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Review

Escherichia coli O104:H4 outbreak — have we learnt a lesson from it?

Sylwia K. Bloch, Agnieszka Felczykowska and Bożena Nejman-Faleńczyk✉

Department of Molecular Biology, University of Gdańsk, Gdańsk, Poland

Shiga toxin-producing *Escherichia coli* (STEC) strains belong to the group of pathogens that cause bloody diarrhea and hemorrhagic colitis with often severe complications. The main problem with human pathogenic *E. coli* strains, including STEC, is a wide spectrum of phenotypes and clinical manifestations. It is related to a variety of exchangeable genetic elements, like plasmids, bacteriophages, transposons and pathogenicity islands, that take part in horizontal gene transfer which influences creation of new dangerous bacterial strains. A good example of this phenomenon is a novel Shiga toxin-producing *E. coli* O104:H4 serotype that was associated with a widespread and severe foodborne disease outbreak in Germany in 2011. The O104:H4 strain was created by a number of horizontal gene transfer events between two distinct pathogens, resulting in the emergence of the new, atypical strain. That outbreak proved that also rare and unusual serotypes of STEC may be a significant risk factor and that the procedures recommended for STEC detection were not suitable to deal with this kind of pathogens. With respect to new combinations of chromosomal and extrachromosomal elements in susceptible bacterial hosts, epidemics and frequent human infections caused by STEC strains, we suggest that more attention should be paid to the development and improvement of diagnostic methods. It is difficult to determine STEC bacteria by general microbiological, biochemical and immunological assays, because strains can vary dramatically in their phenotypic and serotypic properties. It is postulated that standardized genetic tests, based on detection of features most frequently presented by STEC, particularly those located on easily exchangeable elements (such as Shiga toxin-encoding phages), can be more adequate for STEC detection.

Key words: *Escherichia coli* O104:H4, outbreak, STEC, mobile genetic elements, detection methods

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BACKGROUND

Diarrheagenic *Escherichia coli* strains are important causes of diarrhea in humans. They have been divided into different pathotypes according to their virulence attributes and the mechanisms involved in the disease process. Five major groups of intestinal *E. coli* pathogenic strains have been established such as: enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enterohemorrhagic *E. coli* (EHEC) (Donnenberg & Whittam, 2001; Robins-Browne & Hartland, 2002; Bugarel *et al.,* 2011). Sometimes instead of EHEC, a larger group of pathogenic *E. coli* bacteria, known as Shiga toxin-producing *E. coli* (STEC) is listed (Kagkli *et al.,* 2012).

EHEC belong to the group of STEC bacteria which are defined by their ability to produce Shiga toxins (Donnenberg & Whittam, 2001). Production of Shiga toxins depends on the presence of *stx* genes, located in the bacterial genome on lambdoid prophages named Shiga toxin-carrying phages or shortly Stx phages, which can be classified as mobile genetic elements (Nataro & Kaper, 1998; Besser *et al.,* 1999; Schmidt, 2001). Functionally active Shiga toxins may be detected using the Vero cell toxicity test and this is the reason why these bacteria are also called verotoxin- or verocytotoxin-producing *E. coli* (VTEC) (Konowalchuk *et al.,* 1977). The acronyms STEC and VTEC are used interchangeably in the literature, however, in this work the designation STEC will be used.

STEC strains can spread with an alarming ease via food or water and they have started to attract special attention as some highly pathogenic serotypes caused severe epidemic outbreaks with numerous deaths. Production of Shiga toxin molecules by STEC strains results in serious changes in the host cell metabolism due to inhibition of protein synthesis, which cause bloody diarrhea and often severe complications like hemorrhagic colitis and/or hemolytic uremic syndrome, especially dangerous in children (Nataro & Kaper, 1998; Besser *et al.,* 1999; Gyles, 2007; Serna & Boedeker, 2008). Several hundred *E. coli* serotypes that produce Shiga toxins have been described (Scheutz & Strockbine, 2005; Karch *et al.,* 2005) but according to an opinion of the European Food Safety Authority, serogroups O26, O103, O111, O145, and O157 have been the most frequently isolated *E. coli* bacteria from humans and associated with epidemic or/and serious human infections. Surprisingly, the outbreak in northern Germany and several other countries in 2011 brought to light another dangerous, *stx*-positive serotype, the O104:H4 strain (Muniesa *et al.,* 2011).

✉e-mail: b.nejman@biotech.ug.gda.pl

Abbreviations: AAF, aggregative adherence fimbria; CT-SMAC, cefixime tellurite sorbitol MacConkey agar; EAEC, enteroaggregative *Escherichia coli*; EAHEC, enteroaggregative-heamorrhagic *Escherichia coli*; EFSA, European Food Safety Authority; EHEC, enterohemorrhagic *Escherichia coli;* ehly, enterohemolysin; EIEC, enteroinvasive *Escherichia coli;* ELISA, enzyme-linked immunosorbent assay; EPEC, enteropathogenic *Escherichia coli*; ESBLs, extended-spectrum β-lactamases; HPI, high-pathogenicity island; HUS, hemolytic uremic syndrome; LEE, locus for enterocyte effacement; LPF, long polar fimbrie; LPS, lipopolysaccharide; Pic, protease involved in colonization; RPLA, reversed passive latex agglutination; RT-PCR, Real-Time PCR; Set1, *Shigella* enterotoxin 1; STEC, Shiga toxin-producing *Escherichia coli*; TelR, tellurite resistance; VBNC, viable but non-culturable state; VTEC, verotoxin or verocytotoxin-producing *Escherichia coli.*

THE *ESCHERICHIA COLI* O104:H4 PHENOMENON

The Shiga toxin-producing *E. coli* serotype O104:H4 was responsible for one of the largest German epidemic outbreaks, which took place in the summer of 2011. This multidrug-resistant serotype O104:H4 caused over 3000 cases of diarrhea without hemolytic uremic syndrome (HUS) and over 830 cases with HUS, leading to 54 deaths (Muniesa *et al.,* 2011). Furthermore, some infections were observed in 12 other European countries and also in North America and Canada (Muniesa *et al.,* 2011; World Health Organization, 2011). It is known that the source of the epidemic serotype O104:H4 that rapidly spread across the globe were fenugreek seeds that had been imported to Europe from Egypt in 2009 (Razzaq, 2006; Obrig, 2010; World Health Organization, 2011). The fatal cases, many difficulties with diagnosis, and over 3 billion euros in economic losses caused by that outbreak have attracted attention of scientists, who posed a question: Why was O104:H4 such a 'killer', what is the phenomenon of this bacterium?

Researchers, using next generation sequencing technology, explored the genome sequence of the epidemic O104:H4 isolates and determined that this strain has a unique combination of genes present in enteroaggregative as well as enterohemorrhagic *E. coli* types (Table 1) (Mellmann *et al.,* 2011; Ho *et al.,* 2011; Rasko *et al.,* 2011). In addition, investigation of its genome sequence indicated that the German outbreak strain can represent a new pathotype designated as enteroaggregative-heamorrhagic *Escherichia coli* (EAHEC) and that several horizontal gene transfer events took place to create its genome (Brzuszkiewicz *et al.*, 2011).

In the case of the O104:H4 strain, we observe also the specific situation that two mobile elements, a phage and a plasmid, contribute to the main virulence features of the pathogen (Muniesa *et al.,* 2011). Many analyses have indicated that this strain possesses the genetic background of EAEC and harbors a plasmid carrying an aggregative adherence fimbria (AAF) operon *aat* identified as an allelic variant of AAF/I (Rasko *et al.,* 2011), which take part in the initial attachment of bacteria to the intestinal mucosa. However, it lacks the plasmid carrying AAF/III and the *astA* gene encoding the EAEC heat-stable enterotoxin (EAST1). The German outbreak strain has also the same adherence phenotype as EAEC and adheres to HEp-2 cells in culture with a specific "stacked brick" pattern, that distinguishes them from enteropathogenic *E.coli* (Bielaszewska *et al.,* 2011). It should be noted that the *agg*R gene was also found on that plasmid. AggR is one of the transcriptional activators (Nataro *et al.,* 1994) that regulate expression of the *aap* gene encoding dispersin, which in turn interacts with lipopolysaccharide (LPS) and regulates the structure of the AAF filaments (Velarde *et al.*, 2007). The *ipd* gene encoding an extracellular serine protease and a gene encoding serine protease Pet, which are usually localized next to the AAF operon on the pAA plasmid, were also found in the *E. coli* O104:H4 strain. In addition, similarly to EAEC, the O104:H4 strain encodes two important virulence factors: protease involved in colonization (Pic) and *Shigella* enterotoxin 1 (Set1) — a bacterial AB_5-type toxin. It is known that Pic is a multifunctional protein with mucinase activity, and together with Set1 they are involved in enteric pathogenesis (Fasano *et al.,* 1995; Harrington *et al.,* 2009).

In contrast to EAEC strains, the German outbreak strain produced the 2a variant of the Shiga toxin, for which genes are carried on a lambda-like bacteriophage (Herold *et al.,* 2004; Muniesa *et al.,* 2004). However, in contrast to EHEC, it does not contain a plasmid-borne gene for enterohemolysin (*ehly*) and a LEE (locus for enterocyte effacement) pathogenicity island, which is important for adherence in the colon (Brzuszkiewicz *et al.,* 2011; Mora *et al.,* 2011). It is worth to mention that effective production of Shiga toxins is observed only upon prophage induction and its lytic development, which also include replication of the phage genome as an extrachromosomal element (Kimmitt *et al.,* 2000; Schmidt *et al.,* 2001; Wagner *et al.,* 2001, 2002; Herold *et al.,* 2004; Waldor & Friedmann, 2005; Nejman *et al.,* 2009).

During phage induction which can be caused by a wide range of inducers, like low pH, iron ions, antibiotics (Kimmit *et al.,* 1999, 2000) or hydrogen peroxide (Łoś *et al.,* 2009, 2010), many copies of the phage genome are generated and expression of the *stx* genes increases rapidly. The next step of this process is disruption of the bacterial host cell and release of a large amount of Shiga toxins and Stx progeny phages, thus further enhancing severity of infection.

The Stx prophage integration in the German outbreak strain was a recent evolutionary event (Rasko *et al.,* 2011). The new dangerous O104:H4 strain presents on its cell surface a typical receptor conserved in *E. coli*, named YeaT, which is probably recognized by a tail spike protein of the Stx phage (Smith *et al.,* 2007; Rohde *et al.,* 2011). The *stxAB* genes carried on the O104:H4 prophage are located between genes encoding the antitermination protein Q and the S/R lysis proteins of the lambdoid prophage. The same organization is observed

Table 1. Origins of the most significant features acquired by *E. coli* O104:H4 strain

Original strain	Features transferred to the *E. coli* O104:H4	Location of corresponding gene(s)
EAEC (e.g. O125:H21, O44:H18, O44:H44, O78:H2)	pAA plasmid (allelic variant AAFI), *Shigella enterotoxin* 1 (Set1), Pic protein	plasmid chromosome chromosome
EHEC (e.g. O157:H7, O111:H8, O26:H-, O26:H11)	Shiga toxin-converting bacteriophage (variant Stx2a), Tellurite resistance (TeIR), Mercuric resistance plasmid, Long Polar Fimbrie (LPF)	prophage chromosome plasmid chromosome
OTHER (e.g. *Klebsiella pneumoniae, Yersinia pestis*, *E. coli* O6:K2:H1)	TEM-1 and CTX-M resistance plasmid, High-Pathogenicity Island (HPI) and iron uptake systems, IrgA homologue adhesin	plasmid chromosome chromosome

in the case of Stx phage 933W from the O157:H7 strain, whose genome has a high degree of sequence identity with the Stx phage integrated into the O104:H4 chromosome. All these features suggest recent horizontal gene transfer events and also confirm that the high virulence of the German outbreak strain is a direct consequence of the phage acquisition (Muniesa *et al.,* 2012; Boyd *et al.,* 2012). The considerable diversity observed in the prophage elements of STEC strains indicates a highly mobile nature of the phage genomes and their significant role in transferring genetic material between bacterial strains (Brussow *et al.,* 2004; Ahmed *et al.,* 2012).

Similarly to *E. coli* O157:H7, the O104:H4 strain is also resistant to a highly toxic tellurium oxyanion, named tellurite (Zadik *et al.,* 1993; Taylor *et al.,* 2002; Bielaszewska *et al.,* 2005; Orth *et al.,* 2007). Tellurite resistance (TelR) is encoded by the terZABCDEF operon located on the EHEC O157:H7 chromosome. All elements of this operon were identified in the outbreak strain too. Furthermore, the O104:H4 strain acquired also a mercuric resistance plasmid carrying genes encoding mercuric ion transport proteins (MerT, MerP, MerC), the transcriptional regulators MerR and MerD, and mercuric ion reductase MerA (Brzuszkiewicz *et al.*, 2011).

Another feature of the new pathogenic strain is the presence of plasmid-encoded extended-spectrum β-lactamases (ESBLs), CTC-M and TEM1, whose genes seem to be located on a mobile genetic element Tn3-type transposon that often occurs in enteric bacteria (Livermore, 1995). This specific plasmid shares also high similarity with plasmids pEC_Bactec, pCVM29188_10, and pEK204 (Brzuszkiewicz *et al.*, 2011).

In summary, the German outbreak O104:H4 strain is an example of a unique combination of relatively well-known virulence genes derived from two pathogens which, when conquered one organism, lead to the development of a more severe disease and wreak havoc on our health. This specific combination of genes, located mostly on mobile DNA elements, can be achieved by *E. coli* very easily, which suggests that this could not be the last surprise from these bacteria.

METHODS FOR STEC DETECTION IN THE LIGHT OF THE *ESCHERICHIA COLI* O104:H4 OUTBREAK

Detection of STEC strains has been the subject of several studies in recent years, resulting in the development of a number of methodological reports. Since *E. coli* O104:H4, an unusual bacterial strain, was characterized as a hybrid of enteroaggregative and enterohemorrhagic *E. coli* pathotypes, the diagnostic strategies of *stx*-positive strains have received additional attention. The elaborated methods can be grouped into several classes, and some of them should be taken into consideration after the *E. coli* O104:H4 outbreak in 2011.

The first class of methods for STEC detection applies different microbiological and biochemical assays. According to The European Standard EN ISO 16654:2001, published by the European Committee for Standardization, the method for detection of *E. coli* O157 is based on culturing bacteria on cefixime tellurite sorbitol MacConkey agar (CT-SMAC), followed by confirmation of sorbitol-negative colonies from CT-SMAC by indole production and agglutination with *E. coli* O157 antiserum. This standard is based on the early studies which suggested that O157 strains are able to produce indole and are unable to ferment sorbitol. This procedure is recommended in spite of later studies showing that some strains are indole-negative and that some of *E. coli* O157 bacteria and also other *stx*-positive serotypes of *E. coli* (including O104:H4 strain) are sorbitol-positive (Riley *et al.,* 1983; Bopp *et al.,* 1987; Vaishnavi *et al.,* 2001; Corry *et al.,* 2003; Łoś *et al.,* 2011; Scheutz *et al.*, 2011). Moreover, the majority of STEC strains present variable β-D-glucuronidase reactions (Krishnan *et al.*, 1987; Vaishnavi *et al.,* 2001) and not all O157 strains carry the *stx* genes (Schmidt *et al,* 1999). Apart from that, differential media have been elaborated for the isolation of non-O157 STEC serotypes, reported as the most commonly associated with serious human diseases (Posse *et al.,* 2008; Tillman *et al.,* 2012). However, according to an opinion of the European Food Safety Authority (EFSA), there is no microbiological method that can be accepted as an official standard for the detection and isolation of non-O157 STEC. Considering the technical side of microbiological methods, the long long proceduredu-ration of procedure is their main disadvantage, however, an additional serious problem was noticed during the outbreak in Germany. This problem concerns the ability of the outbreak strain *E. coli* O104:H4 to enter a viable but non-culturable state (VBNC). The VBNC state is marked by the presence of viable cells but the inability to grow on routine laboratory media. It was found that *E. coli* O104:H4 bacteria enter the VBNC state in response to stressful conditions such as a nutrient-poor micro-environment, toxic concentrations of copper ions or certain types of tap water (Aurass *et al.,* 2011; Muniesa *et al.,* 2012), which seriously complicated its detection by culture-based methods and following isolation of the pathogen. In the light of the technical disadvantages as well as the occurrence of many phenotypic variants of STEC strains, their detection by microbiological and biochemical assays as part of a routine monitoring system seems to be difficult and even unreliable, therefore application of such methods should be considered carefully with suggestions to use other options.

The second group of methods for STEC detection is based on immunological assays. Serogrouping of pathogenic *E. coli* strains is performed in order to determine the "O" somatic antigen and "H" flagella antigen, characterising each serogroup. STEC strains belong to a large variety of serotypes. Presently, a total of 181 O-antigens and 53 H-antigens are known, but only a limited number of those have been identified as clinically important (Karmali *et al.,* 2003; Blanco *et al.,* 2004; EFSA, 2007). There are various immunoassay formats which include enzyme-linked immunosorbent assays (ELISAs), immunoblot assays, reversed passive latex agglutination (RPLA), direct immunofluorescent filter techniques, and several immunocapture techniques (De Boer & Heuvelink, 2000; Blais *et al.,* 2004; Islam *et al.,* 2006). These assays utilize poly- or monoclonal antibodies specific for the O and H antigens, several of them are commercially available (for instance from Denka-Seiken, Japan, or Remel, USA). However, there is a very limited range of immunoassays which specifically target STEC non-O157 serotypes. Moreover, they target mainly serotypes that have been reported as the most dangerous for humans (Ludwig *et al.,* 1996; Aldus *et al.,* 2003), which makes them useless for detection of rare serotypes (such as *E. coli* O104:H4). An additional problem of such immunoassays is that false-positive results may be generated because of cross-reaction with surface antigens of other bacteria. Taking it into consideration, we conclude that in the light of the huge number of STEC serotypes and dangers arising from rare serotypes (such as the *E. coli* O104:H4 strain), the immuno-based methods that utilize

antibodies towards Shiga toxins (Bettelheim *et al.,* 2003; Parma *et al.,* 2012) seem to be universal and more appropriate for detection of pathogens from the STEC group, at least in the initial phase of the detection process.

Molecular methods (the third group of the methods) for STEC detection are used in various nucleic acid-based assay formats, the most popular and commercially available being those based on PCR and Real-Time PCR (RT-PCR) techniques. Nucleic acid-based assays target various genes, including virulence genes, such as *stx* coding for Shiga toxins or *eae* coding for intimin and serogroup target genes associated with O- as well as H-antigens (Schmidt *et al.,* 2000; Perelle *et al.,* 2004; Anklam *et al*, 2012). Determination of the H-type has been directed mainly at the *fli*C gene whereas the majority of O-type specific assays target different genes of those O-antigens associated with the most commonly isolated strains from humans (such as the *rfbO157* gene of O157, *wzx* gene coding for the flippase in O26, *wzy,* encoding the O-antigen polymerase in O103, or *wbdL* gene encoding sugar transferase in O111) (Paton *et al.,* 1998; Beutin & Strauch, 2007; Bugarel *et al.,* 2010; Fratamico *et al.,* 2011). Some of these targets have been combined into multiplex assays (Paton *et al.,* 1999; Monday *et al.,* 2007; Beutin *et al.,* 2009) and one of such assay, based on multiplex PCR amplification of virulence genes (*stx* and *eae*), is recommended by the European Union Reference Laboratory for STEC identification in food samples (EFSA, 2007; 2009). Another recommended method is a RT-PCR protocol submitted to the International Organization for Standardization (ISO/DTS 13136:2012) in the form of "Technical Specification" by Working Group 6 of the Technical Committee 275 of the European Committee for Standardization (CEN TC275/WG6). The method is presently under evaluation and targets a combination of both virulence genes (including those encoding Shiga toxins and intimin) and serogroup specific genes for O26, O103, O111 and O145 (Nielsen & Andersen, 2003; Perelle *et al.,* 2004, 2005).

Since the appearance of the unusual (deprived of the *eae* gene) bacterial strain *E. coli* O104:H4, diagnostic strategies have attracted additional attention and new methods have been developed. The presence of the *eae* gene was considered as a hallmark of pathogenic STEC, thus the diagnostic methods available and recommended before the German outbreak turned out useless in the case of *E. coli* O104:H4 detection. The majority of methods elaborated for detection of this unusual strain target different O104 antigen-encoding genes, such as *wbwC, wzx*, and *wzy* (Delannoy *et al.,* 2012; Kagkli *et al.,* 2012; Zhang *et al.,* 2012). The European Union Reference Laboratory for STEC has proposed a method that aims at the identification of the presence of O104 antigen in *stx*-positive enrichment cultures. This RT-PCR based assay targets O104 serogroup-associated genes: *wzxO104,* coding for the O-antigen, flippase Wzx, and the gene encoding the flagellar antigen H4, *fliCH4*. The molecular design of this RT-PCR protocol has been partly described in the literature (Bugarel *et al.,* 2010).

ARE THE DIAGNOSTIC TRENDS GOING IN THE RIGHT DIRECTION?

The outbreak in Germany showed that two pathotypes, EHEC and EAEC (considered to be clearly distinct from each other until then) are able to exchange and share virulence genes located on mobile genetic elements such as Stx phages. All the *stx* genes found so far among STEC strains are carried on lambdoid prophages, which after induction are released from the bacterial host and able to infect other susceptible *E. coli* strains, thus further enhancing Shiga toxin production by horizontal gene transfer (Herold *et al.,* 2004; Łoś *et al.,* 2011). Also, bacterial genera other than *Escherichia* can be converted by Shiga toxin-carrying phages to be Shiga toxin producers (Strauch *et al.,* 2001; Herold *et al.,* 2004). The genome of the German outbreak strain was created by horizontal gene transfer events that took place between two distinct pathogens (Table 1), resulting in the emergence of the new atypical organism — a big surprise for the whole medical community. The ease with which Stx phages and other mobile genetic elements can be transferred between *E. coli* strains suggests that this situation may occur again in the future and surprise us one more time. We cannot predict which pathogens will be converted to Shiga toxin producers next time, but we can try to better prepare for this event. Accordingly, modified detection strategies for STEC strains should be considered.

The direction of STEC diagnostic is going towards assumptions that monitoring of STEC should be mainly concentrated on O157 (since this serogroup is associated with severe human illnesses) and those non-O157 serogroups that are most frequently reported as causing human infections. According to the opinion of the BIOHAZ panel on the monitoring of STEC (EFSA, 2007; 2009), the serogroups that should be considered as the most dangerous to humans beside O157 are O26, O91, O103, O111, and O145. Hence, the European Committee for Standardization is currently developing standard methods for their detection. The German outbreak in 2011 brought to light an additional dangerous serotype, *E. coli* O104:H4, indicating that also rare and unusual serotypes of STEC may pose a significant threat to humans. It is important to realize that the appearance of the atypical strain O104:H4 was very surprising for diagnostics and that the detection procedures recommended for STEC were not designed to deal with this kind of pathogens. Moreover, it is essential to realize the danger arising from the fact that such situation can occur again and also other rare strains can be converted into Shiga toxin producers.

Taking it into account, we would like to highlight the necessities of development of STEC diagnostic towards elaboration of more universal methods, essential for the whole STEC group. Determination of STEC bacteria by microbiological, biochemical and serological assays is difficult because strains can vary significantly with respect to their phenotypic and serotypic properties. We suggest that molecular methods based on detection of STEC-specific characteristics, particularly those located on easily exchangeable elements (such as Shiga toxin-converting phages), could be more adequate. Methods targeting various STEC serotypes are significant especially in later steps of detection, such as pathogen characterization, however, serotyping when applied to the wide variety of STEC strains is not an optimal method of identifying public health risk. In the light of the *E. coli* O104:H4 outbreak and a risk of similar situation occurring in the future, expansion of the diagnostic trends towards development and standardization of STEC-specific but serotype-independent methods should be taken into consideration.

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