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

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

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RUNNING HEAD: O76:H19 STEC and aEPEC infection in a household

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 diarrhea, 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 characterised in the household during a five months 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 fertilised 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 among family members, and illustrates the importance of molecular epidemiology in understanding disease clusters.

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

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]. Particularly, non-O157 STEC are considered emerging pathogens, despite being currently underrecognised 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]. Particularly, atypical EPEC (aEPEC) are more prevalent compared to STEC in industrialised 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 May 30, 2012, the clinical microbiological laboratory of the Hospital Complex of Navarre (CHNa) submitted a Stx1-positive stool culture to the Spanish National Reference Laboratory (SNRL) for further STEC diagnostic assays. The sample had been obtained from a 14-month-old girl reporting bloody diarrhoea. At the SNRL, both an O76:[H19] STEC and an O168:H6 aEPEC were recovered. Although STEC infections are not notifiable in Spain, since O76:H19 STEC has been associated with HUS [4] an epidemiological investigation was conducted. The girl's parents were interviewed by telephone, using a structured trawling questionnaire. The questionnaire included questions related to general food handling and hygienic procedures, as well as specific risk factors, including consumption of raw food, especially unpasteurized dairy products and potentially faecally contaminated vegetables, and non-disinfected water, as well as contacts with farm animals or pets and recent history of travel. The hypothesis-generating interview only identified as a potential source of girl's infection consumption of vegetables grown in a family garden irrigated with well water and fertilised with sheep manure. As a consequence, single stool samples from the four household members, consisting of the index girl, her mother (32 years of age), father (33 years) and older sister (3 years), were obtained on days 36, 74, 137 and 201 (counted from the

day the first STEC-positive sample was collected). Stool samples from four other relatives of the family, 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 first streaking area of the culture plate was tested for *stx1*, *stx2* and *eae* genes by PCR [5]. When culture tested positive, individual *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 *fliC* 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 v4.5 (Bio-Rad, United Kingdom).

On day 36, no more 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 stool sample from the mother. 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 the 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 and 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 two to five 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 among 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. Anyway, 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 enterohemorrhagic *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 and 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 characterised in a household during a five months period. Among STEC-infected family members, only the 14-month-old girl developed bloody diarrhoea but neither required hospitalisation nor 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 recognised 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 fertilised 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 among Norwegian children in a day-care centre [3].

The PFGE analysis revealed prolonged carriage in two family members. Concretely, 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 characterised 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 for example 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 among 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, handwashing when handling food or young babies is particularly necessary to prevent STEC and other diarrhoeagenic *E. coli* infections in households.

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DECLARATION OF INTEREST

None.

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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	ND
1898/12	Girl	36	O8:H25	*eae*	aEPEC	3	ND
2188/12	Girl	36	O51:H49	*eae*	aEPEC	6	ND
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	ND
1903/12	Older sister	36	O180:[H2]	*eae*	aEPEC	4	ND
2376/12	Girl	74	ONT:H29	*eae*	aEPEC	8	ND
2378/12	Father	74	O76:H19	*stx1c*, *ehxA*, *subAB*	STEC	1	ST675
3467/12	Girl	137	O51:H49	*eae*	aEPEC	6	ND

PFGE, pulsed field gel electrophoresis; MLST, multilocus sequence typing; STEC, Shiga toxin-producing *Escherichia coli*; aEPEC, atypical enteropathogenic *E. coli*; ST, sequence type; ND, not done; ONT, O antigen non-typeable.

* Days counted from the day the first STEC-positive stool sample was collected.

† Genetic H serotyping results in non-motile isolates are denoted in square brackets ([H]).

‡ Symptomatic when the stool sample was collected.

Fig. 1. PFGE profiles of STEC and aEPEC 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).