

Characteristics of the enteroaggregative Shiga toxin/verotoxin-producing *Escherichia coli* O104:H4 strain causing the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011

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The *Escherichia coli* strain causing a large outbreak of haemolytic uraemic syndrome and bloody diarrhoea in Germany in May and June 2011 possesses an unusual combination of pathogenic features typical of enteroaggregative *E. coli* together with the capacity to produce Shiga toxin. Through rapid national and international exchange of information and strains the known occurrence in humans was quickly assessed. We describe simple diagnostic screening tools to detect the outbreak strain in clinical specimens and a novel real-time PCR for its detection in foods.

Sequence of events

Having received the first Early Warning Response System (EWRS) alert issued by the Robert Koch Institute (RKI) in Germany on 23 May about an increase in the number of patients presenting with haemolytic uraemic syndrome (HUS) and bloody diarrhoea caused by Shiga toxin-producing *Escherichia coli* (STEC) with more than 30 possible cases reported since the second week of May, the World Health Organization Collaborating Centre (WHO CC) for Reference and Research on *Escherichia* and *Klebsiella* at Statens Serum Institut (SSI) in Denmark issued an alert to the Danish *E. coli* network of regional hospitals on the same day. On 24 May, Hvidovre University hospital reported a German patient who had been diagnosed with Shiga toxin/verotoxin-producing *E. coli* (STEC/VTEC) and referred the strain to SSI. The WHO CC found that this first isolate was of serotype O104:H4 and produced Shiga toxin (Stx)/verotoxin (VT) as also reported by RKI. Referral from other regional hospitals identified the German outbreak strain in further patients in Denmark during the next days. This information was immediately shared by postings on the Urgent Inquiry Network (UIN) Epidemic Intelligence Information System (EPIS) hosted

by the Food- and Waterborne Diseases and Zoonoses (FWD) Surveillance Network of the European Centre for Disease Control and Prevention (ECDC), and emails to FWD, the European Union Reference Laboratory for *E. coli* (EU-RL) and the two associated networks including public health (ECDC) and food safety (EU-RL) reference laboratories, the Global Food-borne Infections Network (GFN), Food Safety, WHO Geneva and the WHO Regional Office for Europe, and PulseNet at the United States (US) Centers for Disease Control and Prevention (CDC).

Having verified the specific characteristics of eight of the Danish outbreak strains, the WHO CC sent the index strain and the reference strain for the O104 antigen to the EU-RL in Rome. The strains were received on 31 May and tested positive by the EU-RL using a novel real-time PCR developed at the EU-RL and its network for detection of *E. coli* strains of serotype O104:H4. Thus, within a week, screening tools and a novel PCR protocol for detection of the outbreak strain in clinical specimens and in foods were developed, tested and shared with national as well as international networks. In return, members of the networks contributed with their existing knowledge of *E. coli* strains of serotype O104:H4, thereby collectively adding to the existing knowledge of this pathogen and describing the relevant characteristics of the reported strains for public health investigation.

The outbreak strain

The Danish isolates were PCR-positive for the *aggR* gene, which is typical of enteroaggregative *Escherichia coli* (EAggEC). Further analysis showed that the outbreak strain (first eight isolates from Danish patients)

were also positive for the following genes: *sigA*, *sepA*, *pic*, *aatA*, *aaiC*, *aap*, as well as *aggA*, which encodes the major component of the AAF/I adhesin. AAF/I is a fimbrial organelle usually associated with a strong ability to form biofilms and haemagglutination with human erythrocytes. Preliminary testing at WHO CC showed that the isolates were moderate to good biofilm producers particularly in Dulbecco's minimum essential medium (DMEM) supplemented with 0.45% glucose, which is typical and defining for EAggEC strains. The outbreak strain was a typical *E. coli*: lactose-positive, sorbitol-fermenting and beta-glucuronidase-positive. Furthermore, the strain was positive for *iutA* encoding an aerobactin receptor found in 80% of extraintestinal pathogenic *E. coli* isolated from urosepsis [2] and negative for the STEC-associated adhesin (*saa*) and cytotoxin subtilase (*subAB*).

Taken together, these data indicate that the outbreak strain is indeed a typical EAggEC strain that has acquired the bacteriophage encoding Stx/VT. Using a novel protocol for subtyping of *stx/vtx* genes [3], we have shown that the gene encoding Stx/VT is *stx2a/vtx2a*.

Sequence analysis of the published *stx2a/vtx2a* sequence (SRX067313 on <http://www.ncbi.nlm.nih.gov/sra>) showed 100% amino acid identity of the holotoxin to Stx2a/VT2a from *E. coli* O157:H7 EDL933 isolated from Michigan ground beef in 1983 (accession number X07865 [4]) but differed by one nucleotide at position 867 (C instead of T), making the nucleotide sequence identical to the sequence found in sorbitol-fermenting O157 strains from Germany in 2002 and 2005 (accession numbers AY143336 and AY143337, unpublished), DQ231589 and DQ231590 [5], and Scotland in 2006 (EU526759) [6]. This sequence variant of *stx2a/vtx2a* has also been detected in isolates from seagulls (accession number AB030484, unpublished) and human isolates of different serotypes: *E. coli* O121:H19 from Canada (DQ143182 and DQ143183) [7] and Idaho, US (EF441611) [8], and O111:H8 also from Idaho, US (EF441606) [8].

These findings could explain the unexpectedly high level of virulence in a STEC/VTEC strain negative for the attaching/effacing pathogenicity island. It is indeed conceivable that the enteroaggregative adherence phenotype could have allowed these *E. coli* O104 strains to colonise the intestinal mucosa of the affected patients as efficiently as the typical *eae*-positive STEC/VTEC strains. The different mechanism of adhesion might also explain why this strain is more likely to cause severe disease in adults rather than in children, as would be usual for typical HUS-associated STEC/VTEC: adults and children might differ in their susceptibility to the adherence and/or colonisation properties of this type of EAggEC strain. Obviously, elucidating this aspect requires dedicated studies and we cannot exclude that the different rates of HUS between adults and children observed in the current outbreak just reflect a difference in the exposures.

Screening for the outbreak strain

Plating clinical samples on extended-spectrum beta-lactamase (ESBL) plates, such as commercially available Tryptone Bile X-Glucuronide (TBX) medium will allow growth of the outbreak strain and inhibit the majority of other *E. coli* strains. Excellent growth of the index strain (only one of the strains has been tested so far) from the outbreak has also been observed as light red colonies on cefixime tellurite sorbitol MacConkey (CT-SMAC) plates at 37 °C, 41.5 °C and 44 °C (Jeppe Boel, personal communication, 3 June 2011). Since cefixime belongs to the class of cephalosporins, it seems likely that the strain can overcome the cefixime concentration in CT-SMAC, but apparently it is also able to overcome the tellurite concentration.

For quick screening of clinical samples, K9 antiserum for live slide agglutination can be used in both primary and secondary testing laboratories. This is because the O104 O antigen is identical to the K9 capsular antigen [9]. The K9 antiserum is readily available from SSI Diagnostica, Hillerød, Denmark (ivdorders@ssi.dk) and described on the SSI website [10]. At SSI, we have agglutinated culture from confluent growth but pools of 5 to 10 individual colonies can also be agglutinated. Immediate positive reactions indicating the presence of *E. coli* O104 have all been confirmed by conventional serotyping of O and H antigen, presence of *stx2a/vtx2a* and lack of the *eae* gene. Based on our observations so far, all weak reactions have turned out to be negative for the outbreak strain. The strain can also be detected by a number of methods targeting the *stx2/vtx2* gene by PCR, RT-PCR or commercial Stx/VT detection kits. The strain must also be negative for the *eae* gene and confirmed for O104.

Food samples should be enriched in Buffered Peptone Water (225 ml for 25 g test portion) and incubated for 18 to 24 h at 37 °C ± 1 °C. DNA extracted from a 1 ml aliquot is purified and tested for the presence of *stx/vtx* genes (first step of the real-time PCR procedure described in the ISO/TS 13136:2011(E) method [11]).

Samples positive for *stx/vtx* genes (regardless of the presence of the *eae* gene) are tested for the O104-associated gene (*wzxO104*) [12]. The *wzxO104*-positive enrichment cultures are plated onto two media: (i) MacConkey agar, or TBX, or any other medium suitable for *E. coli* isolation, and (ii) a more selective medium containing an antibiotic supplement. Colonies positive for *stx/vtx* genes are identified for the O104 antigen-associated gene *wzxO104* and the gene encoding the H4 flagellar antigen, *fliCH4* [12]. Conventional serotyping can be performed by standard methods [13]. Other markers can be tested by either conventional or real-time PCR for further characterisation.

DNA from an outbreak strain provided by the Robert Koch Institute to be used as positive control in the PCR assays can be obtained from Istituto Superiore di Sanità (ISS) in Rome (crl.vtec@iss.dk).

To the best of our knowledge, this unusual combination of virulence factors of STEC/VTEC and EAggEC has rarely been described in humans. A strain of serotype O111:H2 [14] caused a small outbreak of HUS in France in 1995, but the episode involved children, as is typical for STEC/VTEC [15]. As in the present outbreak in Germany, the association of the French strains with severe disease (HUS) supports the view that this unusual combination of virulence factors might confer a very high degree of virulence.

Serotype O104:H4

Sporadic cases of *stx2/vtx2*-positive *E. coli* serotype O104:H4 have been reported. These reports include two isolates from patients with HUS in Germany in 2001 [16], one in France in 2004 (data from the dedicated EU surveillance network Enter-net; not including clinical information), one from a case of HUS in Korea in 2005 [17], two HUS cases in the Republic of Georgia in 2009 (unpublished information provided via PulseNet, US CDC), and one uncomplicated case of diarrhoea in Finland in 2010 (reported to FWD on EPIS). The isolates from Germany 2001, Finland 2010 and the Republic of Georgia 2009 were EAggEC and STEC/VTEC.

The strain from the Republic of Georgia had the following characteristics: serotype O104:H4, Shiga toxin subtype *stx2a*, *eae*-negative, haemolysin-negative, *aatA*-positive (EAggEC marker), susceptible to ceftriaxone (unlike the current outbreak strain), sorbitol-, lactose-, and beta-glucuronidase-positive, biochemically consistent with *E. coli*, Shiga toxin production on the low end of the spectrum, similar to that of the German strain (Peter Gerner-Smidt, personal communication 7

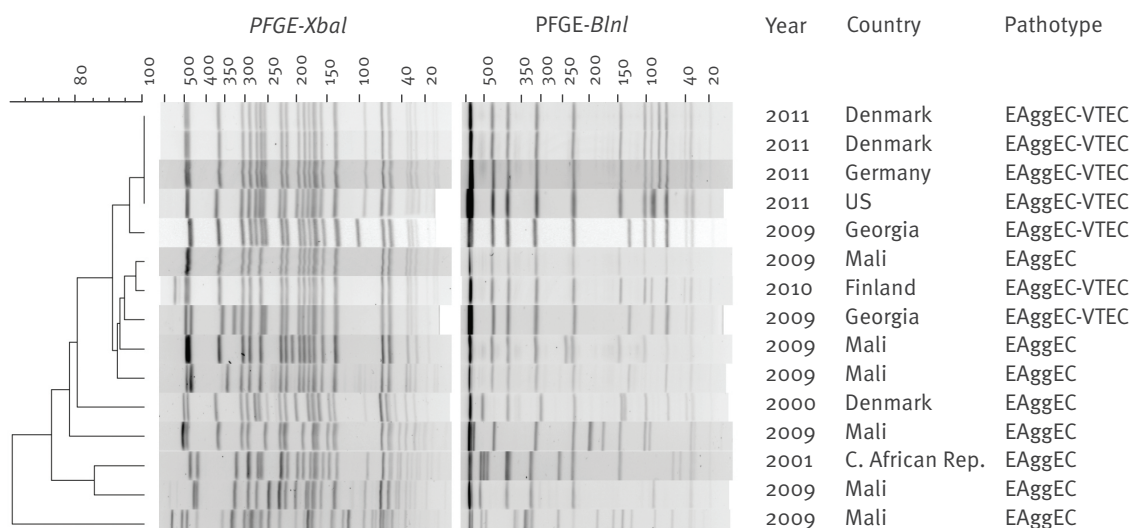
June 2011 from PulseNet, US CDC, and the Georgian team of investigators). At this time, we do not have further information on the remaining O104:H4 STEC/VTEC isolates from France and Korea.

In general, we have limited knowledge on EAggEC of this serotype: The archetype isolate for the aggregative adherence fimbriae type III (AAF/III, encoded by the *agg3A* gene) is strain 55989, which was isolated during a study of EAggEC as a cause of persistent diarrhoea in African patients infected with human immunodeficiency virus (HIV) [18,19]. In a recent study of childhood diarrhoea in Mali, we identified *Stx/VT*-negative EAggEC O104:H4 in three children with moderate to severe diarrhoea and from three healthy controls (unpublished data). The three EAggEC strains isolated from these cases were PCR-positive for different combinations of *aggR*, *aatA*, *aaiC*, *aap*, *astA*, *sepA*, *pic*, *sigA*, *aggA*, *agg3C* and *agg3A*.

We have compared the pulsed-field gel electrophoresis (PFGE) profiles of the available *E. coli* O104:H4 isolates to elucidate the diversity within this serotype, irrespective of the virulence profile. PFGE typing using the enzymes *XbaI* and *BlnI* showed that the serotype O104:H4 is diverse (Figure). For *XbaI*, a high similarity of >95% was seen for the 2011 German outbreak isolates (isolated in Denmark, Germany and the US) and one of the isolates from Republic of Georgia. A large cluster of isolates with >90% similarity included the German outbreak strain, the two Georgian cases from 2009, the isolate from the Finnish patient (all *stx2a/vtx2a* and EAggEC) as well as three of the *stx/vtx*-negative EAggEC isolates from patients in Mali. The profiles of five of the *stx/vtx*-negative EAggEC isolates showed

FIGURE

PFGE profiles (*XbaI* and *BlnI*) of *Escherichia coli* O104 compared with four isolates from the outbreak of haemolytic uraemic syndrome in Germany, May to June 2011



C. African Rep: Central African Republic; CDC: Centers for Disease Control and Prevention; PFGE: pulsed-field gel electrophoresis; RKI: Robert Koch Institute; US: United States.

PFGE profiles (*XbaI* and *BlnI*) using the *E. coli* non-O157 PulseNet protocol (www.pulsenetinternational.com). Dendrogram based on analysis of the *XbaI* profiles. All isolates are EAggEC O104:H4 with and without *stx2/vtx2* gene. German outbreak isolates are from patients infected in May 2011 in Germany and diagnosed in Denmark, the US (profiles provided by PulseNet, US CDC) and Germany (strain provided by RKI, Germany). O104:H4 isolates from Mali are from children with and without diarrhoea.

major differences from the outbreak strain (Figure). The 11 Danish PFGE-typed isolates related to the German outbreak had indistinguishable *Xba*I profiles. One isolate from a case infected in Germany and diagnosed in the US had a minor variation in the *Bln*I profile (profile provided by PulseNet, US CDC) (Figure).

General characteristics of EAggEC

EAggEC is a pathotype of diarrhoeagenic *E. coli* defined as *E. coli* that do not secrete the heat-stable or heat-labile toxins of enterotoxigenic *E. coli* (ETEC), and by its characteristic aggregative or 'stacked brick' pattern (AA) of adherence to HEp2-cells in culture [20]. This property is usually due to the presence of aggregative adherence fimbriae (AAF), whose expression is regulated by the *aggR* gene, located on the large EAggEC virulence plasmid termed pAA [21]. EAggEC infections are usually associated with watery diarrhoea, which is often persistent [20]. Illness results from a complex interaction between pathogen and host, which implicates the initial adherence of the bacteria to the epithelium of terminal ileum and colon, by virtue of the aggregative adherence fimbriae (characteristic aggregative pattern), followed by a damage/secretion stage manifested by cytokine release, mucosal toxicity, intestinal secretion and induction of mucosal inflammation [22-26].

EAggEC is best known for its role in persistent diarrhoea (>14 days) in infants and children in developing countries. Studies in Mongolia [27], India [28], Brazil [29,30], Nigeria [31,32], Israel [33], Venezuela [34], Congo [35] and many other countries, have identified EAggEC as a highly prevalent (often the most prevalent) *E. coli* pathotype in infants. Further, the role of EAggEC as an important pathogen in AIDS patients continues to develop, and EAggEC now ranks among the most important enteric pathogens in this population group [36,37]. In a recent review of all published studies of traveller's diarrhoea, EAggEC was in aggregate second only to ETEC as the most common pathogen [38].

The first reported EAggEC outbreaks occurred in Mexico City before 1993 (year unpublished) where persistent diarrhoea was reported. Five of the infected children died as a consequence of the diarrhoea. Both outbreaks occurred in the malnutrition ward of a paediatric hospital [39], demonstrating that EAggEC is not exclusively a disease of infants under the age of 12 months [40]. Itoh et al. described a massive outbreak of EAggEC diarrhoea among Japanese children in 1993 affecting nearly 2,700 patients [41]. Another EAggEC outbreak was reported in a Serbian nursery in 1995 [42] in which 16 newborn babies (duration of illness 3–9 days) and three infants (18–20 days) developed diarrhoea accompanied by pyrexia and weight loss. Outbreaks have also been reported among adults in the United Kingdom [43] and a small outbreak of EAggEC serotype O92:H33 was reported in Italy in which pecorino cheese (unpasteurised milk) was epidemiologically implicated [44]. As these outbreaks suggest, EAggEC is capable of causing diarrhoea in adults and children, even in the absence of Stx/VT. We believe that this

pre-existing diarrhoeagenic and outbreak potential, coupled with the highly virulent Stx/VT, has resulted in a hypervirulent strain currently circulating in Germany. It should also be noted that EAggEC are common in all populations of the world, industrialised and developing, but that no animal reservoir has been described. This observation suggests the startling possibility that this new O104 strain may have the capacity to persist among human populations, perhaps indefinitely.

Conclusions

The rapid exchange of information, strains and DNA fingerprints within existing national and international public health and food safety networks has been vital in the quick and alternative assessment of the public health significance of the strain causing the outbreak of HUS in Germany in May and June 2011. The combined contributions have resulted in major findings including:

- the characterisation of an unusual combination of pathogenic features typical of EAggEC combined with the capacity to produce Shiga toxin in the outbreak strain;
- recommendations for simple diagnostic screening tools for primary laboratory detection of the outbreak strain in clinical specimens;
- a novel real-time PCR protocol for detection of *E. coli* O104:H4 in foods;
- presentation of the known occurrence and clinical presentation in humans and the likely reservoir.

We hope that this report will help to strengthen existing networks, inspire the development of new networks and improve food safety in the future when new or emerging bacterial pathogens may occur in the food chain.

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