The 2011 German Enterohemorrhagic *Escherichia Coli* O104:H4 Outbreak—The Danger Is Still Out There

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Contents

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Abstract Enterohemorrhagic *Escherichia coli* (EHEC) are Shiga toxin (Stx) producing bacteria causing a disease characterized by bloody (or non-bloody) diarrhea, which might progress to hemolytic uremic syndrome (HUS). EHEC O104:H4 caused the largest ever recorded EHEC outbreak in Germany in 2011, which in addition showed the so far highest incidence rate of EHEC-related HUS worldwide. The aggressive outbreak strain carries an unusual combination of virulence traits characteristic to both EHEC—a chromosomally integrated Stx-encoding bacteriophage, and enteroaggregative *Escherichia coli*—pAA plasmid-encoded aggregative adherence fimbriae mediating its tight adhesion to epithelia cells. There are currently still open questions regarding the 2011 EHEC outbreak, e.g., with respect to the exact molecular mechanisms resulting in the hypervirulence of the strain, the natural reservoir of EHEC O104:H4, and suitable therapeutic strategies. Nevertheless, our knowledge on these issues has substantially expanded since 2011. Here, we present an overview of the epidemiological, clinical, microbiological, and molecular biological data available on the 2011 German EHEC O104:H4 outbreak.

1 Introduction

Commensal *Escherichia coli* (*E. coli*) are part of the human gut microbiota (Human Microbiome Project 2012). Pathogenic *E. coli* strains, however, can cause various intestinal and extraintestinal diseases in humans (Kaper et al. 2004). Among them are the enterohemorrhagic *E. coli* (EHEC), which causes diarrhea, hemorrhagic colitis (bloody diarrhea), and hemolytic uremic syndrome (HUS, characterized by hemolytic anemia, thrombocytopenia, and acute kidney injury) (Karch et al. 2005). The hallmark of EHEC pathogenesis is the production of Shiga toxins (Stx), which irreversibly inhibit host cell protein synthesis and lead to cell death (Karpman et al. 1998; Tarr et al. 2005). The majority of EHEC infections and EHEC-associated HUS have been attributed to the serotype O157:H7 (Karch et al. 2005). Strains belonging to serogroups other than O157, however, have been also recognized as clinically important (Johnson et al. 2006; Mellmann et al. 2008b). For example, almost one-third of the 524 EHEC isolates from HUS patients that were used to generate the German HUS-associated *E. coli* (HUSEC) reference strain collection belonged to non-O157 serotypes (Mellmann et al. 2008b).

The largest EHEC outbreak ever recorded in Germany took place from May to July 2011. Nearly 4000 EHEC gastroenteritis and more than 850 HUS cases were reported, leading to 54 deaths (Robert-Koch-Institut 2011). This was also the largest incidence of EHEC-associated HUS worldwide. Moreover, the infections were characterized by an unusually high rate of progression to HUS (Frank et al. 2011b), further suggesting that the strain responsible for it is highly virulent. EHEC of the rare serotype O104:H4 (EHEC O104:H4) was identified as the causative agent for the outbreak. Interestingly, with respect to virulence gene content the outbreak strain is a hybrid of EHEC and enteroaggregative *E. coli* (EAEC) (Brzuszkiewicz et al. 2011;

Mellmann et al. 2011; Rasko et al. 2011). EAEC is another type of intestinal pathogenic *E. coli* associated with acute and persistent diarrhea (Nataro et al. 1998). Besides having a chromosomally integrated Stx bacteriophage, EHEC O104:H4 carries a pAA plasmid-encoded aggregative adherence fimbriae (a characteristic feature of EAEC), mediating its tight adherence to cultured epithelial cells (Bielaszewska et al. 2011). Up to date, only a few sporadic or small outbreak-related cases of infections associated with other EHEC strains displaying an aggregative adherence phenotype have been reported (Morabito et al. 1998; Mellmann et al. 2008b; Jourdan-da Silva et al. 2012). Therefore, it remains unclear if the severity and dimensions of the 2011 EHEC O104:H4 outbreak were due to a particularly virulent strain or favorable outbreak settings or both.

In this chapter, we aimed to provide an overview of the current knowledge on the 2011 EHEC O104:H4 outbreak and its highly pathogenic causative agent. We summarized the epidemiological and clinical data on the outbreak. Furthermore, we described the genomic organization of EHEC O104:H4, as well as the factors and mechanisms, which were shown to contribute to its virulence. Last, but not least, we reviewed the treatment approaches used during the outbreak and other still experimental therapeutic strategies.

2 The 2011 EHEC O104:H4 Outbreak

2.1 Time Course and Epidemiology of the Outbreak

On May 1, 2011, first symptoms of EHEC infection attributed to the outbreak appeared in patients. Two days later the first patient developed HUS. On May 19, 2011, the Robert-Koch-Institut, Germany's national public health authority, was informed about a cluster of three cases of HUS in children admitted on the same day to the university hospital in the city of Hamburg (Frank et al. 2011b). On 22 May, the peak of the outbreak was reached. After this time point, the numbers of new EHEC infections and associated HUS cases decreased. In the mid of June, only single cases but no disease clusters were reported. The last recognized infection was recorded on 4 July. Three weeks after no additional case was reported, the end of the outbreak was declared on July 26, 2011 (Robert-Koch-Institut 2011) (see also Fig. 1).

In total, over 4000 cases of gastroenteritis, 852 HUS cases, and 54 deaths attributed to the outbreak were reported. Though in every part of Germany cases occurred, the four northern federal states (Hamburg, Bremen, Lower Saxony, and Schleswig-Holstein) reported more than 50% of all cases, with an incidence rate of 10/100,000 (Robert-Koch-Institut 2011). Cases linked to this outbreak were also communicated from other European countries: On May 25, 2011, Sweden reported nine cases of HUS of whom four had traveled to northern Germany from 8 to 10 May. Denmark reported four cases of gastroenteritis, two of them progressed to HUS. Here, all cases had a recent travel history to northern Germany. Another two HUS cases with travel history to northern Germany in the relevant period were

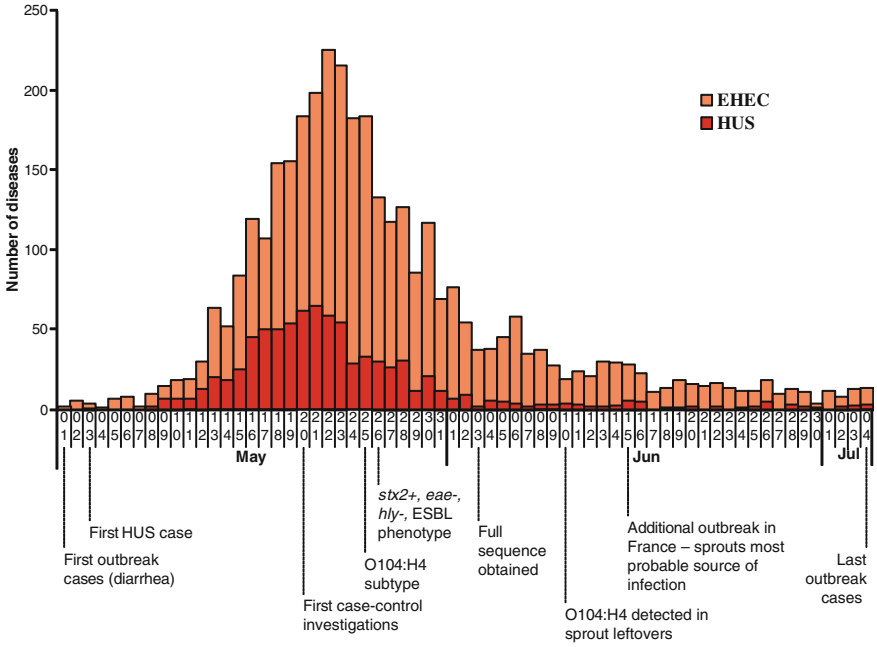

Fig. 1 Time course and epidemiology of the EHEC O104:H4 outbreak. The diagram shows the epidemiological course of the outbreak including 809 HUS and 2717 EHEC cases with known onset of diarrhea, reaching its peak on 22nd May 2011(Robert-Koch-Institut 2011). The bottom line indicates analyses and diagnostic pathways during the outbreak (Karch et al. 2012)

communicated, one by the Netherlands and other by the UK (Frank et al. 2011a). German cases could also be linked to 15 cases of an O104:H4 outbreak in Bordeaux, France, since the pulsed-field gel electrophoresis pattern of the French isolates was identical but different from pre-outbreaks O104-reference strains (Mariani-Kurkdjian et al. 2011).

2.2 *Diagnostic of the Outbreak Strain*

After the HUS clusters were reported to the Robert-Koch-Institute on May 19, 2011, *E. coli* strains were examined in the national reference laboratory for bacterial enteritis. On 23 May, conventional PCRs on two cultured isolates revealed that the outbreak was caused by an stx_1- and *eae*-negative and stx_2-positive EHEC strain (Robert-Koch-Institut 2011). Simultaneous molecular subtyping via partial *gnd*-sequencing, *fliC*-RFLP-typing and multilocus-sequence typing (MLST), performed in the German national consulting laboratory for HUS revealed an O104:H4 serotype of MLST sequence type (ST) 678 (Bielaszewska et al. 2011; Mellmann et al. 2011). On 26 May, an additional ESBL phenotype with resistances against

third-generation cephalosporines was confirmed and the Stx2a subtype was identified (Bielaszewska et al. 2011; Karch et al. 2012) (see also Fig. 1).

A multiplex PCR of the rfb_{O104} (gene from the O104 biosynthetic cluster), $fliC_{H4}$ (gene from the H4 antigen biosynthetic cluster), stx_2 and *terD* (gene from the tellurite resistance cluster) was initially used to identify outbreak isolates (Bielaszewska et al. 2011). A real-time multiplex PCR targeting stx_2, wzy_{O104}, *and* $fliC_{H4}$ was additionally developed to allow for the rapid and sensitive detection of EHEC O104:H4 in human stools (Zhang et al. 2012). To further increase diagnostic specificity, a high throughput alignment-free strategy based on whole genome sequencing data was developed to design PCR primers which could discriminate between the 2011 outbreak strain and the closely related HUSEC041 (Pritchard et al. 2012), which is another *stx2* positive O104:H4 strain isolated from a single HUS patient in 2001 in Germany (Mellmann et al. 2008b).

2.3 Origin, Reservoir, Transmission, and Shedding of the O104:H4 Outbreak Strain

The identification of the outbreak source was initiated while the outbreak was still ongoing. In a case-control study including 26 cases and 87 control patients the source of illness was found to be significantly associated with sprout consumption in a univariable analysis and with sprout and cucumber consumption in a multivariable analysis (Buchholz et al. 2011). Later a study investigating outbreak cases related to a community center event could show that the consumption of fenugreek sprouts was significantly associated with the development of symptoms (King et al. 2012). Fenugreek sprouts origin could be traced back to a common import of seeds from Egypt that arrived in Rotterdam and was distributed to Germany, and then partly redistributed to the UK from where a portion finally made its way to France (Karch et al. 2012). After the indirect identification of fenugreek sprouts as the most probable infection vehicle and establishing a sales stop for this food product at the beginning of June 2011, no clusters of diseases occurred. Nevertheless, the outbreak strain was not detected on any of the sprout samples analyzed and no contamination was found on investigated farms (European-Food-Safety-Authority 2011). The question when and where the potential contamination occurred is not clarified as well. A recent study did not show any exceptional or prolonged survival of culturable EHEC O104:H4 on dry fenugreek seeds. This indicated that the contamination might have not initially occurred in Egypt, but rather at later stages of the seed processing (Knodler et al. 2016).

Initial investigations conducted during the outbreak, revealed cattle is unlikely to be a reservoir of EHEC of serotype O104:H4, different from what was shown for other EHEC variants like O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28 (Wieler et al. 2011; Auvray et al. 2012; Pierard et al. 2012). However, recent investigations based on experimental inoculation of the outbreak strain in calves showed that cattle can at least transiently carry and therefore be a reservoir for

O104:H4 (Hamm et al. 2016). Infections via contaminated sprouts were considered to be more likely, thus classifying O104:H4-associated infections as a foodborne disease (Buchholz et al. 2011; King et al. 2012) even though it remained unclear which was the main mode of transmission during the outbreak. Two studies described that smear infections/secondary transmission can easily occur within household settings, in particular, as shedding of O104:H4 begin prior to development of hemorrhagic colitis or HUS (Aldabe et al. 2011; Kuijper et al. 2011; Diercke et al. 2014). In contrast, trials observing 14 households containing 20 carriers of EHEC O104:H4 could not detect any household transmission in a prospective follow-up-study (Sin et al. 2013). The overall duration of shedding of the outbreak strain after the occurrence of symptoms was found to be 14–15 days in adults and 35–41 days in children (Vonberg et al. 2013).

2.4 *Clinical Characteristics and Outcome of Infected Patients*

Prior to 2011, EHEC was described to mainly affect young children and the elderly equally in both sexes (Rangel et al. 2005; Tarr et al. 2005). During the 2011 outbreak, however, 90% of the cases were recorded in adults (older than 17 years) (Frank et al. 2011b). Studies performed in 13 pediatric departments reveal that median age among children suffering from infections with the outbreak strain was 11.5 (Loos et al. 2012). Interestingly, 58% of the gastroenteritis and 68% of the HUS cases were recorded in female patients (Frank et al. 2011b).

EAEC infections are mostly characterized by watery diarrhea, low fever, and little or no vomiting. However, cases of bloody stools and persistent diarrhea (longer than 14 days) are also recorded (Nataro et al. 1998). Typical symptoms of an EHEC infection include bloody or non-bloody diarrhea, vomiting, and fever. Patients infected with EHEC O104:H4 developed these symptoms after a median incubation time of 8.5 days (Werber et al. 2013). This incubation period is considerably longer when compared to the onset of disease upon infections with O157:H7 (3–4 days) or EAEC 042 (8–18 h) (Nataro et al. 1995; Tarr et al. 2005) (see also Fig. 2). EHEC-associated illness is self-limiting and the majority of patients exhibit spontaneous recovery. The percentage of HUS cases among infected individuals during the 2011 outbreak (22%) was considerably higher than the estimated HUS rate in O157:H7 outbreaks (Gould et al. 2009), suggesting that EHEC O104:H4 was exceptionally virulent. Surprisingly, the development of severe symptoms as hemorrhagic diarrhea and HUS during the 2011 outbreak was shown to steadily increase with age (King et al. 2012; Menne et al. 2012; Kielstein et al. 2013; Soon et al. 2013; Werber et al. 2013).

Long- and short-term follow-up studies investigated outcome of patients after EHEC O104:H4 infection. In pediatric departments, more than two-thirds of patients suffered from renal complications and received different forms of dialysis therapy (hemodialysis, hemofiltration, peritoneal dialysis). Severe neurological

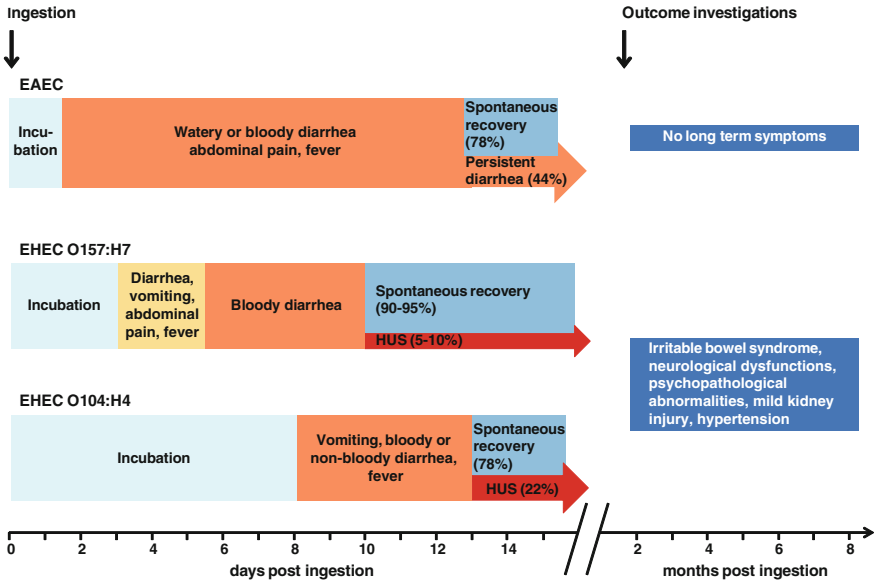

Fig. 2 Clinical course of EAEC, EHEC O157:H7 and EHEC O104:H4 outbreak strain-infections. Short-term manifestations and long-term outcome of patients after EAEC, EHEC O157:H7 and EHEC O104:H4 outbreak strain infections are shown. The clinical course of EAEC is based on numerous strains and studies (Nataro et al. 1998). In contrast to EAEC 042 (Nataro et al. 1995) and EHEC O157:H7 (Tarr et al. 2005), after infection with the O104:H4 outbreak strain (Frank et al. 2011b) incubation time is comparably long and results in HUS in more than 1/5 of all infected patients. Long-term outcome of patients is comparable to disorders observed in patients after EHEC O157:H7 infections (Jandhyala et al. 2013)

complications occurred in 26%. Short-term outcome after 4 months showed a recovery of renal function in 94% and neurological symptoms in 18 out of 23 patients (Loos et al. 2012). Other studies performed in adult patients report that 48% of patients with a severe O104:H4-infection displayed neurological symptoms as disorientation, reduced attention, restlessness, prominent, nervousness, amnestic deficits, aphasia, epileptic seizure, oculomotor disturbance, myoclonus, and headache. Eight months after the outbreak only 3/217 patients still suffered from neurological symptoms (Magnus et al. 2012). Half of the patients with neurological symptoms showed abnormalities within MRI imaging, performed within 20 days after onset of diarrhea. 81% of these are resolved on follow-up investigations (Lobel et al. 2014). Most frequent psychopathological abnormalities in follow-up studies after EHEC O104:H4 infections were feelings of anxiety, formal disorders of thought, disturbances of attention and memory, disturbances of effect, panic attacks, and disorders of drive and psychomotility. Occurrence of these abnormalities was increased with age, family history of heart disease, and higher levels of C-reactive protein (Kleimann et al. 2014). Prospective follow-up of six patients with HUS due to O104:H4 did not show end-stage renal diseases but milder forms of kidney injury including proteinuria (27%), increased serum creatinine (4.4%),

increased cystatin C (47%), and reduced GFR (47%). In 9 out of 36 patients without previous hypertension de novo hypertension occurred (Derad et al. 2016). Additionally, numbers of post-infectious irritable bowel syndromes increased from 9.8% to 23.6% after six months and to 25.3% 12 months after EHEC infection. Incidence of new irritable bowel syndrome was 16.9% (Andresen et al. 2016). In summary, outcome of patients suffering from EHEC O104:H4 infections is similar to patients suffering from O157:H7 diseases (Fig. 2), who presented renal insufficiency, hypertension, psychopathological and neurological disorders and long-term gastrointestinal complications in outcome investigations (Siegler 1994; Siegler and Oakes 2005).

2.5 Predictive and Prognostic Factors

Until recently, clinical trials that address predictive and prognostic factors in EHEC O104:H4 infected patients are rare. Initial investigations show a correlation of higher levels of microRNAs circulating in serum of HUS patients with neurological impairment and thrombocytopenia (Lorenzen et al. 2012). Further studies evaluated CD55 and CD59 expression on peripheral blood cells in EHEC O104:H4 infected patients concerning HUS evolvement. Here, data did not support a role for CD55 and CD59 in HUS development (Dammermann et al. 2013). Parameters reflecting renal perfusion during ultrasound examinations, however, correlated with severity of acute kidney injury in patients after EHEC O104:H4 infection and might have prognostic value within clinical settings (Reising et al. 2016). Further studies in which levels of angiopoietin-2, an antagonistic receptor ligand known to be involved in the development of endothelial dysfunction in HUS, were quantified, showed a predictive relevance for complicated clinical courses in case of early presence of this protein (Lukasz et al. 2015). Moreover, the same group found neutrophil gelatinase-associated lipocalin, a biomarker indicating degree of acute kidney injury, to be significantly increased in patients developing HUS and requiring renal replacement therapy after EHEC O104:H4 infection (Lukasz et al. 2014).

Since patients will continue to have severe disease and complications from EHEC O104:H4 infections, further (prospective) research is necessary to clarify predictive values of mentioned parameters. In particular, studies are needed that evaluate parameters predicting development of HUS and severe long-term disorders.

3 The “Patchwork” Genome Structure of EHEC O104:H4

Early PCR-based genotyping analysis revealed that with respect to virulence gene content the 2011 outbreak strain is a hybrid of EHEC and EAEC (Bielaszewska et al. 2011; Scheutz et al. 2011). Shortly afterward, several next-generation sequencing-based studies further elucidated the “patchwork” genome structure of

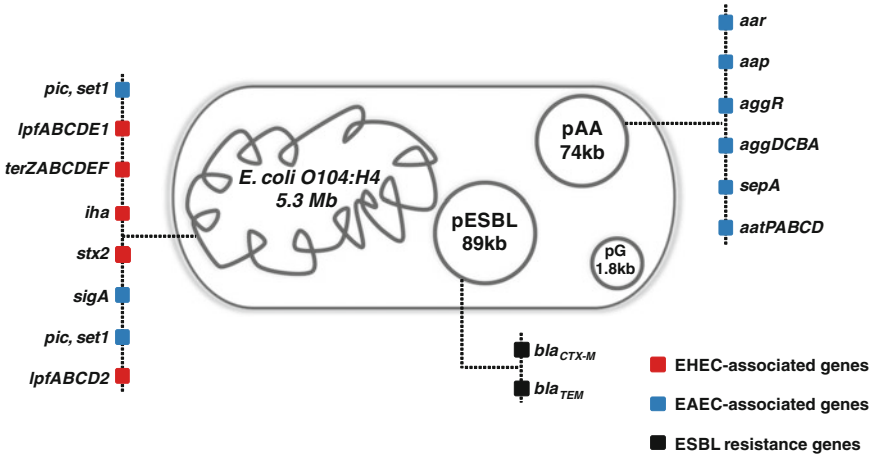

Fig. 3 The virulence-associated loci in the "patchwork" genome of EHEC O104:H4. The EHEC O104:H4 outbreak strain contains both EHEC- (red) and EAEC-associated virulence loci (blue). The additional genetic elements located on the pESBL plasmid (black) mediate the phenotypic resistance against third-generation cephalosporines (Mellmann et al. 2011; Rasko et al. 2011)

EHEC O104:H4 (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011) (Fig. 3), which consists of a chromosome (5.3 Mb) and the plasmids pAA (74 kb), pESBL (89 kb) and pG (1.5 kb small cryptic plasmid).

3.1 The Chromosome

Comparative genomics and phylogenic analysis revealed that the EHEC O104:H4 chromosome has closest sequence relationship to the one of the EAEC strain 55989 (also of serotype O104:H4) and is only distantly related to one of the commonly isolated EHEC strains (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011). However, the 2011 EHEC O104:H4 outbreak strain also harbors a chromosomally integrated bacteriophage encoding Stx, which is not present in the majority of EAEC strains (including the EAEC strain 55989) and is the primary virulence factor involved in EHEC pathogenesis. Stxs are classified as Stx1 or Stx2 type, and further divided into subtypes (Stx1a,c,d and Stx2a–g) (Scheutz et al. 2012), with Stx2a being the one most often associated with the severity of illness and development of HUS (Friedrich et al. 2002). The Stx2a phage in EHEC O104:H4 was found to be closely related to the one in the EHEC O157:H7 strains EDL933 and Sakai and also integrated into the *wrbA* site (Mellmann et al. 2011; Rohde et al. 2011). In contrast to the majority of EHEC strains associated with HUS (Karch et al. 2005), the chromosome of the 2011 outbreak strain does not encode the LEE (locus of enterocyte effacement)

pathogenicity island, which is responsible for the intimate bacterial attachment to the intestinal mucosa and the formation of the characteristic attaching and effacing lesions (Donnenberg et al. 1993; McDaniel et al. 1995).

Other chromosomally encoded EHEC O104:H4 loci linked to EHEC virulence are the operons encoding the long polar fimbriae (Lpf) 1 and 2. The *lpf* operons were extensively characterized in EHEC O157:H7 and reported to contribute to the intestinal adherence and colonization in vivo (Jordan et al. 2004; Torres et al. 2004). In addition, EHEC O104:H4 carries a gene coding for the IrgA homologue adhesin (Iha), which was shown to confer adherence to non-adherent *E. coli* and thus proposed to function as a novel adhesin in EHEC O157:H7 (Tarr et al. 2000). The *lpf* and *iha* loci, however, are also present in the closely related EAEC 55989 strain.

The chromosome of EHEC O104:H4 also carries several EAEC (and *Shigella*) – specific virulence traits. Similar to EAEC strain 55989, the 2011 outbreak strain has two chromosomal copies of *pic*, which encode a SPATE (serine protease autotransporters of *Enterobacteriaceae*) with mucinase activity described to be involved in EAEC pathogenesis by promoting intestinal colonization and mucus hypersecretion (Henderson et al. 1999; Harrington et al. 2009; Navarro-Garcia et al. 2010). Interestingly, *set1AB* coding for the two subunits of Shigella enterotoxin 1 (ShET1) is found on the opposite strand within the *pic* coding region. The *Shigella* ShET1 homologue is able to elicit an immune response (Fasano et al. 1995), but its role in EAEC virulence still remains to be elucidated. Moreover, EHEC O104:H4 encodes SigA, another SPATE which is highly prevalent among EAEC strains (Boisen et al. 2009) and characterized in *S. flexneri* to be cytotoxic to cultured human epithelial cells (Al-Hasani et al. 2009).

The whole genome sequencing revealed the presence of several chromosomal resistance markers in EHEC O104:H4. The 2011 outbreak strain displays tellurite resistance encoded by the gene cluster *terZABCDEF*, which is typically found in EHEC O157:H7 strains but missing in the EAEC strain 55989 (Bielaszewska et al. 2005; Mellmann et al. 2011). The *ter* operon is located in close proximity to *iha* on the TAI (tellurite resistance and adherence conferring island) (Tarr et al. 2000). In addition, the outbreak strain carries also genomic island-encoded resistance determinants to mercury (the *mer* operon), ethidium bromide, sulfonamides, beta-lactams, and tetracyclines (Brzuszkiewicz et al. 2011; Grad et al. 2013).

3.2 The PAA Plasmid

The 2011 EHEC O104:H4 outbreak strain carries a 74 kb pAA virulence plasmid—another characteristic feature of EAEC strains. Different pAA-encoded aggregative adherence fimbriae variants (AAF/I to V) confer the distinct "stacked-brick" adherence of EAEC to cultured human epithelial cells (Nataro et al. 1992; Czeczulin et al. 1997; Bernier et al. 2002; Boisen et al. 2008; Jonsson et al. 2015). Both ex vivo and in vivo experiments suggest that the AAF-mediated adherence is a key step in EAEC pathogenesis (Tzipori et al. 1992; Hicks et al. 1996). The tight

aggregative adherence of the 2011 outbreak strain to cultured cells is conferred by the *aggDCBA* cluster coding for AAF/I (Bielaszewska et al. 2011). In contrast to EHEC O104:H4, the EAEC strain 55989 carries a different pAA plasmid encoding another AAF variant, the AAF/III. Even though there is considerable sequence heterogeneity among the pAA plasmids of these strains, the majority of their virulence-associated features are found conserved (Rasko et al. 2011).

The pAA of EHEC O104:H4 harbors several other EAEC-specific virulence loci. The *aap* gene is coding for the surface protein dispersin, which facilitates proper fimbrial extension from the bacterial surface and thus contributes to EAEC adhesion and intestinal colonization (Sheikh et al. 2002; Velarde et al. 2007). The Aat secretion system, encoded by the *aatPABCD* operon, was characterized as the ABC (ATP-binding cassette) transporter responsible for dispersin secretion out of the bacterial cell (Nishi et al. 2003). The *sepA* locus, which is not shared between EHEC O104:H4 and the EAEC strain 55989, encodes the serine protease SepA. This SPATE is the major extracellular protein of *S. flexneri* and a *sepA* deletion was associated with reduced mucosal inflammation in vivo (Benjelloun-Touimi et al. 1995). The *aggR* gene is coding for the EAEC master virulence gene regulator AggR. AggR is an AraC-type transcriptional activator and regulates the expression of AAF/I, dispersin, Aat and SepA, as well as other pAA- and chromosomally encoded loci (Nataro et al. 1994; Sheikh et al. 2002; Nishi et al. 2003; Morin et al. 2013; Berger et al. 2016). Recently, it was shown that pAA of EAEC strain 042 also encodes Aar (AggR-activated regulator), which negatively regulates AggR expression (Santiago et al. 2014). The *aar* locus is also found transcribed in the pAA of EHEC O104:H4 (Berger et al. 2016).

3.3 The pESBL Plasmid

The extended-spectrum beta-lactamase (ESBL) phenotype of the EHEC O104:H4 outbreak strain is conferred by the presence of an 89 kb plasmid (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011; Rohde et al. 2011). pESBL is a conjugative IncI1 (incompatibility group I1) plasmid with high sequence similarity to the pEC_Bactec plasmid isolated from a horse with arthritis (Smet et al. 2010). pESBL carries the genes $bla_{\text{CTX-M-15}}$ and bla_{TEM} coding for the beta-lactamases CTX-M-15 and TEM-1, which hydrolyze and confer resistance to penicillins and extended-spectrum cephalosporins (Shaikh et al. 2015).

3.4 Evolution of EHEC O104:H4

Phylogenetic analyses and comparative genomics based on whole genome sequencing data led to the formulation of two hypotheses about the evolution of the EHEC O104:H4 outbreak strain. Due to its high genome sequence similarity to the

EAEC strain 55989 and the missing LEE pathogenicity island, which is characteristic to some EHEC strains, it was hypothesized that the 2011 outbreak strain has originated from an EAEC progenitor that acquired an Stx2 phage (Brzuszkiewicz et al. 2011). Since the loss of Stx-encoding genes has been described as a frequent event both in vitro and in vivo (Bielaszewska et al. 2007a, b), another evolutionary model was proposed, in which the 2011 outbreak strain and the EAEC 55989 strain have evolved from a common Stx producing ancestor with an EAEC genotype (Mellmann et al. 2011). Whole genome phylogenetic comparison of 53 *E. coli* and Shigella strains revealed that the 2011 outbreak strain was present within the distinct clade formed by the analyzed EAEC strains of O104:H4 serotype and thus further supported the evolutionary model of Stx phage acquisition by an EAEC ancestor (Rasko et al. 2011). Similarly, the EHEC strain EHEC O157:H7 strain is believed to have evolved from enteropathogenic *E. coli* (another diarrheagenic *E. coli* pathotype) of serotype O55:H7 through a series of horizontal gene transfer events including the acquisition of Stx1- and Stx2-encoding bacteriophages (Feng et al. 1998).

There are several reports describing that Stx phages can lysogenize *stx*-negative *E. coli* strains (Schmidt et al. 1999; Toth et al. 2003; Mellmann et al. 2008a). Recently, it was shown that regions characteristics to the Stx-encoding phage isolated from a 2011 outbreak strain were also present in several bovine EHEC isolates. Moreover, the phage isolated from the outbreak strain and one from the bovine EHECs were able to form lysogens on an *stx*-negative EAEC O104:H4 strain by integrating into its *wrbA* locus. This led to the conclusion that the 2011 EHEC O104:H4 outbreak strain could have evolved by acquisition of an Stx phage from a bovine origin (Beutin et al. 2013). However, only transient lysogens were observed in a study in which strains belonging to different diarrheagenic *E. coli* pathotypes, among them EAEC, were infected by a panel of Stx2 bacteriophages suggesting that the event of a stable Stx phage acquisition is rather uncommon (Tozzoli et al. 2014).

The evolutionary path of the 2011 *E. coli* outbreak strain from either a hypothetical EAEC strain 55989 progenitor or Stx producing O104:H4 progenitor is assumed to involve several other horizontal gene transfer events, such as the exchange of an AAF/III-encoding plasmid for the pAA plasmid coding for AAF/I and the acquisition of pESBL (Brzuszkiewicz et al. 2011; Mellmann et al. 2011; Rasko et al. 2011). Interestingly, the ESBL phenotype is a characteristic feature only of the 2011 EHEC O104:H4 outbreak strain and not present in other sequenced *stx*-positive O104:H4 isolates, suggesting that the plasmid might have recently been acquired by the outbreak strain or have been unstable in the other genetic backgrounds (Rasko et al. 2011; Ahmed et al. 2012; Grad et al. 2013).

4 Virulence Factors and Mechanisms of EHEC O104:H4

4.1 Shiga Toxin—The Cardinal Virulence Factor of EHEC

The link between Stx production and the development of hemorrhagic colitis and *E. coli*-associated HUS was established in the 80 s (Karmali et al. 1983; Riley et al. 1983). Stxs are AB_5 cytotoxins composed of a 32 kDa enzymatically active A subunit noncovalently associated with five identical 7.7 kDa B subunits. The A subunit is a N-glycosidase, while the B pentamer mediates the binding of the Stx holotoxin to a eukaryotic membrane glycosphingolipid receptor globotriaosylceramide (Gb3Cer) (Donohue-Rolfe et al. 1991). The amount of Gb3Cer present on the host cell surface appears to correlate with the clinical complications of EHEC infections. The highest Gb3Cer content is found in the microvascular endothelium of the kidney, as well as in the colonic microvascular endothelia and the endothelial vasculature of the cerebellum (Müthing et al. 2009; Bauwens et al. 2013), and is thus consistent with the observed renal pathology, hemorrhagic colitis and neurologic symptoms, respectively (Jacewicz et al. 1999; Ren et al. 1999; O'Loughlin and Robins-Browne 2001). A recently published report suggests that Stx is capable of direct injury of erythrocytes at certain developmental stages of erythropoiesis (Betz et al. 2016). Stx-mediated damage of erythrocyte progenitor cells may therefore contribute to anemia observed in EHEC-caused extraintestinal complications and furthermore explains the huge demand for blood transfusion during onset of HUS. External Stx is not cytotoxic to macrophages, but it is stimulating the release of pro-inflammatory cytokines (Tesh et al. 1994; van Setten et al. 1996), which increase the susceptibility of endothelial cells to Stx by enhancing Gb3 synthesis and expression on the membrane (Louise and Obrig 1991; van de Kar et al. 1992). However, both cell-free Stx and Stx produced by ingested bacteria are cytotoxic to the phagocytic single-celled protozoan *Tetrahymena thermophila* (Lainhart et al. 2009; Stolfa and Koudelka 2012). Bacterial Stx production was shown to function as a defense mechanism against predators and to confer a survival advantage over those bacteria that do not encode Stx (Lainhart et al. 2009), which suggests that Stx toxicity to humans may have evolved accidentally.

Upon binding to the Gb3Cer receptor, Stx is internalized in a clathrin-dependent or independent manner and transported by the retrograde pathway from the endosomes via the Golgi apparatus to the endoplasmic reticulum (ER) (Sandvig et al. 1992; Romer et al. 2007; Sandvig et al. 2010). The Stx holotoxin dissociates in the reducing environment of the ER and the enzymatically active A subunit is translocated to the cytosol, where the A1 portion depurinates an adenine of the 28S rRNA (Obrig et al. 1987; Endo et al. 1988; Lee et al. 2016) and thus irreversibly inhibits protein synthesis and induces cell death, inflammatory response or activation of the ribotoxic stress response (Obrig et al. 1988; Thorpe et al. 2001). From the cytosol, Stx can reach the nucleus and a body of evidence has been provided that Stx (like other ribosome-inactivating proteins) is able to remove adenine moieties not only from rRNA in the cytosol, but can also efficiently depurinate

DNA in the nucleus. This effect leads to DNA damage observed in cell cultures and is likely to result from direct DNA-damaging activities and/or indirect DNA repair inhibition (Brigotti et al. 2002; Sestili et al. 2005) indicating the existence of more than one retrograde pathway.

Animal experiments have been successfully employed to study EHEC O104:H4 pathogenesis in vivo. Infection of germ-free mice with the 2011 outbreak strain resulted in the development of acute renal tubular necrosis [ATN; (Al Safadi et al. 2012)]. Such renal damage was previously described in EHEC O157:H7 infection experiments of streptomycin-treated mice and mainly attributed to Stx2 production (Wadolkowski et al. 1990). Interestingly, mice infected with EHEC O104:H4 were characterized by a delayed development of ATN in comparison to the ones infected with EHEC O157:H7 (at 13–15 days post-infection vs. 5 days (Wadolkowski et al. 1990; Al Safadi et al. 2012), which is consistent with the observed longer incubation time during the 2011 German outbreak (Frank et al. 2011b). Another study with EHEC O104:H4 and *stx*-negative variants demonstrated that the 2011 outbreak strain causes weight loss and mortality in ampicillin-treated mice and that Stx2 is the key virulence factor responsible for the observed pathogenesis (Zangari et al. 2013). Moreover, similar observation linking Stx2 production of EHEC O104:H4 to disease progression was made in two rabbit models (Zangari et al. 2013; Munera et al. 2014).

The *stx2* operon is located downstream of the phage late genes and its expression is solely dependent on phage induction and the resulting transcription from the phage late promoter (Karch et al. 1999; Wagner et al. 2001). Therefore, the release of free Stx2 from the bacteria cells is mainly attributed to phage-mediated lysis (Waldor and Friedman 2005). In addition, Stx2 is detected together with other virulence factors in outer membrane vesicles (OMVs) shed by EHEC (Kolling and Matthews 1999; Kunsmann et al. 2015; Bielaszewska et al. 2017). EHEC OMVs were shown to bind to, get internalized by and be cytotoxic to human intestinal epithelial cells (EHEC O104:H4 and O157 OMVs) and brain and renal microvascular endothelial cells (*E. coli* O157 OMVs; (Kunsmann et al. 2015; Bielaszewska et al. 2017). Moreover, the Stx2 was found to be the main factor for the observed OMV cytotoxicity in EHEC O104:H4 (Kunsmann et al. 2015). Thus, OMVs provide an alternative means for bacterial Stx2 release. Even though this mechanism would allow for a Gb3 independent cellular uptake of Stx2, it was recently shown that similar to free Stx2, after liberation from OMVs interaction of the OMV-delivered Stx2 with the Gb3 receptor is essential for its retrograde transport and cytotoxicity (Bielaszewska et al. 2017).

Interestingly, the EHEC O104:H4 outbreak strain was shown to produce in culture and in cell culture infection experiments significantly less Stx2 than the prototypical EHEC O157:H7 strains EDL933 and Sakai. (Laing et al. 2012). Moreover, the Stx translocation rates across an epithelial monolayer during microaerobic human colonic infection were found significantly lower in O104:H4 than that of O157:H7 (Tran et al. 2014). Thus, one could argue that the enhanced virulence of the EHEC O104:H4 in comparison to typical EHEC strains could not be accredited to increased Stx2 expression and transcytosis. However, upon induction with mitomycin C, the 2011 outbreak strain was shown to produce in

culture significantly more Stx2 than both EHEC O157:H7 strains EDL933 and Sakai (Laing et al. 2012). It remains to be further elucidated if the conditions in the human gut could induce a similar response in EHEC O104:H4 to that obtained with mitomycin C in vitro.

4.2 The Importance of the PAA Plasmid to EHEC O104:H4 Virulence

EHEC O104:H4 expresses pAA-encoded AAF/I and displays tight "stacked-brick" adherence both to cultured Hep2 epithelial cell and cecal mucosa in germ-free mice (Bielaszewska et al. 2011; Al Safadi et al. 2012). This adherence pattern is characteristic for EAEC strains (Nataro et al. 1992; Tzipori et al. 1992) but unusual for EHEC, which often colonize in single layers displaying LEE-mediated intimate attachment to the epithelia (Donnenberg et al. 1993). Thus, the increased virulence of the 2011 outbreak strain was hypothesized to be attributed to the AAF/I-mediated intestinal adherence, which could facilitate the absorption of Stx from the gut to the systemic circulation (Bielaszewska et al. 2011).

EHEC O104:H4 can sporadically lose the pAA plasmid during the course of the disease. Interestingly, pAA loss was correlated with a significantly reduced HUS progression in patients, which speaks for an attenuated virulence of the pAA-negative isolates (Zhang et al. 2013). In contrast, the pAA plasmid was found not to be essential for the colonization and intestinal pathology in a rabbit model (Munera et al. 2014). Nevertheless, it was shown that the AAF/I indeed contribute not only to the tight adherence of the outbreak strain but also to translocation of the Stx2 across an epithelial cell monolayer, further suggesting that the pAA plasmid has a crucial importance to EHEC O104:H4 virulence (Boisen et al. 2014). Mutant analysis revealed that disruption of the actin cytoskeleton and the reduction of trans-epithelial resistance, which accompany EHEC O104:H4 infection of polarized T84 cells, depends on AggR and AggA (the major AAF/I subunit) but not on Stx2. On the other hand, the prototype EHEC O157:H7 failed to disrupt the polarized T84 cell monolayer and did not lead to significant levels of Stx2 transport from the apical to the basolateral side of the cells. Moreover, the expression of AAF/I alone in an *E. coli* K12 strain was found to enhance the translocation of exogenous Stx2 across the epithelial monolayer and thus demonstrated a direct effect of the fimbriae on epithelial permeability. Interestingly, the inflammatory response to the outbreak strain in the T84 system was dependent on both Stx production and the EAEC virulence factors AggR, AggA, and SepA (Boisen et al. 2014).

A recent study sheds light on the EHEC O104:H4 pAA transcriptional organization and gene regulation (Berger et al. 2016) (Fig. 4). The pAA transcriptome was analyzed using differential RNA-seq that allows for the high throughput mapping of transcription start sites (TSS; 5′-PPP ends of primary transcripts) and processing sites (PS, 5′-P ends of processed transcripts) (Sharma and Vogel 2014) (Fig. 4, Track I and II). TSS were detected for the majority of pAA-encoded

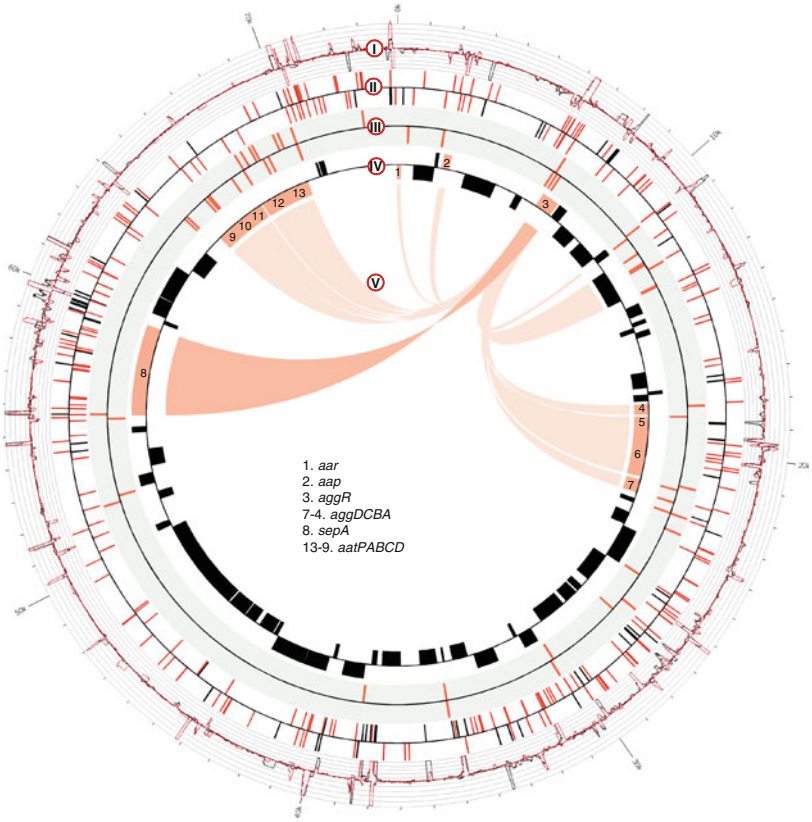

Fig. 4 The transcriptional organization and gene regulation in the pAA plasmid of EHEC O104:H4. Track I: Differential RNA-seq data on the pAA plasmid. The graphs represent the normalized number of pAA reads mapped per nucleotide in terminator exonuclease (TEX) + (red) and TEX- (black) libraries (y-axis = abundance relative score, max. 100). Track II. TSS (red) and PS (black) candidates annotated by dRNA-seq. Track III. Computationally predicted AggR (red) binding sites. Track IV. Annotated ORFs in pAA. Virulence-associated genes are colored in orange and a gene legend is given in the middle of the circle. Track V. AggR regulon. The AggR regulon is based on Morin et al. 2013 (light orange) and Berger et al. 2016 (dark orange). The figure was modified from Berger et al. 2016

virulence genes, suggesting that they were expressed at least on the mRNA level in EHEC O104:H4 (Berger et al. 2016). Interestingly, operon-internal TSS were detected within the AAF/I gene cluster, which could allow for the transcriptional uncoupling of the secreted AAF/I protein subunits AggA and AggB from the outer membrane usher protein AggC and periplasmic chaperone AggD. Moreover, numerous antisense RNA candidates mapped in this analysis were found to be associated with virulence genes, suggesting that also post-transcriptional regulation may be important for their appropriate expression. In addition, a computational-based screen for AggR binding sites followed by experimental

validation revealed that the EHEC O104:H4 pAA-encoded serine protease SepA is a new member of the AggR regulon (Berger et al. 2016), which was previously characterized in the EAEC strain 042 (Morin et al. 2013) (Fig. 4, Track III and IV). The AggR-dependent regulation coordinates SepA expression with other important EAEC virulence factors and may be a hint for a role of the serine protease in EHEC O104:H4 pathogenicity (Berger et al. 2016).

4.3 Other Factors of Importance to EHEC O104:H4 Virulence

Along with Stx2 and pAA-encoded factors, several other determinants have been suggested to play an important role in the virulence of the 2011 outbreak strain. EHEC O104:H4 encodes the serine proteases Pic, SigA, and SepA—a number and combination of SPATEs which has been rarely reported in EAEC strains (Boisen et al. 2009). The chromosomally–encoded Pic and SigA, but not the pAA plasmid, were found to be critical for the EHEC O104:H4 colonization and disease severity in an infant rabbit model. Surprisingly, SigA release rather than its protease activity contributed to EHEC O104:H4 pathogenicity (Munera et al. 2014).

Lpf1 and Lpf2 were shown to be important virulence factors in EHEC (Jordan et al. 2004; Farfan et al. 2013). Therefore, it was also relevant to address their role in the adhesion and colonization of EHEC O104:H4. An *lpf2* deletion mutant was characterized only by a significant reduction in its ability to colonize polarized cells. The loss of functional Lpf1, however, resulted in a reduced ability of O104:H4 to adhere to both polarized and non-polarized cells, as well as to form a stable biofilm. In addition, the *lpf1* mutant showed a reduced capacity to colonize the cecum and large intestines in a murine model, thus suggesting that even in the presence of AAF/I, Lpf1 is an important colonization factor of EHEC O104:H4 (Ross et al. 2015).

In contrast to EHEC O157:H7, the outbreak strain is a strong biofilm producer in vitro and in vivo. Its extensive in vivo biofilm formation was found to correlate with an enhanced *stx2* and other virulence gene expression and increased kidney damage in a germ-free mouse model (Al Safadi et al. 2012). The second messenger c-di-GMP stimulates the production and secretion of the biofilm-associated polysaccharide PGA, as well as activates the expression of the positive transcriptional biofilm regulator CsgD, which in turn is regulating the expression of the extracellular matrix component curli (Hengge 2009). Curli fibers are involved in adhesion and biofilm formation, and their expression is more pronounced below 30 °C (Barnhart and Chapman 2006). Typical for EAEC genotypes, the 2011 outbreak strain was shown to produces high levels of c-di-GMP. Moreover, EHEC O104:H4 and the EAEC strain 55989 displayed high CsgD expression levels and strong curli production not only at 28 °C but also at 37 °C (Richter et al. 2014). In contrast to EAEC 55989, however, the outbreak strain produced no cellulose (Richter et al. 2014), which is known to counteract the adhesive and pro-inflammatory properties of curli fibers (Wang et al. 2006). Interestingly, the

closely related HUSEC041 strain (Mellmann et al. 2008b) was characterized by low CsgD and curli synthesis at 37 °C and high cellulose production. Thus, the EHEC O104:H4 unique biofilm-related properties have been proposed to additionally contribute to its enhanced virulence (Richter et al. 2014).

5 Treatment

In general, one has to differentiate between treatment of EHEC infections and HUS, even though both lack a causative therapy. Current guidelines recommend measures preventing EHEC infected persons from developing HUS after the onset of diarrhea. During HUS conventional supportive treatment (see below) is state of the art (Wurzner et al. 2014). Evaluation of treatment strategies in HUS patients infected with O104:H4, however, revealed results contrary to the current guidelines in particular by calling into question benefits from plasmapheresis and harmful effects of antibiotic treatment (Menne et al. 2012).

5.1 Supportive and Symptomatic Therapy

Symptomatic treatment options during EHEC triggered HUS have diversely been discussed. Trials evaluating fresh frozen plasma transfusion (Loirat et al. 1988; Rizzoni et al. 1988), heparin (Vitacco et al. 1973) with or without urokinase (Loirat et al. 1984) or dipyridamole (Van Damme-Lombaerts et al. 1988) and steroids as anti-inflammatory substances (Perez et al. 1998) in young children with post-diarrheal HUS did not show an outcome superior to classical supportive therapy.

Recent recommendations deal with supportive therapy as state of the art including fluid management, treatment of hypertension, renal replacement and ventilatory support (Bitzan et al. 2010; Wurzner et al. 2014). In this context, early volume expansions can have positive effects on both, short- and long-term disease outcomes (Ardissino et al. 2016). Early recognition of and parenteral volume expansion during EHEC O157:H7 infections have been associated with attenuated renal injury failure (Ake et al. 2005). In an observational cohort study, Hickey et al. determined that intravenous fluid therapy during the pre-HUS phase prevents oligoanuric HUS significantly (Hickey et al. 2011). Renal replacement therapy, however, is recommended to be performed according to clinical manifestations of HUS as oligo- or anuria. Peritoneal dialysis and hemodialysis are equivalent options in these cases, preferring peritoneal dialysis in infants <4 years and hemodialysis in older children and patients receiving higher amounts of blood products as platelets or PRBC (Bitzan et al. 2010). Best clinical practices involve rapid and accurate clinical and microbiological identification of infected patients, volume expansion, and support of the intestinal and extraintestinal complications that can ensue during acute enteric infection and associated HUS (Tarr 2009).

5.2 Antibiotics

The use of antibiotics during EHEC infections is currently not recommended in most countries, as it is believed to increase the risk for HUS. However, the majority of studies leading to that assumption are of retrospective nature and limited to a surprisingly few antibiotics. In addition, none of them systematically excludes antibiotics, that are known to induce Stx production in vitro, e.g. trimethoprim-sulfamethoxazole (Karch et al. 1986; Proulx et al. 1992; Wong et al. 2000, 2012). Moreover, a meta-analysis of nine high-quality studies did not show a higher probability of HUS development upon administration of antibiotics (Safdar et al. 2002). A recent meta-analysis of seventeen reports also showed no significantly increased risk of developing HUS associated with antibiotic administration. However, including only the studies ($n = 5$) which were with low risk of bias and meeting an acceptable definition of HUS, a significant association was reached (Freedman et al. 2016). Notably, also these five studies did not omit antibiotics which induce Stx production in vitro.

The experience with the hypervirulent strain EHEC O104:H4 outbreak strain made it very clear that a rational approach on the question which antibiotics may be beneficial in EHEC infections is of utmost importance, especially as the capacities for symptomatic treatment may become rapidly limited, if a future outbreak is just an order of magnitude larger. Therefore, in order to finally obtain an unbiased picture of the usefulness of antibiotics in the therapy of EHEC infections (i) the effects of inhibitory and sub-inhibitory concentrations of antimicrobial substances on Stx production should be determined in vitro and (ii) only those antimicrobial substances that do not stimulate Stx production in vitro should be afterward tested in vivo, ideally in prospective, randomized, placebo-controlled studies.

In vitro data for the effects of antibiotics on Stx production is already available, even though it would be desirable to systemically include more clinically relevant bacterial genetic backgrounds in the future (Karch et al. 1986; Kimmitt et al. 2000; McGannon et al. 2010; Bielaszewska et al. 2012; Corogeanu et al. 2012). According to these studies, antibiotics that inhibit bacterial transcription and translation appear to be very promising candidates for therapy. In addition, the translational inhibitor azithromycin was shown to be effective in reducing the elevated Stx levels detected in presence of phage sensitive *E. coli*, which may play an underestimated role in overall toxin production and the individual course of illness during EHEC infections (McGannon et al. 2010). Even though not routinely recommended by the authorities, antibiotic therapy administered during the 2011 outbreak also proved to be beneficial. A retrospective case-controlled study on the German EHEC O104:H4 outbreak showed that an aggressive antibiotic therapy (meropenem + ciprofloxacin ± rifaximin) applied once the disease had progressed to HUS was associated with significantly lower mortality rate, duration of shedding and seizure frequency (Menne et al. 2012). Moreover, azithromycin treatment resulted in a lower frequency of long-term carriage of the outbreak strain in patients (Nitschke et al. 2012).

5.3 Eculizumab

The use of a group of monoclonal antibodies, namely eculizumab, targeting the complement component 5 (C5) was controversially discussed. Some studies found eculizumab to have beneficial effects on the recovery from O104:H4-associated HUS during the French and German outbreak (Greinacher et al. 2011; Delmas et al. 2014). Due to this treatment, neurological disorders and renal function but also laboratory parameters as hemoglobin, platelets, lactate hydrogenase could be rapidly improved in HUS patients infected with the O104:H4 outbreak strain or O157:H7 (Greinacher et al. 2011; Lapeyraque et al. 2011; Delmas et al. 2014; Saini et al. 2015). On the other hand, different studies could not prove any benefit of eculizumab therapy compared to conventionally performed therapeutic regimens like supportive care, therapeutic plasma exchange, hemodialysis or antibiotic treatment (Kielstein et al. 2012; Menne et al. 2012; Ullrich et al. 2013), calling into question this new therapeutic approach. Even though, short- and long-term outcome in some critically ill patients with eculizumab could be improved, demonstrating no obvious side effects after application, further randomized controlled trials are needed before a beneficial effect can be assigned to this therapeutic agent.

5.4 Probiotics

Although probiotics do not provide therapeutic options in the acute phase of disease, they might have a relevant preventive function. Multiple studies have been performed verifying the beneficial effect of probiotics in vitro and in experimental animal models. Promising candidate as *Lactobacillus* and *Bifidobacterium* spp. showed protecting effects and decreasing cytotoxic activity after co-incubation with EHEC O157:H7 in vitro (Mogna et al. 2012; Kakisu et al. 2013) and in vivo (Asahara et al. 2004; Eaton et al. 2011; Chen et al. 2013), most likely mediated by lactic acid production, which directly correlates to bacteriostatic/bactericidal effects (Ogawa et al. 2001) and level of *stx2a* expression in EHEC O157:H7 (Carey et al. 2008). Recent studies concentrate on different *E. coli* strains mediating protective activity, namely *E. coli* 1307 (Reissbrodt et al. 2009) and *E. coli* strain Nissle 1917 (EcN). EcN, first used in 1917, is one of the most investigated probiotics, known to significantly improve various dysfunctions within the intestinal tract as e.g. ulcerative colitis and inflammatory bowel disease (Kruis 2004). Antagonistic effects of EcN could be proved for the mouse intestine colonized by EHEC O157:H7 (Leatham et al. 2009). In addition, in an investigation including two EHEC O104:H4 isolates derived from the German outbreak EcN showed a very efficient antagonistic activity regarding adherence of these pathogenic strains to human gut epithelial cells, their growth, and their Stx2 production in vitro (Rund et al. 2013), which confirms that commensal *E. coli* strains can provide a barrier to infection by intestinal pathogenic *E. coli* including the O104:H4 outbreak strain.

5.5 Stx Receptor Analogs and Stx Neutralizing Molecules

Different agents, which imitate Stx receptor properties, can reduce the amount of cellular bound Stx. Receptor analogs consisting of or harboring the Gb3 trisaccharide were shown to bind Stx in the circulation, to exert neutralizing effects in vitro and to significantly reduce brain damage in animal models after application of a fatal dose of EHEC O157:H7 (Kitov et al. 2000; Nishikawa et al. 2002; Mulvey et al. 2003; Watanabe et al. 2004; Nishikawa et al. 2005). Synthetic Stx receptors were effective in vitro but could not prove this promising effect in a multicentre randomized placebo-controlled clinical trial in children aged 6 months to 18 years with diarrhea-associated HUS (Trachtman et al. 2003). Other strategies concentrated on constructing a recombinant bacterium that displayed a Stx receptor mimic on its surface. High efficiency in adsorption and neutralizing Stx were shown in vitro and mice were completely protected from consequences of Stx producing *E. coli* infections (Paton et al. 2000, 2001). There are several experimental approaches concentrating on partially cell-permeable agents neutralizing Stx. MMA-tet protected mice from fatal doses of EHEC O157:H7 after oral application and did not affect vesicular transport mechanisms (Tsutsuki et al. 2013). Intravenous administration of the cell-permeable peptide TVP in animals resulted in the absence of acute kidney injury and reduction of thrombocytopenia, but did not alter anemia (Stearns-Kurosawa et al. 2011). Two peptides TF-1 and WA-8, which specifically block the binding of Stx2 to target cells, protected mice from toxicity by significantly decreasing the concentration of Stx2 in the bloodstream (Li et al. 2016). Small molecules, inhibiting retrograde toxin trafficking from the early endosomes to the trans-Golgi network, showed first promising protecting effects in vitro (Stechmann et al. 2010; Noel et al. 2013).

5.6 Phages

Another therapeutic concept concentrates on controlling Stx producing *E. coli* via lytic phages, specifically reducing their absolute number. Several phages have been investigated up to now, showing promising results in reduction of EHEC O157:H7 and other pathogenic serogroups in vitro, on surfaces, fruits, vegetables, beef, and in milk (Abuladze et al. 2008; Niu et al. 2009; Sharma et al. 2009; Alam et al. 2011; Patel et al. 2011; Viazis et al. 2011; Carter et al. 2012; Ferguson et al. 2013; Hudson et al. 2013; McLean et al. 2013; Liu et al. 2015). In vivo experiments in animal models could mostly confirm these findings showing less disease complications and reduced shedding after phage therapy (Tanji et al. 2005; Raya et al. 2006; Sheng et al. 2006; Rozema et al. 2009; Rivas et al. 2010; Coffey et al. 2011). These effects could be observed even in intestinal pathogenic *E. coli* showing a high profile of antibiotic resistances (Viscardi et al. 2008). Candidate therapeutic phages

efficiently lysing the EHEC O104:H4 outbreak strain could be identified by Merabishvili et al. (Merabishvili et al. 2012). Nevertheless, in vivo studies are still pending until these phages can be considered a therapeutic option in the future.

6 Conclusion

In 2011 EHEC O104:H4 caused the largest EHEC outbreak in German history and the highest incidence rate of EHEC-related HUS ever recorded worldwide. The highly aggressive strain carries virulence loci characteristic to both EHEC and EAEC and showed therefore not only Stx production and but also an aggregative adherence phenotype. The dimensions and severity of the 2011 outbreak demonstrated the catastrophic potential of this rare combination of pathogenic traits. In addition, not knowing the natural reservoir of the strain makes it harder to minimize the risk of future exposure to EHEC O104:H4. The major challenges remain improving diagnostic speed and treatment. The latter is of utmost importance, as EHEC infections are one of the few bacterial infections for which still no causative, but only a few symptomatic therapies exist. Moreover, there is the danger that new strains of similar or even greater pathogenic potential may arise in the future, which could cause even larger outbreaks or higher incidence rates of HUS. The handling of an EHEC outbreak larger than the one in 2011 in Germany solely on the basis of currently available therapies will become problematic even in developed countries with state of the art health care system.

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