International Journal of Medical Microbiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Genetic characterization of Shiga toxin producing *Escherichia coli* belonging to the emerging hybrid pathotype O80:H2 isolated from humans 2010–2017 in Switzerland

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ARTICLE INFO

Keywords:
STEC O80:H2
Extraintestinal
Virulence
Hybrid
Core genome

ABSTRACT

Shiga toxin-producing *E. coli* (STEC) O80:H2 is an uncommon hybrid pathotype that has recently emerged in France. We analysed 18 STEC O80:H2 isolated from humans in Switzerland during 2010–2017. All isolates carried *stx2a* or *stx2d*, the rare *eae* variant *eae*-ξ and at least seven virulence genes associated with pS88, a plasmid that is found in extraintestinal pathogenic *E. coli* (ExPEC). Whole genome sequencing (WGS) identified additional chromosomal extraintestinal virulence genes encoding for type 1 fimbria (*fimA, fimC and fimH*), aerobactin (*iuc/iutA*) and afimbrial adhesins (*afaA/C/D/E*-VIII). Core genome multi-locus sequence typing (cgMLST) detected two closely related but distinct subclusters with different *stx2* and *iuc/iutA* genotypes. All isolates were multidrug resistant (MDR), but susceptible to third generation cephalosporins and azithromycin. STEC/ExPEC hybrid pathotypes such as STEC O80:H2 represent a therapeutical challenge in the event of extraintestinal infection.

1. Introduction

Shiga toxin (Stx)-producing *Escherichia coli* (STEC) are important foodborne pathogens and responsible for gastrointestinal illnesses which may involve non-bloody or bloody diarrhea, haemorrhagic colitis (HC), and the haemolytic uremic syndrome (HUS) (Karch et al., 2005). The primary virulence trait of STEC is Stx, which includes two major groups, Stx1 and Stx2, whereby Stx2a, Stx2c and Stx2d are mainly associated with severe disease (Fuller et al., 2011). An additional virulence trait that may be present in STEC includes intimin, an outer membrane protein which is responsible for the ability to form attaching and effacing lesions in the human intestinal mucosa (Jerse et al., 1990). Intimin is encoded by the chromosomal gene *eae*, which is part of a pathogenicity island termed the locus for enterocyte effacement, LEE (Kaper et al., 2004). Differentiation of *eae* subtypes represents a valuable tool for typing STEC in the clinical setting as well as for epidemiological studies. At present, 30 distinct *eae* subtypes have been identified and appended by lower case Greek letters and Roman numbers α1, α2, α8, β1, β2, β3, γ1, γ2, ε1, ε2, ε3, ε4, ζ, ζ3, η, η2, θ, ι1, ι2, κ, λ, μ, ν, ξ, ο, π, ρ, σ, τ, and υ, respectively (Ooka et al., 2012). *E. coli* O157:H7 is reportedly the most common STEC serotype in the European Union and in Switzerland, nonetheless, non-O157 STEC serogroups, in particular O26, O91, O103, O111, O121 and O145, are also frequently detected (EFSA, 2017; Fierz et al., 2017). By contrast, reports of STEC O80:H2 strains are rare. However, this pathotype has recently emerged in France and is associated with severe cases of HUS, as well as HUS asociated with bacteremia (Mariani-Kurkdjian et al., 2014; Soysal et al., 2016). A further case of STEC O80:H2 induced lethal complication of HUS was very recently reported in the Netherlands (Wijnsma et al., 2017). This unusual STEC serotype features the rare eae-ξ (xi), and genetic determinants encoded by the pS88 plasmid which is associated with extraintestinal-virulence pathogenic *E. coli* (ExPEC) (Peigne et al., 2009).

This study aimed to examine the molecular characteristics of 18 human STEC O80:H2 isolates collected during 2010–2017 at the National Centre for Enteropathogenic Bacteria and *Listeria* (NENT) in Zürich, Switzerland, using conventional PCR methods and whole genome sequencing. Moreover, the genetic relatedness of the strains was determined using core genome multilocus sequence typing.

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https://doi.org/10.1016/j.ijmm.2018.05.007
Received 10 April 2018; Received in revised form 10 May 2018; Accepted 31 May 2018

2. Materials and methods

2.1. Bacterial strains

For this study, we analysed 18 STEC O80:H2 human isolates received between 2010 and 2017 at the NENT in Zürich, Switzerland. Ten strains (55.6%) were from female, and eight (44.4%) from male patients. The median age was 28 years (range < 1 – 81 years). Six (33.3%) strains were isolated from patients ≤5 years of age. Twelve (66.6%) of the infections occurred during the summer–early autumn season. The majority (n = 13, 72.2%) of the cases were registered in the western parts of Switzerland that share borders with the high-incidence regions of France (Soysal et al., 2016). Aggregate clinical data was attainable for 10 patients. Thereof, one (10%) developed HUS, and four (40%) were hospitalised.

2.2. Ethics statement

All the clinical isolates were collected from stool samples in the course of diagnostic procedures and were processed at the NENT. This study was approved by the local ethics committee of Zürich (BASEC-Nr.Req-2016-00374).

2.3. Serotyping

The O80 serogroup was determined by O80-specific PCR using primers and conditions described previously (Soysal et al., 2016). The H2 type was identified by PCR targeting the *flic*$_{H2}$ gene with primers described elsewhere (Alonso et al., 2017).

2.4. Detection of virulence genes

The presence of *stx* genes was initially determined by real-time PCR (LightCycler

R 2.0 Instrument, Roche Diagnostics Corporation, Indianapolis, IN, USA) (EURL, 2013a). PCR-based identification of *stx1* and *stx2* subtypes was carried out as described in a previous study (Scheutz et al., 2012). The presence of *eae* and the identification of the *eae*-ξ variant was verified using methods described previously (Blanco et al., 2005; EURL, 2013a). The strains were further screened by PCR for the presence of *hlyA* encoding enterohemolysin (Schmidt et al., 1995), *iha*, encoding an iron acquisition protein (Schmidt et al., 2001), the subtilase cytotoxin gene, *subAB* (Funk et al., 2013), *ipaH*, characteristic for enteroinvasive *E. coli* (EIEC) (Persson et al., 2007), *aggR* coding for a transcriptional regulator in enteroaggregative *E. coli* (EAEC) (EURL, 2013b), and the pS88 related genes *sitA*, *eitB, cia, iss, iucC*, *iroN*, *hlyF*, *etsC*, *cvaA*, and *ompT*$_p$ (Peigne et al., 2009).

2.5. Multi locus sequence typing (MLST)

MLST was performed by PCR amplification of internal fragments of seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icdF*, *mdh*, *purA*, and *recA*) (Wirth et al., 2006). Custom sequencing of the alleles was performed by Microsynth (Balgach, Switzerland). Sequence types (STs) were assigned in accordance with the *E. coli* MLST database website (https://pubmlst.org/databases.shtml).

2.6. Whole genome sequencing (WGS) and in silico analysis

Whole genome sequencing was performed using a MiSeq Illumina platform with 2 × 300 nt pair-end sequencing as previously described (Meinel et al., 2014). Reads were *de novo* assembled using SPAdes (version 3.11.1) (Bankevich et al., 2012) and the resulting assembly was polished using Pilon (version 1.22) (Walker et al., 2014). Mean coverage of the sequenced genomes was more than 50-fold.

We carried out *in silico* genome analysis using the virulence factor database (VFDB) (Chen et al., 2005), to determine the presence of virulence genes. Furthermore, we performed a core genome MLST to assess the genetic relatedness among the isolates. The core genome MLST is based on ATCC 25,922 and was generated using Ridom SeqSphere Software (version 4.1.9, available at http://www.ridom.de/seqsphere/cgmlst/).

Antimicrobial resistance genes were searched for using the RGI tool (version 3.2.1) that is based on the CARD database (Jia et al., 2017).

2.7. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk-diffusion method and the antibiotics ampicillin (AM), amoxicillin-clavulanic acid (AMC), cefazolin (CZ), cefotaxime (CTX), cefepime (FEP), nalidixic acid (NA), ciprofloxacin (CIP), gentamicin (GM), kanamycin (K), streptomycin (S), sulfamethoxazole/trimethoprim (SXT), fosfomycin (FOS), azithromycin (AZM), nitrofurantoin (F/M), chloramphenicol (C) and tetracycline (T) (Becton Dickinson, Heidelberg, Germany). Results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) performance standards (CLSI, 2016). For azithromycin, an inhibition zone diameter of ≤ 12 mm was considered resistant. Multidrug resistance (MDR) was defined as resistance to three or more classes of antimicrobials, counting ß-lactams as one class.

3. Results

3.1. Detection of virulence genes

Of the 18 STEC O80 strains, nine (50%) harboured *stx2a*, and 9 further (50%) *stx2d* (Table 1). All isolates harboured the rare variant of the intimin gene, *eae*-ξ. Fourteen isolates encoded *hlyA*, and 13 *iha*, respectively (Table 1). All 18 isolates contained at least seven pS88-related virulence genes (Table 1).

In silico genome analysis revealed that all 18 isolates carried fimbria associated genes *fimA, fimC* and *fimH* (Table 1). Further, 9 isolates contained the aerobactin encoding genes *iucA*, *iucB*, *iucC*, and *iutA*. Finally, *afa*-VIII genes encoding for afimbrial adhesins were detected in three isolates (Table 1).

3.2. Clonal relationship among the STEC O80:H2 isolates

MLST by PCR assigned all 18 isolates to ST301. Using core genome data, we identified two distinct but highly related clusters of the STEC O80:H2-ST301 strains (Table 1 and Fig. 1). Cluster 1 consisted of nine isolates that harboured *stx2a* (Fig. 1). Cluster 2 contained nine isolates that contained *stx2d* (Fig. 1). Furthermore, cluster 2 consisted of the isolates containing the *iucA*, *iucB*, *iucC*, and *iutA* genes, and contained the three isolates carrying *afa*-VIII genes (Table 1 and Fig. 1). Finally, in contrast to isolates from cluster 1, all isolates belonging to cluster 2 harboured pS88 associated *etsC* (Table 1).

3.3. Antimicrobial susceptibility

Antimicrobial drug susceptibility testing revealed that all strains were MDR, i.e., resistant to three or more classes of antimicrobials, counting ß-lactams as one class (Supplementary Material Table 1). Rates of resistance were 100% for ampicillin, streptomycin, and sulfamethoxazole/trimethoprim. Fourteen (77.8%) of the isolates were resistant to nalidixic acid, 13 (72.2%) to tetracycline, and nine (50%, all belonging to cgMLST cluster 2) to chloramphenicol. None of the isolates were resistant to third-generation cephalosporins, ciprofloxacin, fosfomycin, azithromycin or nitrofurantoin (Supplementary Material Table 1).

In correlation to the phenotypic profiles, the genotypical presence of *bla*$_{TEM-1}$ and *aph(6)-Id* was confirmed *in silico* for all isolates, whereas