

Shiga toxin 2a and Enteroaggregative *Escherichia coli* – a deadly combination

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In 2011, a Shiga toxin (Stx) type 2a-producing enteroaggregative *E. coli* (EAEC) strain of serotype O104:H4 caused a large lethal outbreak in Northern Europe. Until recently, the pathogenic mechanisms explaining the high virulence of the strain have remained unclear. Our laboratories have shown that EAEC genes encoded on the pAA virulence plasmid, particularly the AggR-regulated AAF/I fimbriae, enhance inflammation and enable the outbreak strain to both adhere to epithelial cells and translocate Stx2a across the intestinal epithelium, possibly explaining the high incidence of the life threatening post-diarrheal sequelae of hemolytic uremic syndrome. Epidemiologic evidence supports a model of EAEC pathogenesis comprising the concerted action of multiple virulence factors along with induction of inflammation. Here, we suggest a model for the pathogenesis of the O104:H4 outbreak strain that includes contributions from EAEC alone, but incorporating additional injury induced by Stx2a.

Shiga Toxin (Stx)-producing Enteroaggregative *E. coli* (Stx-EAEC)

Infectious diarrhea is one of the most frequent causes of human illness. The greatest burden of diarrhea is in children under 5 years of age in developing areas. In fact, childhood diarrhea accounts for almost one million directly attributable deaths per year globally.¹ Additionally, morbidity associated with repeated episodes of childhood diarrhea can be long-

lasting.² Collectively, the diarrheagenic *Escherichia coli* (DEC) represent the most common bacterial pathogens worldwide.^{3–5} Some of the DEC pathotypes represent a major cause of morbidity and mortality in developing and low-income countries; these pathotypes include the enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC) and enteroaggregative *E. coli* (EAEC).^{3,6} In contrast, the Shiga toxin (Stx)-producing *E. coli* (STEC), a DEC which can cause bloody diarrhea, constitute a challenge for public health systems in industrialized countries. The epidemiology of infections caused by DEC ranges from sporadic endemic diarrhea to small or large outbreaks of food- or water-borne diarrhea. Some of the most severe and well-known DEC outbreaks have been due to STEC, particularly the O157:H7 serotype.^{7,8} O157:H7, referred to by some authors as enterohemorrhagic *E. coli* (EHEC), are known to cause sporadic cases and outbreaks of hemorrhagic colitis and a potentially lethal sequelae: hemolytic uremic syndrome (HUS).^{9,10} HUS is defined clinically by the triad of hemolytic anemia, thrombocytopenia, and renal failure.

HUS is the most frequent cause of renal failure in children,¹¹ and Shiga toxin is the critical STEC virulence factor associated with the development of HUS.¹² Shiga toxins are classified as belonging to one of 2 types, Stx1 or Stx2, and within the 2 types there exist several subtypes.¹³ However, O157:H7 *E. coli* strains that produce Stx2a are more likely to be associated with HUS.

From May through June 2011, an unprecedented outbreak of gastroenteritis

occurred in Germany, with 4,137 known affected individuals and 54 deaths.¹⁴⁻¹⁶ The responsible organism was an unusual Stx2a-producing strain of serotype O104:H4. In addition to the Stx2a-encoding phage, the O104:H4 isolate carried the extended-spectrum β -lactamase (ESBL) antibiotic resistance plasmid.¹⁶ The consumption of fenugreek-sprouted seeds imported from Egypt was identified as the most likely source of infection for primary outbreak cases.¹⁷ Implication of O104:H4 in a massive outbreak was remarkable, but equally remarkable was the high proportion of cases developing HUS: 22% of the recognized O104:H4 cases were diagnosed with this complication contemporaneously. In contrast, historical rates of HUS after O157:H7 infection typically range from 6%-15%.^{15,18,19} Although passive clinical surveillance cannot accurately ascertain the rate of any complication, it is indisputable that the outbreak strain was unusually virulent for a non-O157:H7 strain.

Interestingly, whereas the O104:H4 strain carried a Stx2a phage characteristic of HUS associated STEC strains, it did not possess other O157:H7 virulence traits, most notably the locus of enterocyte effacement (LEE) pathogenicity island,¹⁶ which promotes the distinctive attaching and effacing epithelial lesion associated with LEE positive STEC.^{20,21} Instead, the O104:H4 outbreak isolate harbored genes characteristic of a completely different DEC pathotype, EAEC. Such an unusual combination of Stx with virulence factors typically found in EAEC has been documented prior to the 2011 German outbreak. This include reports of sporadic small outbreaks of HUS attributed to Stx-producing EAEC of the O111:H2, O111:H21 and, yes, O104:H4 serotypes. Nevertheless, these cases or outbreaks were localized to small populations in France, Ireland, and the Republic of Georgia and were not spread throughout Europe, as seen with the German outbreak strain.²²⁻²⁴

No standard nomenclature has been put forward for Stx-producing EAEC strains. We will argue that they are not in fact “hybrid strains,” but are rather EAEC that have been infected with Stx-encoding phage and should be designated Stx-EAEC.

An Unusual Combination of Virulence Genes

EAEC is a cause of acute and persistent diarrhea in multiple settings²⁵⁻³¹ and EAEC strains express a heterogeneous array of putative virulence factors³²⁻³⁶ encoded on the bacterial chromosome or on the EAEC-specific pAA plasmid. EAEC strains often harbor a transcriptional activator of the AraC/XylS class called AggR,³⁷ which controls expression of genes on both the plasmid and the chromosome. Genes under AggR control include those that encode the aggregative adherence fimbriae (AAF), of which at least 5 variants exist (AAF/I-V).³⁸⁻⁴³ AAF adhesins are essential for EAEC adherence to human intestinal explants, and for both cytokine release and opening of epithelial tight junctions in a polarized epithelial model.^{44,45} AggR is also required for expression of genes encoding dispersin (the *aap* gene), the Aat dispersin translocator⁴⁶ and a chromosomal cluster termed Aai, encoding a type VI secretion system.⁴⁷ Recently, Santiago et al. described a novel EAEC regulator called Aar (AggR-activated regulator), which is a member of a previously unrecognized large class of regulators in pathogenic Gram negative bacteria.⁴⁸ The identified *aar* gene is activated by AggR, however when *aar* is deleted, *aggR* and the AggR regulon remain persistently activated. Thus, Aar could act directly or indirectly as a virulence suppressor as it down-regulates the expression of the positive regulator AggR. However, Aar is present in the German O104:H4 outbreak strain, so the exact contribution of Aar to EAEC pathogenesis remains enigmatic. Additionally, EAEC strains also often harbor a variable number of serine protease autotransporters of *Enterobacteriaceae* (SPATEs) that are implicated in immune evasion, mucosal damage, secretogenicity, and colonization.⁴⁹

Limited knowledge exists on EAEC of the O104:H4 serotype. In a recent study of children’s diarrhea in Mali, we identified Stx-negative EAEC O104:H4 in 6 children with and without diarrhea.³² Whole-genome sequencing of the Stx2a+ O104:H4 outbreak strain, Stx-negative O104:H4 Malian EAEC isolates and

other O104:H4 isolates confirmed that the outbreak strain was indeed genetically EAEC.⁵⁰ Genomic forensics suggested that the O104:H4 outbreak strain was an EAEC that had acquired the Stx2a phage because the outbreak strain was phylogenetically clustered with typical EAEC of O104:H4 and other serotypes. In addition, the genome analysis demonstrated that, other than Stx2a, the outbreak strain contained no virulence factors not already described in EAEC strains, including the AAF variant I (AAF/I), Aap/dispersin, the Aat translocator, the Aai type VI secretion system, the AggR regulator, and 3 SPATE proteases (Pic, SigA and SepA).⁵⁰ That said, SPATE proteases are present in pathogenic *E. coli* strains and *Shigella* spp,⁵¹ however, the confluence of Pic, SigA, and SepA is a combination typically found among *Shigella flexneri* 2a strains,⁵⁵ a finding that suggests that the German outbreak strain shares characteristics with that highly invasive and inflammatory diarrheagenic pathogen.

The Pathogenesises of the O104:H4 Outbreak Strain

So why was the O104:H4 EAEC outbreak strain so virulent? And which genes or combinations of genes could explain the high HUS incidence rate in patients? Answering these questions is challenging, given that EAEC are human-specific pathogens and that few animal infection models mimic human disease. However, Zangari et al. recently investigated the virulence of the German O104:H4 outbreak strain in a mouse model and showed that Stx2a from the outbreak strain was associated with weight loss, renal impairment, and death.⁵² By contrast, those factors associated specifically with “typical” EAEC virulence are less clear because EAEC is a highly heterogeneous group of pathogens. EAEC as currently defined most likely encompasses both pathogenic and non-pathogenic *E. coli* strains.⁵³ Nonetheless, EAEC has unmistakably been associated with diarrhea in some individuals (e.g. in volunteers and outbreak patients),^{15,54-58} subclinical colonization in endemic areas is common, urinary tract infections and sepsis, and recently with poor growth in children.⁵⁹⁻⁶¹

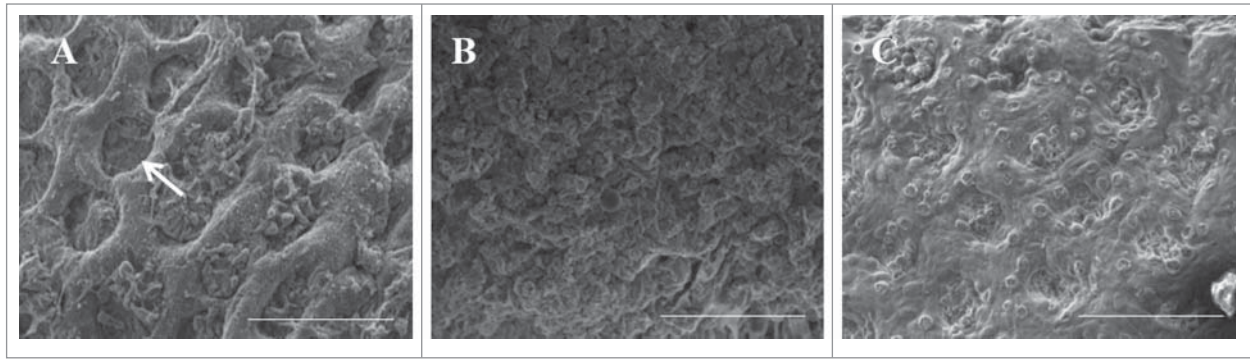


Figure 1. Adherence to *Macaca fascicularis* intestinal explants. Sections of proximal colon from freshly euthanized cynomolgus monkeys were incubated with strain C227-11 (A), with HS (B) or with media alone for 3 hours (C) and then visualized by Scanning Electron Microscopy (SEM).⁶⁸ In panel (A), the arrow indicates dilation of the crypts. Scale bar is 100 μm .

The essential differences between pathogenic and non-pathogenic strains are mostly unknown, but pathogenesis both *ex vivo* and *in vivo* suggest that the current pathogenic scheme of EAEC is comprised of these 3 stages: (1) after ingestion and passage through the stomach, EAEC adheres to the intestinal mucosa within a mucus-containing biofilm by virtue of AAF and possibly other adherence factors such as Pic mucinase and dispersin^{62,63}; (2) stimulation of mucus production following biofilm formation at the surface of the mucosa; and (3) inflammation of the mucosa, manifested by cytokine release, cell exfoliation, and intestinal secretion.^{45,60,64,65} The final pathogenic step described previously is mediated as follows: both AAF and flagellin induce an inflammatory response in intestinal epithelial cells,^{45,66-68} manifested by increased cytokine production, as well as fecal lactoferrin in stools of patients with EAEC-induced diarrhea. SPATE toxins (such as Pet, SigA, Sat) cause cytotoxicity due to alteration of cytoskeletal elements.^{51,69}

Based on the above outline of the stages of EAEC-mediated gastroenteritis, we hypothesized that the plasmid-borne virulence factors of EAEC contributed to the high pathogenicity of the German outbreak strain by promoting strong adherence to the epithelium and/or by opening epithelial tight junctions and facilitating Stx2a translocation. We used isolate C227-11 from the German outbreak, and investigated the potential contribution of pAA with mutants of C227-11, either

cured of the pAA plasmid or deleted individually for known pAA-encoded virulence-associated genes *aggR*, *aggA*, or *sepA*.

As expected, lack of the pAA plasmid abolished the capacity of C227-11 to adhere to viable colonic tissue harvested from the cynomolgus monkey *Macaca fascicularis*.⁶⁸ In contrast, C227-11 adheres in an aggregative manner and forms heavy biofilms with a thick mucus layer upon interaction with the monkey colonic tissue (Fig. 1A) compared to commensal strain HS and uninfected control (Fig. 1B and C). Adherence is followed by mucosal toxicity, including crypt dilation,

microvillous vesiculation, and epithelial cell extrusion. We also investigated the role of AAF/I in biofilm formation and epithelial cell adherence for C227-11, and found that like variants AAF/II, IV and V, expression of the AggA fimbrial adhesin was necessary and sufficient for adherence and biofilm formation (not shown⁶⁸). Fig. 2 shows an electron micrograph of C227-11 in which AAF/I can be visualized.

We next interrogated cytoskeletal rearrangement in cells infected with C227-11, because such rearrangement has been demonstrated for other EAEC and has

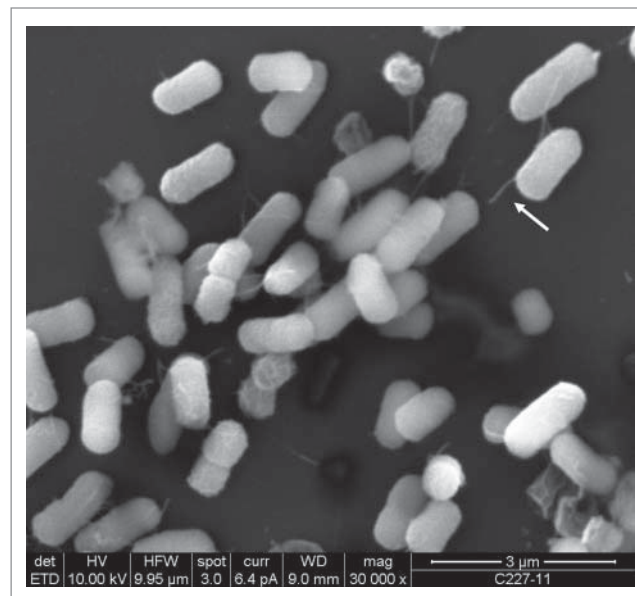


Figure 2. Scanning Electron Microscopy of C227-11 and biofilm formation after 18 hours on glass cover slips. Arrow indicates aggregating bacteria and AAF/I fimbriae.

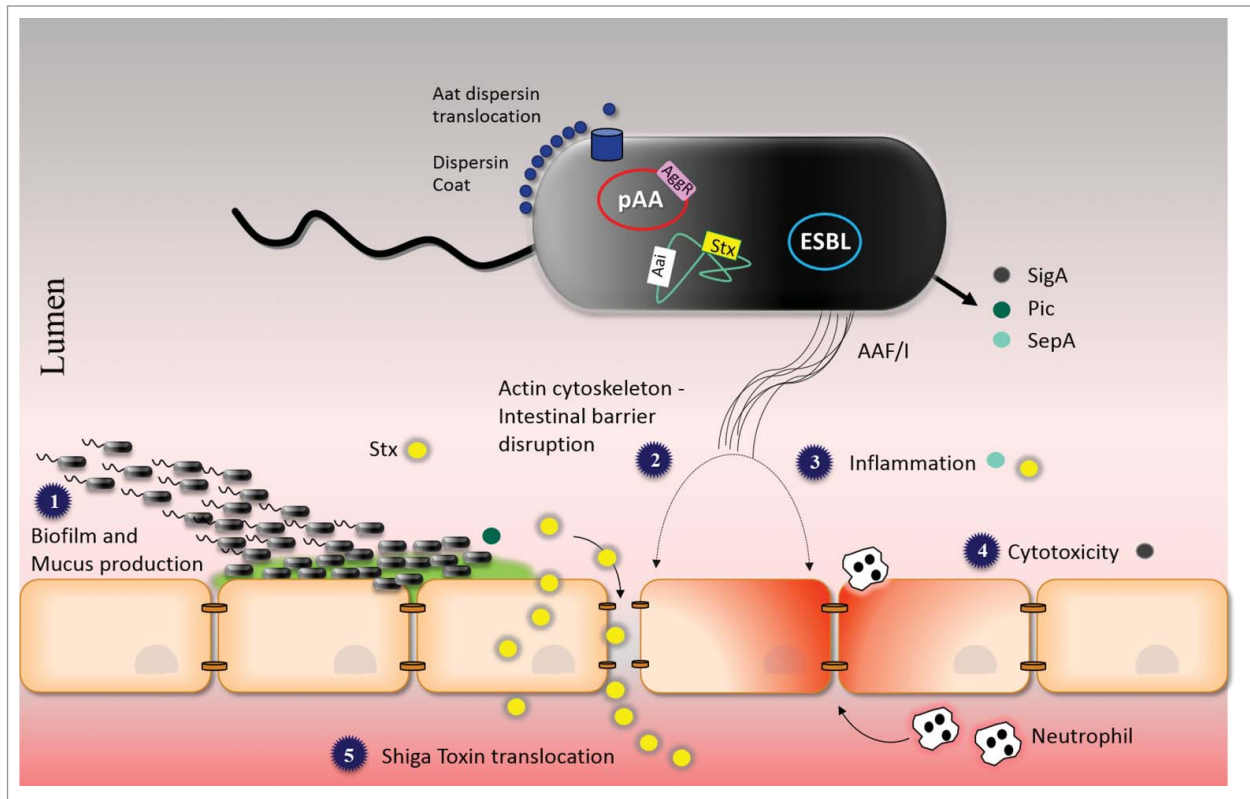


Figure 3. Model of the Pathogenesis of the O104:H4 outbreak strain. See text for explanation. Abbreviations: AAF/I; aggregative adherence fimbriae type I, pAA; virulence plasmid of C227-11, ESBL; extended-spectrum β -lactamase antibiotic resistance plasmid, AggR; AraC/XylS family activator, Aai; Type VI secretion system, Aat; dispersin translocator, Stx; Shiga Toxin, SigA; IgA protease-like homolog, Pic; Serine protease precursor, and SepA; Shigella extracellular protease.

been linked to the presence of AAF fimbriae.⁴⁴ In addition, disruption of the epithelial barrier coincides with perturbation of the actin cytoskeleton for STEC strains that carry the LEE island.^{70,71} However, as mentioned previously, C227-11 does not harbor the LEE island. We found that disruption of the actin cytoskeleton and reductions in transepithelial resistance in T84 cells infected with C227-11 were dependent on AggR and AggA but not on the Stx2a phage. Thus, the contribution of AggR in altering actin arrangement (and reducing adherence) is likely due to its role in the expression of AAF/I. We also found that a prototype O157:H7 failed to decrease transepithelial resistance to the same extent as EAEC, supporting the notion that it is AAF or AAF-dependent factors rather than Stx2a that are responsible for epithelial barrier loss in this model. These observations were confirmed by expressing the AAF/I fimbrial adhesion in an *E. coli* K12 background, which proved sufficient to

enhance translocation of Stx2a across the epithelial monolayer.⁶⁸ STEC strains associated with HUS possess several T3SS-associated effector proteins that can disrupt the integrity of the epithelial junctions and possibly contribute to Stx penetration. However, we suggest that AAF-associated tight junction disruption caused by the German outbreak strain more plausibly explains the increased rate of Stx-related complications induced by this strain.

We also observed that incubation of C227-11 with infected cell monolayers released the chemokine interleukin 8 (IL-8), and that cytokine release was significantly lower when the cells were infected with the strain cured of the Stx2a phage (C227-11 Φ cu). In addition, deletion of *aggA*, *aggR*, or *sepA* significantly decreased secretion of IL-8 into the basolateral compartment. Furthermore, when expressed in K12, the *agg*-fimbrial cluster from C227-11 was sufficient to induce a pro-inflammatory response, as previously

reported for the EAEC AAF/I prototype strain, JM221.⁶⁷ Taken together these results suggest that induction of an inflammatory response by C227-11 is multifactorial and depends on both Stx2a and EAEC factors.

We suggest a model for the pathogenesis of the O104:H4 outbreak strain that include steps listed above for EAEC alone, but incorporates additional features explained by the expression of Stx2a (Fig. 3). (1) O104:H4 adheres to the intestinal mucosa within a mucus-containing biofilm, by virtue of AAF and possibly other adherence promoting factors such as the Pic mucinase and dispersin. (2) Interactions between AAF/I and the epithelial monolayer causes disruption of the epithelial barrier and actin cytoskeletal rearrangement. (3) AAF/I, SepA and Stx2a triggers release of inflammatory cytokines and recruitment of neutrophils.^{67,68,72} Such transepithelial migration of neutrophils may also lead to

further Stx2a penetration.⁷³ (4) SigA causes cytotoxicity possibly due to alteration of cytoskeletal elements.⁵¹ (5) AAF/I-mediated disruption of the epithelial barrier in addition to para-cellular passage of neutrophils facilitates the delivery of Stx2a to the lamina propria, with increased access to intestinal vasculature and, ultimately, to the target organs that express high levels of the Stx2a globotriaosylceramide receptor, Gb₃ (particularly, kidneys and brain).

Our findings thus suggest a mechanism whereby the O104:H4 outbreak was associated with unusually high rates of HUS. Our studies demonstrate the importance of the pAA plasmid in the pathogenesis of the O104:H4 outbreak strain, both in ways typical of EAEC (adherence and inflammation), and in a unique and sinister new synergy (barrier dysfunction that augments Stx2a translocation). Consistent with our results is the observation that a proportion of O104:H4 isolates during the outbreak had lost the pAA plasmid, and patients whose initial isolate expressed the plasmid were significantly more likely to develop HUS than those infected with a pAA-negative strain.⁷⁴

Given the virulence of the Stx-EAEC O104:H4 strain it would be relevant for the health community to consider, if the acquisition of the Stx2 phage by an EAEC is an uncommon event or something likely to occur more frequently. As mentioned, the German outbreak was not the first clinically linked instance of a Stx-EAEC, but it is an example of such a strain causing a large-scale outbreak of disease. However, whether the German outbreak was due to a particularly virulent Stx-EAEC, a rare epidemiologic opportunity, or both is unclear.

Concluding, the emerging of Stx-EAEC strains possesses a major challenge for the public health, which needs to implement diagnostic procedures that identify the most virulent clones and this is particular challenging with this heterogeneous pathotype.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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