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Characteristics of Shiga toxin producing- and enteropathogenic Escherichia coli of the emerging
serotype O80:H2 isolated from humans and diarrheic calves in Belgium

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477 50 15.

Running title: E. coli O80 Belgium
ABSTRACT

Objectives
Recently a highly virulent *E. coli* O80:H2 pathotype carrying Shigatoxin genes, the intimin subtype *eaeξ*, and genes associated with the ExPEC pS88 plasmid was described in France. In this study we examined the relatedness of Belgian *E. coli* O80:H2 isolated from humans and diarrheic calves as well their similarities with the French pathotype.

Methods
Eighteen Belgian *E. coli* O80:H2 strains (9 human STEC (2008-2016), 2 bovine STEC (1987) and 7 bovine aEPEC (2009-2015)) were characterized with conventional PCR, disk diffusion susceptibility testing and whole genome sequencing.

Results
Only nine sporadic human STEC O80:H2 cases were detected in Belgium so far. All patients were female, just two of them suffered from haemolytic uremic syndrome (HUS). All studied strains had the *eaeξ* subtype, belonged to the MLST ST-301, and carried virulence genes associated to the type III secretion system and non-LEE encoded effectors. Multiple genes of the pS88 plasmid were detected in all but two strains (1 human and 1 calf STEC). The Shigatoxin subtypes *stx1a* (n=3; 1 human, 2 calf), *stx2a* (n=2) and *stx2d* (n=6) were detected. All strains were multidrug resistant, two were ESBL positive. cgMLST revealed some human and calf *E. coli* differed by only 22 loci.

Conclusions
The STEC/ExPEC O80:H2 pathotype was present in calves in Belgium as early as 1987, but human infections have been rare and mostly mild. The human STEC and bovine aEPEC cluster together and have the potential to be as virulent as the French isolates as shown by their similar gene content.
INTRODUCTION

A new pathotype of *E. coli* O80:H2 was first described in France in 2014. These strains carry the Shiga toxin (stx) genes of Shiga toxin-producing *E. coli* (STEC), the uncommon intimin subtype *eaeξ* gene, as well as genes usually present on the extraintestinal pathogenic *E. coli* (ExPEC) pS88 plasmid. This highly virulent pathotype was associated with haemolytic uremic syndrome (HUS) as well as bacteremia (1;2). Similar strains have been identified in humans and animals in other European countries; but its actual source still needs to be elucidated (2-4). A recent study revealed that 40% of 104 atypical enteropathogenic *E. coli* (aEPEC) isolated from diarrheic Belgian calves between 2009 and 2015 belong to the O80:H2 serotype and carry the *eaeξ* gene (5).

In this paper we characterized eighteen Belgian human and bovine cases of *E. coli* O80:H2 using whole genome sequencing to examine their mutual relatedness and the similarities with the French pathotype.

METHODS

Nine human STEC O80:H2 isolated at the Belgian National Reference for STEC were compared to seven out of 42 aEPEC and two STEC O80:H2 originating from diarrheic calves (5). Epidemiological data were collected anonymously in the frame of Decision No 2119/98/EC concerning the epidemiological surveillance and control of communicable diseases in the Community, as completed by Decision No 1082/2013/EU.

PCR was executed for the detection of the *E. coli* virulence genes *eae*, *ehxA*, *aaiC*, *aggR* and *stx* subtyping (6). Disk diffusion susceptibility testing and determination of extended spectrum β lactamases (ESBL) production was performed using antibiotics selected by the Programme Food- and Waterborne Diseases and Zoonoses (Supplemental table 1).

DNA for whole genome sequencing was extracted using the Dneasy blood & tissue kit (Qiagen) and DNA libraries were prepared via the KAPA Hyper Plus kit (Kapa Biosystems). All libraries were
sequenced on a MiSeq instrument (Illumina) using the v2 (2 x 250 bp) and v3 (2 x 300 bp) reagent kits. De novo assembly was performed using SPADes. Subsequent identification of virulence genes, antibiotic resistance genes, serotypes, multi-locus sequence types (MLST), and analysis of custom databases designed to detect eae subtypes and pS88-related virulence genes was done using tools available from the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/) (VirulenceFinder 1.5, ResFinder 2.1, SerotypeFinder 1.8, MLST 1.1, MyDbFinder 1.1) (Supplemental table 2)(7-10). All tools were run with a %ID threshold of 85% and minimum length of 60%. Core genome MLST (cgMLST) was performed using the Enterobase scheme v2 (2513 loci) and a minimum spanning tree was created in the BioNumerics 7.6.2 software (Applied Maths, Biomérieux).

RESULTS

i. Case description

Between 2008 and 2016 nine unrelated Belgian patients were diagnosed with STEC O80:H2 infection. All the patients were female, their ages ranged from 11 months to 78 years, and they resided in different parts of Belgium. The source of infection was unknown for all. Most of the patients had uncomplicated diarrhea (n = 4), one patient had bloody diarrhea, and two suffered from HUS. Bacteremia was not reported for any of the patients. All bovine strains were collected from the feces or the intestinal contents after necropsy of calves (ages 16 days – 6.5 months) suffering from diarrhea with no clinical evidence of septicemia (Table 1)(5).

ii. Virulence genes

All isolates were eaeα positive, 16 E. coli were ehxA positive, and no enteroaggregative genes (aaiC, aggR) were detected in any of the isolates. Five human STEC were stx2d positive, three were stx2a positive and one was stx1a positive. The stx1a subtype was found in two calf strains (Table 1). All isolates carried genes associated to the type III secretion system (espA, espB, espF, espP) and non-LEE encoded effectors (nleA, nleB, nleC), as well as the Glutamate decarboxylase (gad) gene. The EHEC factor for adherence gene (efa1) was present in all E. coli while the iha adhesion gene was detected
in all bovine strains but only 3/9 human isolates. Microcins-associated genes (mchf, cma, mchB, mchC) were more found in the bovine E. coli than in the human strains. aEPEC strain ARSIA 23 carried the iron-regulated ireA gene (Supplemental Table 3). We screened for ten genes related to the ExPEC pS88 plasmid and at least six genes were found in 16 isolates: cia (16/18), cvaA (18/18), eitB (0/18), etsC (8/18), hlyF (16/18), iroN (16/18), iss (17/18), iucC (8/18), ompT (13/18), and sitA (16/18). Calf strain FMV 36819_5 and human strain EH2549 carried only 3/10 and 1/10 genes, respectively (Table 1).

iii. Antibiotic resistance profiles

All strains analyzed in this study were resistant to seven or more tested drugs, which was reflected by the presence of several acquired resistance genes (Supplemental tables 1 and 4). The production of ESBL by EH2549 and ARSIA 3088 was confirmed by the presence of the blaCTX-M-1 gene in the former and blaCTX-M-14 in the latter. All isolates were resistant to nalidixic acid and susceptible to ciprofloxacin, but no quinolone resistance genes were detected. Azithromycin was not tested phenotypically, but two strains possessed Macrolide-Lincosamide-Streptogramin (MLS) resistance genes.

iv. Multi locus sequence typing

All isolates belonged to the MLST ST-301. The number of loci difference in the cgMLST MST ranged from 9 - 50 between the E. coli isolated from 2008 to 2016. Three human STEC (EH2262, EH2549, and EH2786) were closer related to the calf aEPEC than to the other STEC. FMV 36819_5 and FMV 36819_3, the two STEC isolated from a diarrheal calf in 1987, were the most distant and differed 160 and 201 loci, respectively, from the human STEC EH2400 (Figure 1).

DISCUSSION

Until now human STEC O80:H2 infections are uncommon in Belgium (only nine isolates since 2008, without increasing frequency) and their clinical outcome mild, in contrast to the severe French and
Dutch cases (2;3). No STEC O80:H2 were identified in healthy cattle at slaughterhouses in 2014 and only two stx1a-positive STEC strains in one diarrheic calf in Belgium going back to 1987 (11). Conversely aEPEC O80:H2 are isolated from diarrheic calves with increasing frequency since 2009 (5).

The genetic content of the studied strains is similar to that of previously described O80:H2. All strains carry the rare intimin eaeξ subtype gene, all but two isolates (EH2549 and FMV 36819_5) contain several pS88-related virulence genes, and they all belonged to MLST ST-301 (Table 1). Additionally non-LEE encoded effector genes, the gad gene, microcins-encoding genes, and the adherence genes efa1 and iha were also detected (Supplemental table 3). The efa1 gene is one of the leading candidates as a virulence marker for aEPEC as it has been shown to contribute to diarrheal illness (12). Similar to the French strains, all studied Belgian STEC and aEPEC O80:H2 are resistant to at least seven antibiotics, including two strains producing an ESBL (Supplemental tables 1 and 4).

So far the sources and the transmission routes of STEC O80:H2 to humans are unclear, but the close genetic relatedness of the human and calf isolates in this study indicate that contact with these animals may indeed be a source or way of transmission, more than food contamination. Interestingly the two calf STEC isolated in 1987 are the most distantly related suggesting an evolution of this serotype over time. The aEPEC O80 could be precursors of the human STEC or the aEPEC could be STEC that have lost their stx phages, as already observed in other serotypes. However, this study was conducted on a limited number of strains and further research is necessary to elucidate the source and transmission routes of this emerging pathotype in order to prevent its spreading.
TRANSPARANCY DECLARATION

Klara De Rauw, Ben Caljon, Damien Thiry, Marc Saulmont, Jacques Mainil and Denis Piérard have nothing to disclose.

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Klara De Rauw contributed to the conceptualization, the execution and analysis of the experiments, and the writing of the manuscript. Damien Thiry contributed to the execution and analysis of the experiments and the writing of the manuscript. Ben Caljon contributed to the execution of the experiments and the writing of the manuscript. Marc Saulmont contributed to the execution of the experiments and the writing of the manuscript. Jacques Mainil contributed to the analysis of the experiments and the writing of the manuscript. Denis Piérard contributed to the conceptualization, the analysis of the experiments and the writing of the manuscript.


REFERENCES


(12) Slinger R, Lau K, Slinger M, Moldovan I, Chan F. Higher atypical enteropathogenic Escherichia coli (a-EPEC) bacterial loads in children with diarrhea are associated with PCR detection of
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<th>Strain</th>
<th>Source</th>
<th>Year</th>
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<th>pS88 related genes</th>
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<sup>a</sup>strains FMV 36849_3 and FMV 36819_5 originate from the same calf


<sup>c</sup>ESBL: extended spectrum β lactamase production