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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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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.

Keywords

STEC O80:H2, extraintestinal, virulence, hybrid, core genome

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. 2007). 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 associated 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.

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).

90 **2.3. Serotyping**

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

94

95 **2.4. Detection of virulence genes**

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

107

108 **2.5. Multi locus sequence typing (MLST)**

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

114

115 **2.6. Whole genome sequencing (WGS) and *in silico* analysis**

116 Whole genome sequencing was performed using a MiSeq Illumina platform with 2x 300nt
117 pair-end sequencing as previously described (Meinel et al., 2014). Reads were *de novo*
118 assembled using SPAdes (version 3.11.1) (Bankevich et al., 2012) and the resulting assembly
119 was polished using Pilon (version 1.22) (Walker et al., 2014). Mean coverage of the
120 sequenced genomes was more than 50-fold.
121 We carried out *in silico* genome analysis using the virulence factor database (VFDB) (Chen
122 et al., 2005), to determine the presence of virulence genes. Furthermore, we performed a core
123 genome MLST to assess the genetic relatedness among the isolates. The core genome MLST
124 is based on ATCC 25922 and was generated using Ridom SeqSphere Software (version 4.1.9,
125 available at http://www.ridom.de/seqsphere/cgmlst/).
126 Antimicrobial resistance genes were searched for using the RGI tool (version 3.2.1) that is
127 based on the CARD database (Jia et al., 2017).
128

129 **2.7. Antimicrobial susceptibility testing**

130 Antimicrobial susceptibility testing was performed using the disk-diffusion method and the
131 antibiotics ampicillin (AM), amoxicillin-clavulanic acid (AMC), cefazolin (CZ), cefotaxime
132 (CTX), cefepime (FEP), nalidixic acid (NA), ciprofloxacin (CIP), gentamicin (GM),
133 kanamycin (K), streptomycin (S), sulfamethoxazole/trimethoprim (SXT), fosfomycin (FOS),
134 azithromycin (AZM), nitrofurantoin (F/M), chloramphenicol (C) and tetracycline (T) (Becton
135 Dickinson, Heidelberg, Germany). Results were interpreted according to Clinical and
136 Laboratory Standards Institute (CLSI) performance standards (CLSI, 2016). For
137 azithromycin, an inhibition zone diameter of ≤ 12 mm was considered resistant. Multidrug
138 resistance (MDR) was defined as resistance to three or more classes of antimicrobials,
139 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). All isolates harboured the rare variant of the intimin gene, *eae*-ξ. Fourteen isolates encoded *hlyA*, and 13 *iha*, respectively (Table). All 18 isolates contained at least seven pS88-related virulence genes (Table).

In silico genome analysis revealed that all 18 isolates carried fimbria associated genes *fimA, fimC* and *fimH* (Table). 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).

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 and Figure). Cluster 1 consisted of nine isolates that harboured *stx2a* (Figure). Cluster 2 contained nine isolates that contained *stx2d* (Figure). 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 and Figure). Finally, in contrast to isolates from cluster 1, all isolates belonging to cluster 2 harboured pS88 associated *etsC* (Table).

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 *sul-1*, *sul-2*, genes were detected in 16 isolates (Supplementary Material Table 2). The RGI resistance gene tool did not detect any genes that predict resistance to tetracycline or chloramphenicol.

3.4. GenBank accession numbers

DNA sequences are available under the accession numbers PYSA00000000 to PYSF00000000, and PYRO00000000 to PYRZ00000000, respectively.

4. Discussion

In this study, we characterised 18 STEC O80:H2 isolates that were collected from humans during 2010-2017 in Switzerland. This rare serotype has been described as an emerging STEC in eastern regions of France, and the demographic characteristics of the patients in this study are suggestive of a possible common source or route of infection. However, the source of this serotype has so far not been identified (Soysal et al., 2016).

STEC O80:H2 is associated with high extraintestinal virulence potential, due to the presence of virulence genes encoded on plasmid pS88. This plasmid was first detected in neonatal meningitis *E. coli (*NMEC) O45:H7 ST95, a major etiological agent of meningitis and urosepsis in infants in France (Bonacorsi et al., 2003). The pS88 sequence comprises several virulence regions homologous to plasmids pAPEC-O2-ColV and pAPEC-O1-ColBM from avian pathogenic *E. coli* (APEC) O2:K1 and O1:K1, strains that cause colibacillosis in

chicken (Johnson et al., 2006a; Johnson et al., 2006b). Peigne et al. (2009) have demonstrated that this plasmid sustains high level bacteremia in the neonatal rat model and that pS88-like plasmids are widely distributed among MNEC clones, uropathogenic *E. coli* strains (UPEC), and avian pathogenic E. coli strains (APEC), including *E. coli* O18, O1, O2 and O83 and *E. coli* belonging mostly to ST95. By contrast, STEC O80:H2 appears so far to be the only instance of pS88 found among *E. coli* belonging to ST 301. Nevertheless, within ST301 a number of further *E. coli* serogroups type have been registered in the EcMLST databank and described in the literature, including an STEC O4:H– strain from diarrheic calves (Wieler et al., 1996), an *E. coli* O5 strain from a human infection (Gangiredla et al., 2017), NMEC O7 (Peigne et al., 2009), clinical isolates *E. coli* O132:H2 and O55:H9 (Chattaway et al., 2017), STEC O180:H2 (Joensen et al., 2014), and *E. coli* O186 (Weimer, 2017). Whether any of these strains harbour pS88, and whether ST301 serogroups represent a particular genetic background for the acquisition of pS88-like plasmids remains to be elucidated.

Moreover, it remains unclear to what extent pS88 may be involved in the pathogenicity of other STEC/ExPEC strains described previously. Such hybrids predominantly include UPEC/STEC hybrid strains such as the urovirulent O2:H6 ST141 (Bielaszewska et al., 2014), strains involved in HUS associated with urinary tract infections (UTI), including O157:H7, O17:H18, O103:H2, O174:H2, O145:H28, and O5:H–, some of which lacked identifiable uropathogenic virulence factors such as *papA* (Starr et al., 1998). STEC/ExPEC strains involved in bacteremia have been described for STEC O128ab:H2 (Buvens et al., 2013), O157:H2 (Chiurchiu et al., 2003), and O138:H– (Nguyen et al., 2007), however, characterisations of the genetic factors involved in extraintestinal pathogenicity are lacking.

In addition to pS88, the STEC O80:H2 isolates in this study harboured further genes implicated in extraintestinal virulence. All isolates carried *fimA, fimC* and *fimH*, genes involved in biosynthesis of type 1 fimbria which are crucial for *E. coli* adhesion to epithelial

host cells as well as intracellular survival in phagocytes (Avalos Vizcarra et al., 2016). Furthermore, some of isolates containing the *iucA*, *iucB*, *iucC*, and *iutA*. These genes are involved in the biosynthesis of aerobactin, an iron uptake system that is associated with pathogenesis in extraintestinal *E. coli* strains and frequently present in EAEC clinical isolates, including the 2011 hybrid STEC/EAEC O104:H4 outbreak strain in Germany (Garcia-Angulo et al., 2013). A minority of the isolates carried *afa*-VIII genes encoding for afimbrial adhesins, which are present in both diarrheal and uropathogenic *E. coli* strains and also widespread among bovine pathogenic *E. coli* strains associated with diarrhoea and septicaemia (Antão et al., 2009). Notably, EPEC O80:H2 has recently been identified as an emerging pathogen in young calves and could be a precursor of STEC O80:H2 (Thiry et al., 2017). Further characterisation of these isolates would be desirable in order to establish any common virulence traits between human and calf strains and to attempt an identification the source of STEC O80:H2.

Taken together, our findings provide further evidence for the high pathogenicity and the extraordinary hybrid STEC/ExPEC characteristics which distinguishes STEC O80:H2 from other STEC serotypes.

Although the strains in this study are closely related, cgMLST indicated a trend of subclonal divergence into distinct clusters with different virulence genotypes. While both clusters 1 and 2 include strains carrying *stx2* variants associated with severe disease (*stx2a* or *stx2d*), and genes for type 1 fimbria, cluster 2 comprises strains with potentially higher extraintestinal virulence due to the additional virulence gene *etsC* encoded on pS88, the presence of aerobactin genes *iuc*/*iutA*, and in some cluster 2 isolates, the afimbrial adhesion genes *afaA*/*C*/*D*/*E*-VIII. These data suggest a genetic plasticity of STEC O80 regarding the acquisition of extraintestinal virulence factors. Notably, as opposed to the STEC O80:H2 strains isolated in France (Soysal et al., 2016), none of the strains from this study harboured

240 *stx2a/2d* or *stx2c/2d* combinations. Moreover, in France, 74% of the STEC O80:H2
241 harboured *stx2c/2d* and these accounted for the majority (56.9%) of the HUS cases. Among
242 the 10 patients for whom clinical data was available in this study, one (10%) had HUS. The
243 absence of the *stx2c/2d* genotype among the strains isolated in Switzerland may account for
244 the lower prevalence of HUS.
245 The clinical significance of extraintestinal virulence potential is exemplified by a case report
246 of STEC O80:H2 associated bacteraemia (Mariani-Kurkdjian et al., 2014), raising the
247 controversial question about antibiotic therapy during invasive STEC infection (Freedman et
248 al., 2016). Antimicrobial drug susceptibility testing revealed that all strains were multidrug
249 resistant (MDR), i.e., resistant to three or more classes of antimicrobials (Supplementary
250 Material Table 1). Nevertheless, all isolates remained susceptible to third-generation
251 cephalosporins and azithromycin. Our data therefore lend support to a therapeutical approach
252 suggested by Soysal et al. (2016), which involves the combination of ceftriaxone with
253 azithromycin to treat invasive infections of STECO80:H2. This hypervirulent, MDR hybrid
254 pathotype exemplifies the need to monitor antimicrobial resistance in STEC as well as in
255 other *E. coli* pathotypes. Finally, STEC O80:H2 may represent a threat in terms of public
256 health. Surveillance and characterization of STEC isolates from severe cases of human
257 disease using culture-based methods and WGS to supplement non-culture methods such as
258 PCR based *stx* detection may improve the identification and source tracking of STEC
259 O80:H2 infections.
260
261
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Conflicts of interest

None to declare.

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446 **Table**: Genetic backgrounds of 18 Shiga toxin producing *Escherichia coli* serotype
447 O80:H2-ST301, Switzerland, 2010-2017.
448

Gene*	Description of gene product	No.(%) isolates	cgMLST[1] cluster
EHEC associated markers			
stx2a	Shiga toxin variant	9 (50)	1
stx2d	Shiga toxin variant	9 (50)	2
eae- ξ	Intimin variant (attaching and effacing protein)	18 (100)	1, 2
hlyA	Enterohemolysin	14 (77.8)	1, 2
iha	Iron acquisition protein	13 (72.2)	1, 2
subAB	Subtilase cytotoxin	0 (0)	–
EIEC associated marker			
ipaH	Invasion plasmid antigen	0 (0)	–
EAEC associated marker			
aggR	Transcriptional activator of aggregative adherence fimbria I	0 (0)	–
ExPEC associated markers			
sitA	Periplasmic iron transport protein	18 (100)	1, 2
eitB	*E. coli* iron transport protein	0 (0)	–
cia	Colicin Ia (bacteriocin)	17 (94.4)	1, 2
iss	Increased serum survival protein	18 (100)	1, 2
iroC	ATP binding cassette	18 (100)	1, 2
iroN	Salmochelin siderophore receptor	18 (100)	1, 2
hlyF	Hemolysin	18 (100)	1, 2
etsC	Putative type I secretion outer membrane protein	9 (50)	2
cvaA	Colicin V secretion protein	18 (100)	1, 2
ompTp	Outer membrane protease (omptin)	18 (100)	1, 2
fimA	Type-1 fimbrial protein	18 (100)	1, 2
fimC	Chaperone protein for the biogenesis of type 1 fimbriae	18 (100)	1, 2
fimH	Type 1 fimbrial adhesion	18 (100)	1, 2
iucA	Aerobactin siderophore biosynthesis enzyme	9 (50)	2
iucB	Aerobactin siderophore biosynthesis enzyme	9 (50)	2
iucC	Aerobactin siderophore biosynthesis enzyme	9 (50)	2
iutA	Outer membrane receptor for the ferric-siderophore complex	9 (50)	2
afaA-VIII	Afimbrial adhesion	3 (16.7)	2
afaC-VIII	Afimbrial adhesin usher protein	3 (16.7)	2
afaD-VIII	Afimbrial invasion	3 (16.7)	2
afaE-VIII	Afimbrial adhesion	3 (16.7)	2

449
450 *EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EAEC, enteroaggregative
451 *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*.
452 [1]core genome multilocus sequence type.
453

454

455

Figure legend

Figure: Core genome multilocus sequence type (cgMLST) based minimum spanning tree of 18 human Shiga toxin producing *Escherichia coli* (STEC) O80:H2-ST301 isolates. Each circle contains the strain ID(s). Year of isolation is indicated in square brackets. Blue circles represent the *stx-2a* genotype, red circles indicate the *stx2d* genotype. Cluster 1 is shaded in light green. Cluster 2 is shaded in light yellow and contains strains with the *iuc*/*iutA* genotype. Strains within cluster 2 with the *afa*-VIII genotype are shaded in lime. The numbers on connecting lines represent the number of allelic differences between two strains.