

## Enterohemorrhagic *Escherichia coli* with particular attention to the German outbreak strain O104:H4

### *Enterohemorragische E. coli met speciale aandacht voor de bij de Duitse uitbraak betrokken stam O104:H4*

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#### ABSTRACT

This review deals with the epidemiology and ecology of enterohemorrhagic *Escherichia coli* (EHEC), a subset of the verocytotoxigenic *Escherichia coli* (VTEC), and subsequently discusses its public health concern. Attention is also given to the outbreak strain O104:H4, which has been isolated as causative agent of the second largest outbreak of the hemolytic uremic syndrome worldwide, which started in Germany in May 2011. This outbreak strain is not an EHEC as such but possesses an unusual combination of EHEC and enteroaggregative *E. coli* (EAggEC) virulence properties.

#### SAMENVATTING

In dit overzichtsartikel worden de epidemiologie en de ecologie van de enterohemorragische *Escherichia coli* (EHEC), een subset van de verocytotoxigene *E. coli* (VTEC) besproken en wordt het belang ervan voor de volksgezondheid toegelicht. Speciale aandacht wordt besteed aan de uitbraakstam O104:H4 die geïsoleerd werd als verwekker van de tweede grootste uitbraak van het hemolytische uremisch syndroom wereldwijd. Deze uitbraak begon in mei 2011 in Duitsland maar hield heel Europa in de ban. Deze stam is echter geen EHEC als dusdanig, maar bezit een ongewone combinatie van virulentiefactoren van zowel EHEC als enteroaggregatieve *E. coli*.

#### INTRODUCTION

Most *Escherichia coli* are harmless commensals of the gastrointestinal flora of warm-blooded animals and humans. However, some subsets of *E. coli* have acquired virulence properties that render them capable of causing a variety of clinical outcomes in humans and animals. Most acquired virulence factors that distinguish pathogenic *E. coli* from harmless *E. coli* are encoded on mobile genetic elements capable of horizontal gene transfer or on elements that were once mobile and subsequently evolved to be a stable part of the genome (Kaper *et al.*, 2004). The intestinal *E. coli* pathogens can be divided into six well defined pathotypes: enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), diffusely adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC) (Nataro and Kaper, 1998). The differences between these pathotypes are not discussed in this review since the focus is on the EHEC pathotype and the enteroaggregative verotoxin-producing *E. coli* O104:H4 outbreak strain.

#### PATHOGENIC FEATURES AND VIRULENCE FACTORS OF EHEC

EHEC denotes strains that are associated with hemorrhagic colitis and the hemolytic uremic syndrome in humans, and express verocytotoxins and colonize the intestine by causing typical attaching-effacing (A/E) lesions (Nataro and Kaper, 1998). The majority of clinical cases have been caused by strains belonging to the O157:H7 serotype. However, there are a number of non-O157 serogroups of which the 4 deemed most important in terms of clinical infections are O26, O103, O111 and O145. Nevertheless, there are many other serogroups, such as O91, O121 and recently O104, which have also caused infections.

#### Verocytotoxins

The cardinal trait of EHEC is the production of verocytotoxins. The definition is based upon the production of toxins with a cytotoxic activity against vero cells. There are two main types, namely VT1 and VT2 that can be further divided into subtypes based on their

sequence analyses. The nomenclature is not definite and new variants are constantly being described. The VT1 family can be divided into three subtypes, namely VT1, VT1c and VT1d (Muthing *et al.*, 2009). The VT2 is more heterogeneous and up to now, it has been divided into 7 subtypes, namely VT2, VT2b, VT2c, VT2d, VT2e, Vt2f and VT2g (Mainil and Daube, 2005; Persson *et al.*, 2007). The VT-encoding genes (*vtx*) are generally encoded by a heterogeneous group of temperate lambdoid bacteriophages and are expressed when the lytic cycle is activated (Allison, 2007; Herold *et al.*, 2004). The VT-encoding *vtx2e* was initially thought to be chromosomally encoded (Paton and Paton, 1998). However, a *vtx2e* converting bacteriophage has been isolated (Muniesa *et al.*, 2000). Several studies have demonstrated a correlation between the toxin subtypes, severity of clinical diseases and seropathotypes. In general, EHEC producing VT2 only, generally cause more severe disease than those producing lonely VT1 or both VT1 and VT2 (Table 1).

Although the production of VT is considered a main virulence factor of EHEC, strains of non-verocytotoxin producing *E. coli* O157:H<sup>-</sup> have been isolated from patients with HUS or diarrhoea (Schmidt *et al.* 1999b). However, Joris *et al.* (2011) have demonstrated the spontaneous loss of *vtx* genes during isolation. This might complicate the characterization of the virulence patterns of these strains. They can appear to be *vtx* ne-

gative but clinically and epidemiologically they are originally *vtx* positive strains.

All VT belong to the AB<sub>5</sub> family of toxins, with the A subunit carrying the enzymatic activity and a pentameric receptor-binding B subunit. The pentameric B subunit binds specifically to the globotriaosylceramide (Gb3) and globotetraosylceramide (Gb4) receptor. The Gb4 receptor is preferred by the VT2e, whereas all other VT variants prefer Gb3 (Boyd *et al.*, 1993; Lingwood *et al.*, 1998). After binding to the receptor, Vt are internalized by a clathrin-mediated endocytosis and transported via the Golgi-apparatus to the endoplasmic reticulum (Sandvig *et al.*, 1992). In the cytosol, the A subunit is cleaved by a protease, furin, into a catalytically active A1 fragment and an A2 fragment. The A1 fragment exerts tRNA N-glycosidase activity that specifically removes an adenine residue from the 28S rRNA of the 60S ribosome (Endo *et al.*, 1988). This process inhibits protein synthesis and leads to cell death.

### Locus of enterocyte effacement

The hallmark of EHEC strains is the induction of A/E lesions, which are characterized by localized destruction of brush border microvilli and intimate attachment to the plasma membrane of host epithelial cells. EHEC strains share the induction of A/E lesions

**Table 1. Different types of VT (adapted from Gyles, 2007).**

| VT variants | Characteristics                                                                                                                                                   | Reference                                                                                    |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------|
| Vtx1        | VT produced by VTEC and almost identical to Stx produced by <i>Shigella dysenteriae</i> serotype 1                                                                | (Strockbine <i>et al.</i> , 1986)                                                            |
| Vtx1c       | Variant of Vtx1 that is found in ovine and caprine strains but not in bovine strains and in some eae-negative VTEC; associated with mild diarrhoea or no symptoms | (Brett <i>et al.</i> , 2003; Zhang <i>et al.</i> , 2002)                                     |
| Vtx1d       | A variant of Vtx1 isolated from bovine and human strains; associated with asymptomatic infections                                                                 | (Burk <i>et al.</i> , 2003)                                                                  |
| Vtx2        | Prototype of non-Vtx1 toxins; associated with severe disease in humans                                                                                            | (Strockbine <i>et al.</i> , 1986)                                                            |
| Vtx2c       | Associated with diarrhoea and HUS in humans; common in bovine and ovine VTEC                                                                                      | (Friedrich <i>et al.</i> , 2002; Pierard <i>et al.</i> , 1998; Schmitt <i>et al.</i> , 1991) |
| Vt2d        | Associated with eae-negative VTEC and mild disease in human                                                                                                       | (Friedrich <i>et al.</i> , 2002; Pierard <i>et al.</i> , 1998)                               |
| Vtx2dact    | Vero cell cytotoxicity is increased by elastase in intestinal mucus; these strains are highly virulent                                                            | (Kokai-Kun <i>et al.</i> , 2000)                                                             |
| Vtx2e       | A variant responsible for oedema disease of pigs; rare in human disease and associated with mild diarrhoea or asymptomatic infections in humans                   | (Gyles <i>et al.</i> , 1988; Sonntag <i>et al.</i> , 2005a)                                  |
| Vtx2f       | A variant frequently isolated from pigeon droppings; rare in human disease                                                                                        | (Sonntag <i>et al.</i> , 2005b)                                                              |
| Vtx2g       | A variant isolated from bovine strains; to date not associated with human disease                                                                                 | (Leung <i>et al.</i> , 2003)                                                                 |

with EPEC strains. The locus of enterocyte effacement (LEE), which is constituted of three functionally different components, is the genetic element responsible for the formation of A/E lesions. The first encodes a type three secretion system (TTSS) that exports effector proteins. The second encodes the structural components of the type three secretion apparatus, namely the secreted proteins EspA, B and D. The third encodes the 94-kDA outer-membrane protein intimin, encoded by *eae*, which mediate the intimate attachment of EHEC to the intestinal epithelial cells and its translocated receptor Tir, which is directly inserted in the host cell membrane.

## EHEC IN HUMANS

EHEC infections in humans are associated with a broad spectrum of clinical outcomes ranging from symptom-free infection through watery diarrhoea, to severe hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The incubation period is three days (ranging between 1-12 days) (Tarr *et al.*, 2005). Characteristically, patients suffer the first three days from watery diarrhoea, abdominal cramps and occasionally nausea and vomiting. In 90% of the cases, this diarrhoea becomes hemorrhagic within one to three days. When bloody diarrhoea develops, the patients has a normal platelet count, creatinine concentration and packed-cell volume with no erythrocyte fragmentation (Tarr *et al.*, 2005). In most cases, recovery from illness usually occurs spontaneously over approximately one week. However, the infection can evolve to the life-threatening HUS. The classic triad of features for HUS consists of acute renal failure, microangiopathic hemolytic anemia, and thrombocytopenia. The major risk factors for acquiring HUS include extremes of age (<15 years or > 65 years) (Dundas and Todd, 2000), early neutrophilia, hypoalbuminemia (Dundas *et al.*, 2001), increased C reactive protein level, fever within the first three days of illness (Ikeda *et al.*, 2000) and the administration of antibiotics and antimotility agents (Wong *et al.*, 2000). The mortality from HUS is between 3-17%, however in the elderly, it is as high as 87% (Griffin and Tauxe, 1991). While the kidney is the organ most commonly affected in HUS, evidence of central nervous system, pancreatic, skeletal and myocardial involvement may also be present (Richardson *et al.*, 1988; Sebbag *et al.*, 1999; Siegler, 1994). Pancreatic involvement, indicated by insulin-dependent diabetes mellitus and the increase of pancreatic enzymes, arises in less than 10% of the cases (Andreoli and Bergstein, 1982). The involvement of the central nervous system occurs in up to 25% of the cases and can lead to irritability, learning disabilities, lethargy and seizures and in sporadic cases to cerebral edema and coma (Amirlak and Amirlak, 2006; Elliott and Robins-Browne, 2005).

## RESERVOIR HOSTS OF EHEC

EHEC are usually found in the colon epithelium of ruminants, among which cattle are recognized to be the

main reservoir. Carriage rates in cattle appear to be particularly high (ranging from 0.2 to 70.1%) (Hussein and Bollinger, 2005) and contamination of foodstuffs from these animals has been highlighted as a major source of human infections. However, EHEC has been isolated from other food-producing, domestic and wild animals. In addition to the potential for foods derived from animals becoming contaminated with EHEC, animals themselves can act as vectors for the spreading of EHEC in the environment and to humans.

## Cattle

Cattle are generally regarded as the main natural reservoir of EHEC. Although most of the EHEC strains do not cause disease in cattle, some serogroups such as O5, O26 and O118 are associated with diarrhoea in calves (Mainil, 1999). The apparent resistance of cattle to systemic effects of VT may be due to the distinct pattern of Gb<sub>3</sub> receptors in their kidneys and the absence of receptors on vasculature (Hoey *et al.*, 2002). Shedding of EHEC in cattle has been shown to be intermittent. Most animals that test positive for EHEC become fecal culture negative within two or three months (Besser *et al.*, 1997; Rahn *et al.*, 1997). Nevertheless, while shedding among cattle appears transiently, EHEC infection maintains in cattle herds (Wells *et al.*, 1991). All ages of cattle are susceptible to colonization with EHEC, although peak shedding is observed in subadult cattle from weaning to 24 months of age (Hussein and Sakuma, 2005). The prevalence can also be affected by the season, with higher rates being reported during spring and summer. This seasonal effect has long been theorized to be related to the increased proliferation of EHEC in the environment during warm weather (Hancock *et al.*, 1994; Heuvelink *et al.*, 1998b). However, a recent hypothesis has emerged that day length and physiological responses within the animal to changing day length may explain the seasonal shedding patterns (Edrington *et al.*, 2006). The study showed that after a period of 60 days, there was a significant difference in shedding in the lighted pens compared to control groups with no light-treatment. Once the light-treatment was removed from the test group, shedding decreased to levels equivalent to the control group.

## Other ruminants

Small ruminants, such as sheep and goats, are also known carriers of *E. coli* O157 and non-O157. Compared with cattle, higher prevalence rates for EHEC have been found in sheep and goats. Beutin *et al.* (1993) demonstrated prevalence rates of 56% in goats, 67% in sheep compared to 18% in cattle. In Australia, in 88% of fecal samples from sheep grazing on pasture *vtx* genes were detected (Fegan and Desmarchelier, 1999). Ovine strains belong in general to six serogroups, namely O6, O91, O117, O128, O146 and O166 (Heuvelink *et al.*, 1998a; Urdahl *et al.*, 2003). The water buffalo is another potential source of EHEC in-

fections. A recent survey conducted in Italy has demonstrated that buffalo herds are often colonized by *E. coli* O157 (Galiero *et al.*, 2005). Based on the current preliminary prevalence rates, it is reasonable to assume small ruminants to be a potential source of human EHEC infections. However, given the preliminary character of the prevalence rates, further research may be warranted.

### Non-ruminants

EHEC have been occasionally isolated from animals other than ruminants. In many cases, it is not clear whether they are actual hosts of the bacteria or merely act as vectors after contact with contaminated feces (Wasteson, 2008; Wasteson *et al.*, 1999; Wasteson *et al.*, 1992). Johnsen *et al.* (2001) isolated *E. coli* O157 in 0.1% from pigs raised at farms that also breed cattle. In the United States, a recovery rate of 2% was observed from colon fecal samples of pigs. *E. coli* O157 has also been isolated from horses, cats and dogs. In all cases, these animals are housed on farms which also house cattle (Chalmers *et al.*, 1997; Trevena *et al.*, 1996).

### Wild animals

Besides from domestic animals, EHEC have been isolated from a variety of wild animals, such as rabbits and deer. In the United Kingdom, visitors to a wildlife park became infected with EHEC O157. This outbreak was associated with feces from wild rabbits, living in an adjacent field together with *E. coli* O157 positive cattle. This outbreak revealed that wild rabbits can also act as vectors. Fischer *et al.* (2001) detected EHEC O157 in 0.6% of the feces of white-tailed deer. The consumption of jerky made from deer meat has also been associated with human infections (Keene *et al.*, 1997). These outbreaks associated with wild animals highlight the potential role that wild animals can play in the transmission of EHEC.

## TRANSMISSION OF EHEC

There are four main transmission routes identified through which EHEC can be transmitted to humans: (1) foodborne transmission; (2) waterborne transmission; (3) person-to-person transmission; and (4) direct contact with animals.

### Foodborne transmission

Most outbreaks of EHEC infections are caused by inadequately cooked hamburgers or other beef products, and unpasteurized milk. Dairy products associated with outbreaks are those that are unpasteurized, had a pasteurization failure or were contaminated after pasteurization. The latter can be illustrated by a ice cream-related outbreak in Belgium (Buvens *et al.*, 2011). Over the last years, the contribution of other food vehicles has increased. For example, outbreaks have been associated with fermented sausages, apple juice, mayonnaise and

yoghurt. These outbreaks highlight the acid tolerance and the ability to survive the process of fermentation and drying of EHEC O157 strains. Fresh produce can be contaminated by direct contact with fecally contaminated soil, agricultural run-off or irrigation water. Noteworthy, the use of agricultural run-off water for irrigation is prohibited in Europe. In 2006, a multistate outbreak of *E. coli* O157 occurred in the United States. Fresh baby spinach, contaminated by feces of wild boars, was identified as the source of the outbreak, involving 183 persons (Jay *et al.*, 2007).

### Waterborne transmission

Water is a very efficient vehicle for the dissemination of EHEC. Surface waters may be subjected to EHEC contamination through run-off from pastures and from direct deposition of fecal material on the agricultural land. Water close to cattle herds may therefore present a potential reservoir of EHEC allowing the pathogen to spread in the environment. For example, in many countries, the river water is contaminated with a huge load of treated and untreated manure. Recently, *E. coli* O157:H7 has been detected in the Ganges River (Hamner *et al.*, 2007). Contaminated water as a source of EHEC infection may occur as a result of drinking (Bopp *et al.*, 2003; Olsen *et al.*, 2002) and swimming water (Samadpour *et al.*, 2002). The largest waterborne outbreak occurred in Canada in 2000. The outbreak led to seven deaths and more than 2300 illnesses (Hrudey *et al.*, 2003). The drinking water supply was contaminated by rainwater run-off containing cattle feces. Several of the drinking water outbreaks occurred in water systems without proper chlorination.

### Person-to-person transmission

Since the infectious dose is low (1 to 100 cells) person-to-person can easily occur through fecal-oral transmission following a primary case). Person-to-person transmission has emerged in day-care facilities as the predominant route of EHEC outbreaks (O'Donnell *et al.*, 2002; Reida *et al.*, 1994). It has been demonstrated that infected humans may shed EHEC for several weeks following resolution of the clinical features (Mead and Griffin, 1998). This prolonged shedding tends to be more pronounced in young children, explaining the outbreaks in day-care facilities. Furthermore, EHEC infections can occur asymptotically. Secondary transmission by the primary case in a household is therefore a particular concern.

### Contact with animals

Several EHEC outbreaks have been associated with animal exhibits at fairs, zoos and other venues resulting from direct contact with the animals and their environment followed by inadequate hand washing. Contact with pets has also been a route of EHEC infection. In Germany, a 2-year-old girl with bloody diarrhoea was found to excrete EHEC. Repeated stool samples

from her cat yielded a strain of EHEC O145:H– that showed the identical pathogenicity gen pattern as the girl's isolate. Although the cat had no symptoms, it excreted this strain for several months and was apparently the source of the girl's original infection and/or reinfection (Busch *et al.*, 2007).

#### OUTBREAK IN GERMANY, MAY 2011

Early May 2011, an unusually high number of HUS cases were reported in Germany. The outbreak strain was in fact not an EHEC strain as repeatedly reported in popular media, but shared characteristics of EAEC and VTEC. Strains with combinations of virulence properties from different pathotypes have been described before, but the size and severity of the outbreak have highlighted the importance and unpredictability of the consequences of genetic exchange between pathotypes.

#### The outbreak strain

The outbreak strain has been characterized very thoroughly at the Robert Koch Institute. The strain is of serotype O104:H4 and evolves from a progenitor that belongs to the enteroaggregative pathotype. The emergence of the outbreak strain depends on the acquisition of a *vtx2* prophage and of a plasmid encoding CTX-M-15 ESBL (Rohde *et al.*, 2011). The outbreak strain possesses therefore an unusual combination of virulence factors of EAEC and EHEC. This combination is very rare and has previously been described in strains of serotype O111:H2 involved in a small outbreak of HUS in children in France (Morabito *et al.*, 1998). It remains unclear why this strain has proven to be so virulent. However, it is conceivable that the enteroaggregative phenotype rendered these O104:H4 strains to colonize the intestinal mucosa as efficiently as the typical *eae*-positive EHEC strains. The augmented adherence of the strain to the intestinal epithelium might facilitate the systemic absorption of the verocytotoxins and could explain the high progression to HUS (Bielaszewska *et al.*, 2011).

#### Outbreak description

On May 22th 2011, Germany reported a significant increase in patients with bloody diarrhoea and HUS. During the succeeding month, thousands of infections occurred resulting in 3128 non-HUS cases, 782 cases of HUS and 46 deaths. The Robert Koch Institute stated on July 26th the outbreak in Germany as officially over, as the last onset of disease to be attributed to the outbreak was reported on the 4<sup>th</sup> of July 2011. Up to 125 infections with 49 cases of HUS caused by the outbreak strain have been reported in other European countries including, Austria, the Czech Republic, Denmark, France, Greece, Luxembourg, the Netherlands, Norway, Poland, Spain, Sweden and the United Kingdom. Most patients appeared to be travellers returning from Germany.

Of particular interest, O104:H4 cases showed an uneven sex distribution, with a preponderance of women in both non-HUS infections (59%) and HUS (68%) cases. In addition, people over 20 years of age account for the vast majority of the cases (88%). This sex and age predominance might be related to gender-specific differences in dietary habits, namely vegetables are generally more often consumed by adult women. Other particular features of this outbreak are the high percentage of HUS cases (20-25% instead of 5-6%), common severe neurological complications and non-HUS deaths.

On June 24th, France also reported a cluster of *E. coli* O104:H4 infections among people who attended an open day at a children's community center in Bordeaux. All patients reported eating sprouts served at the event. The European Food Safety Agency (EFSA) conducted a comprehensive investigation to identify the source of the two outbreaks (EFSA, 2011). The analysis of the investigation identified a single lot of fenu-greek seeds, from an exporter in Egypt, as the most likely source of the sprouts linked to the two outbreaks.

#### CONCLUSION

The knowledge of the epidemiology and ecology of EHEC is far from complete. To date, EHEC research has focused mainly on EHEC O157 in cattle. However, EHEC O157 and non-O157 have been reported in many other animal species. New routes of transmission have also emerged, such as contact with animals during farm visits, contact with pets and a wide variety of environment-related exposures. In order to inform risk assessment, further research into non-bovine animal species, foodstuffs or environmental vehicles should be considered and tested.

The main conclusion from the German outbreak is that *E. coli* strains belonging to different pathotypes allowed for the emergence of the highly virulent verocytotoxin-producing enteroaggregative *E. coli* O104:H4 strain. Epidemiologists and microbiologists face many challenges of detecting strains belonging to different pathotypes and in preventing and managing future outbreaks of such strains.

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