (BFP) [2]. In particular, atypical EPEC (aEPEC) are more prevalent compared to STEC in industrialized countries, where aEPEC are frequently identified both in children with diarrhoea and in healthy children [2, 3]. Although there is no evidence of direct transmission from animals to humans, animal carriers have been suggested to be reservoirs for aEPEC infecting humans [2].

On 30 May 2012, the clinical microbiological laboratory of the Hospital Complex of Navarre (CHNa) submitted a Stx1-positive stool culture to
STEC isolates were tested for the additional virulence API 20E system (bioMérieux, France). All recovered further con to obtain the STEC or EPEC isolate, which was E. coli by PCR [5]. When culture tested positive, individual sequence, single stool samples from the four household members, consisting of the index girl, her mother (age 32 years), father (33 years) and older sister, were submitted to the SNLR and screened the stool samples from the follow-up on the family members, not sharing the same household but consuming the suspected vegetables, were also screened for STEC and EPEC on day 74. However, neither the suspected vegetables nor the sheep herd providing manure for the family garden could be sampled and no further action was taken.

At the CHNa, the production of Stx1 and Stx2 toxins in the stool culture from the index girl was investigated by using the Duopath Verotoxins immunochromatographic rapid test (Merck, Germany). The stool culture from the index girl, as well as all the stool samples from the follow-up on the family members, were submitted to the SNLR and screened for STEC and EPEC. For this purpose, samples were cultured on MacConkey agar (Becton Dickinson, USA) after a broth enrichment step. Bacterial growth from the sample was cultured on MacConkey agar (Becton Dickinson, USA) after a broth enrichment step. E. coli-like colonies were tested using the same PCR to obtain the STEC or EPEC isolate, which was further confirmed biochemically as E. coli by the API 20E system (bioMérieux, France). All recovered STEC isolates were tested for the additional virulence genes ehxA and subAB by PCR [5], and the identification of stx1 and stx2 subtypes was performed using a recently developed PCR-based method [6]. All recovered EPEC isolates were tested for the presence of bfpA gene [7], in order to classify them as typical or atypical EPEC. STEC and EPEC isolates were further typed by conventional O:H serotyping, genetic H serotyping by PCR amplifying and sequencing the fiIC gene [8] in non-motile isolates (results denoted in square brackets) and pulsed-field gel electrophoresis (PFGE) with XbaI according to the PulseNet protocol for E. coli O157:H7 [9]. Additionally, STEC isolates were typed by multilocus sequence typing (MLST) [10]. Cluster analysis was performed using the Dice coefficient and the unweighted pair-group method with arithmetic averages (UPGMA) in InfoQuestFP v. 4-5 (Bio-Rad, UK).

On day 36, no further STEC were isolated from the girl’s stool sample, but EPEC isolates were obtained. STEC and EPEC isolates were obtained from the father’s stool sample and a single STEC isolate was identified in the mother’s stool sample. A single EPEC isolate was obtained from the older sister (Table 1). During the follow-up period, on day 74 the father still presented with STEC and the girl with EPEC. On day 137, only the girl with EPEC remained positive (Table 1). Finally on day 201, stool samples from all four family members tested negative for both STEC and EPEC. All other relatives were found to be negative for STEC and EPEC on day 74. All recovered STEC isolates tested negative for eae but positive for ehxA and subAB and belonged to serotype O76:H19/H19 (Table 1). Subtyping of the stx genes resulted in the detection of subtypes stx2b and/or stx1c (Table 1). The EPEC isolates belonged to multiple serotypes (O8:H25, O51:H49, O168:H6, O180:H2, ONT:H6, ONT:H29) and were classified as aEPEC, as all of them tested negative for bfpA (Table 1).

PFGE results showed two different profiles for the O76:H19 STEC isolate from the symptomatic girl (profile 2) and for the three O76:H19 STEC isolates from her asymptomatic parents (profile 1) (Fig. 1). It has been widely demonstrated that the loss of stx genes due to spontaneous curing of stx-carrying phages in STEC clinical isolates involves changes in the PFGE patterns, with isolates differing by 2–5 bands [11]. As the STEC O76:H19 isolates in the present study differed only by five bands (88.4% similarity), the two different PFGE profiles found in them could be explained by the loss of the
stx2b-carrying phage from profile 2 (stx2b-positive) to profile 1 (stx2b-negative). Nevertheless, STEC O76:H19 isolates also differed in their motility (the single profile 2 isolate was non-motile while all three profile 1 isolates were motile), thus contradicting the idea that all STEC O76:H19 isolates in the present study could belong to a single strain. Moreover, MLST analysis classified all O76:H19/[H19] STEC isolates as belonging to sequence type 675 (Table 1), as do the O76:H19 reference strain (HUSEC039) in the German collection of representative HUS-associated enterohaemorrhagic E. coli (HUSEC) [4]. The seven aEPEC isolates revealed six different PFGE profiles, with one being identified on two occasions, 101 days apart, in the girl’s stool samples (profile 6) (Table 1, Fig. 1).

This study represents the first description of both an O76:H19 STEC infection and a mixed infection with aEPEC in Spain. In total, two different STEC strains and six aEPEC strains were isolated and characterized.

Table 1. Characteristics and molecular typing results for STEC and aEPEC isolates from symptomatic and asymptomatic family members

| Isolate | Family member | Day collected* | Serotype† | Virulence genes profile | Pathogenic group | PFGE profile | MLST |
|---------|---------------|----------------|-----------|-------------------------|-----------------|-------------|------|
| 1482/12 Girl‡ | 0 | O76:[H19] | stx1c, stx2b, ehxA, subAB | STEC | 2 | ST675 |
| 1545/12 Girl | 0 | O168:H6 | eae | aEPEC | 5 | n.d. |
| 1898/12 Girl | 36 | O8:H25 | eae | aEPEC | 3 | n.d. |
| 2188/12 Girl | 36 | O51:H49 | eae | aEPEC | 6 | n.d. |
| 1899/12 Mother | 36 | O76:H19 | stx1c, ehxA, subAB | STEC | 1 | ST675 |
| 1901/12 Father | 36 | O76:H19 | stx1c, ehxA, subAB | STEC | 1 | ST675 |
| 2189/12 Father | 36 | ONT:H6 | eae | aEPEC | 7 | n.d. |
| 1903/12 Older sister | 36 | O180:[H2] | eae | aEPEC | 4 | n.d. |
| 2376/12 Girl | 74 | ONT:H29 | eae | aEPEC | 8 | n.d. |
| 2378/12 Father | 74 | O76:H19 | stx1c, ehxA, subAB | STEC | 1 | ST675 |
| 3467/12 Girl | 137 | O51:H49 | eae | aEPEC | 6 | n.d. |

STEC, Shiga toxin-producing Escherichia coli; aEPEC, atypical enteropathogenic E. coli; PFGE, Pulsed-field gel electrophoresis; MLST, multilocus sequence typing; ST, sequence type; n.d., not done; ONT, O antigen non-typable.

* Days counted from the day the first STEC-positive stool sample was collected.
† Genetic H serotyping results in non-motile isolates are given in square brackets [H].
‡ Symptomatic when the stool sample was collected.

Fig. 1. Pulsed-field gel electrophoresis (PFGE) profiles of Shiga toxin-producing Escherichia coli and atypical enteropathogenic E. coli isolates obtained from the stool samples of a girl and her asymptomatic family members. The scales at the top indicate the similarity indices (in percentages) and molecular sizes (in kilobases).
in a household during a 5-month period. Of the STEC-infected family members only the 14-month-old girl developed bloody diarrhoea but did not require hospitalization or antibiotic treatment, and her symptoms resolved between the first and second stool sampling. None of the other STEC-infected family members developed clinically symptomatic disease. The O76:H19 isolate from the index girl carried both stx1 and stx2 while O76:H19 isolates from the parents only carried stx1, shown to be less frequently associated with severe human disease than stx2 [1]. Both serotypes were eae-negative and ehxA-, subAB-positive. Despite intimin production representing a common feature of STEC strains associated with severe human disease, eae-negative STEC strains have also been implicated in outbreaks and serious disease [12]. Moreover, it has been reported that the subtilase cytotoxin, encoded by subAB, might contribute to the virulence of eae-negative STEC strains in synergy with Shiga toxins [13], which could explain the clinical relevance in our index case. Additionally, STEC O76:H19 has been recognized to be an important non-O157 STEC associated with human illness and in particular with causing HUS [4]. Apart from the index girl, her older sister was the only aEPEC-infected family member reporting diarrhoea (before the first STEC-positive stool sample was collected), but symptoms rapidly resolved and she did not required medical care. Although the epidemiological association of aEPEC with diarrhoea is still controversial, their high prevalence worldwide and involvement in diarrhoeal outbreaks [3] support the idea that some aEPEC strains are diarrhoeagenic. The questionnaire identified consumption of vegetables fertilized with sheep manure as a likely source of infection. Sheep have been reported as a common reservoir for STEC infection and O76:H19 STEC strains with the same virulence profiles have previously been isolated from sheep [13]. Although there is no evidence of direct transmission from animals to humans, aEPEC have also been isolated from sheep and exposure to faecal pollution from a sheep herd was the suspected source of infection in a recently reported outbreak of mixed STEC and aEPEC infection in Norwegian children at a day-care centre [3]. The PFGE analysis revealed prolonged carriage in two family members. It was confirmed that the father asymptomatically shed STEC (profile 1) at least for 38 days (from day 36 to day 74), with the mother being infected with the same strain on day 36 (Table 1). The index girl asymptomatically shed aEPEC (profile 6) for 101 days after resolving her STEC-associated bloody diarrhoea episode (Table 1). Prolonged asymptomatic STEC carriage has been best characterized in children, but also reported in adults, even over a 1-year period [14, 15]. Family clusters of STEC infection have been reported to be common, with up to 50% of STEC infections being family-related, e.g. in Finland [16]. In addition, both family clusters and outbreaks of mixed STEC and EPEC infection have previously been reported [3, 14]. Although there was no conclusive evidence regarding the source of infection in this family cluster, prolonged asymptomatic carriage and close household contact between the family members pose a risk of cross-infections. This circumstance is underlined by the fact that those relatives who consumed the same vegetables but did not share the same household were not infected. Therefore, hand-washing when handling food or young babies is particularly necessary to prevent STEC and other diarrhoeagenic E. coli infections in households.

ACKNOWLEDGEMENTS

We thank José Manuel Luquin and Gemma Poignon for facilitating the follow-up sampling of the household members and relatives. We thank Daniel Eibach for critically reviewing the manuscript. We also thank Flemming Scheutz for conventional O:H serotyping the stains. Sergio Sánchez acknowledges the Juan de la Cierva programme from the Ministerio de Economía y Competitividad for his research contract. This study was supported by the Madrid Regional Government (P2009/AGR-1489).

DECLARATION OF INTEREST

None.

REFERENCES

1. Caprioli A, et al. Enterohaemorrhagic Escherichia coli: emerging issues on virulence and modes of transmission. Veterinary Research 2005; 36: 289–311.
2. Trabulsi LR, Keller R, Tardelli Gomes TA. Typical and atypical enteropathogenic Escherichia coli. Emerging Infectious Diseases 2002; 8: 508–513.
3. Wahl E, et al. Investigation of an Escherichia coli O145 outbreak in a child day-care centre – extensive sampling and characterization of eae- and stx1-positive E. coli
yields epidemiological and socioeconomic insight. *BMC Infectious Diseases* 2011; 11: 238.

4. Mellmann A, *et al.* Analysis of collection of hemolytic uremic syndrome-associated enterohemorrhagic *Escherichia coli*. *Emerging Infectious Diseases* 2008; 14: 1287–1290.

5. Sánchez S, *et al.* Subtilase cytotoxin encoding genes are present in human, sheep and deer intimin-negative, Shiga toxin-producing *Escherichia coli* O128:H2. *Veterinary Microbiology* 2012; 159: 531–535.

6. Schutz F, *et al.* Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *Journal of Clinical Microbiology* 2012; 50: 2951–2963.

7. Gunzburg ST, Tornieporth NG, Riley LW. Identification of enteropathogenic *Escherichia coli* by PCR-based detection of the bundle-forming pilus gene. *Journal of Clinical Microbiology* 1995; 33: 1375–1377.

8. Machado J, Grimont F, Grimont PA. Identification of *Escherichia coli* flagellar types by restriction of the amplified flIC gene. *Research in Microbiology* 2000; 151: 535–546.

9. PulseNet. One-day (24–28 h) standardized laboratory protocol for molecular subtyping of *Escherichia coli* O157:H7, *Salmonella* serotypes, *Shigella sonnei*, and *Shigella flexneri* by pulsed field gel electrophoresis (PFGE) (http://www.pulsenetinternational.org/SiteCollectionDocuments/pfge/51_52_54_PNetStand_Ecoli_with_Sflexneri.pdf). Accessed 1 February 2013.

10. Wirth T, *et al.* Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology* 2006; 60: 1136–1151.

11. Bielaszewska M, *et al.* Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Applied and Environmental Microbiology* 2007; 73: 3144–3150.

12. Newton HJ, *et al.* Shiga toxin-producing *Escherichia coli* strains negative for locus of enterocyte effacement. *Emerging Infectious Diseases* 2009; 15: 372–380.

13. Michelacci V, *et al.* A new pathogenicity island carrying an allelic variant of the Subtilase cytotoxin is common among Shiga toxin producing *Escherichia coli* of human and ovine origin. *Clinical Microbiology and Infection* 2013; 19: E149–156.

14. Staples M, *et al.* Prolonged and mixed non-O157 *Escherichia coli* infection in an Australian household. *Clinical Microbiology and Infection* 2012; 18: E140–143.

15. Kuusi M, *et al.* Prolonged shedding of Shiga toxin-producing *Escherichia coli*. *Pediatric Infectious Disease Journal* 2007; 26: 279.

16. Lienemann T, *et al.* Shiga toxin-producing *Escherichia coli* serotype O78:H+ in family, Finland, 2009. *Emerging Infectious Diseases* 2012; 18: 577–581.