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INTRA-ABDOMINAL INFECTIONS, HEPATITIS AND GASTROENTERITIS (T. STEINER, SECTION EDITOR)

Enterotoxigenic *Escherichia coli* Infections

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Abstract

Purpose of Review Review recent developments pertaining to the epidemiology, molecular pathogenesis, and sequelae of enterotoxigenic *Escherichia coli* (ETEC) infections in addition to discussion of challenges for vaccinology.

Recent Findings ETEC are a major cause of diarrheal illness in resource poor areas of the world where they contribute to unacceptable morbidity and continued mortality particularly among young children; yet, precise epidemiologic estimates of their contribution to death and chronic disease have been difficult to obtain. Although most pathogenesis studies, and consequently vaccine development have focused intensively on canonical antigens, more recently identified molecules unique to the ETEC pathovar may inform our understanding of ETEC virulence, and the approach to broadly protective vaccines.

Summary ETEC undeniably continue to have a substantial impact on global health; however, further studies are needed to clarify the true impact of these infections, particularly in regions where access to care may be limited. Likewise, our present understanding of the relationship of ETEC infection to non-diarrheal sequelae is presently limited, and additional effort will be required to achieve a mechanistic understanding of these diseases and to fulfill Koch's postulates on a molecular level. Precise elucidation of the role played by novel virulence factors, the global burden of acute illness, and the contribution of these pathogens and/or their toxins to non-diarrheal morbidity remain important imperatives.

Keywords Enterotoxigenic *E. coli* (ETEC) · Diarrhea · Tropical sprue · Environmental enteric dysfunction · Vaccine

Introduction

Enterotoxigenic*E. coli* (ETEC) are a pathogenic variant or pathovar of *E. coli* defined by production of diarrheagenic heat-labile (LT) and heat-stable (ST) enterotoxins. These bacteria, originally identified as a cause of cholera-like watery diarrhea nearly five decades ago [1, 2], have persisted as a major global health threat, particularly among young children in resource-limited areas of the world. Here, it is estimated that children under the age of five suffer over a billion cases of diarrheal illness annually [3], with ETEC alone linked to hundreds of millions of episodes of diarrhea [4]. While the overall mortality from diarrheal diseases appears to have decreased considerably over the past several decades, ETEC remains a leading cause of death among young children [5, 6•]. The attack rate for ETEC illness appears to be highest during the first 2 years of life in endemic areas [7], with substantial declines thereafter suggesting that protective immunity develops following infection. Notably, these pathogens are thought to cause substantial disease as well as tens of thousands of deaths in older children, adolescents, and adults in areas of high endemicity for diarrheal illness including Africa and Asia [8].

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Challenges in Defining the Global Burden of ETEC

Enterotoxigenic *E. coli* are ubiquitously distributed throughout all resource poor areas of the world. While ETEC are

clearly recognized as a globally important cause of diarrheal illness, regional estimates of death and morbidity associated with ETEC and other enteric pathogens vary widely in recent large epidemiologic assessments in LMIC [4, 9, 10•]. An accurate global accounting of the diarrheal disease burden has generally been confounded by the fact that assessment of the attributable mortality and morbidity, particularly at the level of individual enteric pathogens, may be most difficult in regions with limited infrastructure and high endemicity [4, 11••, 12, 13]. Multiple factors contribute to the considerable variability in disease estimates reported in these studies including the definition of diarrhea, local disease prevalence, access to care, and methods used for detecting enteric pathogens [13–15]. Indeed, use of molecular techniques such as quantitative PCR to attribute causation to individual pathovars leads to ~ 1.5-fold increase in ETEC pathogen-specific disease burden relative to asymptomatic control subjects [6•]. Neither the burden of diarrheal illness nor access to life-saving treatment is equitably distributed, and progress in implementing preventative measures has lagged in some high prevalence areas [16]. Despite apparent overall declines in diarrheal mortality, the incidence of diarrheal infections and their associated morbidity [15, 17] has continued unabated.

ETEC is without question one of the most common causes of diarrheal illness in travelers and in military deployed to endemic areas [18–21]. In 11 separate studies performed between 2010 and 2016, ETEC was the most common pathogen identified in traveler's diarrhea (TD) accounting for an average of 42% and 28% of cases in travelers to Latin America and Asia, respectively [22].

Although ETEC infections in travelers are typically self-limited, antibiotics can hasten the resolution of symptoms. Unfortunately, these and other pathogens associated with TD have become increasingly resistant to antibiotics [23–25], and a number of recent studies have expressed concerns about the potential for acquisition and subsequent carriage [26] of multidrug-resistant *Enterobacteriaceae* including *E. coli* [27] during travel and through the selective pressure of antibiotics for treatment or prophylaxis.

Interestingly, with the advent of molecular testing, ETEC have increasingly been identified in both sporadic cases, and in diarrheal outbreaks in the USA. Nevertheless, as ETEC cannot be distinguished from commensal *E. coli* or other pathovars without molecular testing, they often go unrecognized unless there is a recognized cluster of cases that leads to testing in specialized public health laboratories [28–34]. Notably, application of molecular techniques to pathogen identification in diarrheal stool demonstrated that ETEC were as common as most other enteric bacterial pathogens [35••], suggesting that these pathogens may be commonly missed by culture-dependent methodologies in common use in clinical microbiology laboratories.

ETEC Molecular Pathogenesis

Cellular Action of Enterotoxins

Genes encoding heat-labile and heat-stable toxins [36–40] are encoded on virulence plasmids and were among the first bacterial virulence factors to be cloned, sequenced, and characterized on a molecular level. Indeed, these early findings form the basis of the molecular detection assays presently in use [6•]. LT shares substantial homology with cholera toxin (CT), and like CT, LT is a heteroheximer composed of a single A subunit and a pentameric B subunit. LT binds via the pentamer to GM-1 ganglioside on the surface of intestinal epithelial cells, followed by uptake of the toxin, and liberation of the biologically active A subunit. LT and CT belong to a large family of bacterial ADP-ribosylating toxins which act by transferring ADP-ribose to target substrate molecules [41]. LT-A catalyzes the ADP-ribosylation of GSα leading to formation of an ADP-ribose-GSα-GTP complex that activates adenylate cyclase leading to formation of cAMP.

ST is found in two different forms: STh and STp. Both ST molecules are small cysteine-rich peptides of 18–19 amino acids that share homology with native endogenous peptides, guanylin and uroguanylin, and all four of these molecules bind to guanylate cyclase C (GC-C) on the surface of intestinal epithelia [42••], leading to the production of cGMP.

Both cyclic nucleotides cAMP and cGMP activate intracellular protein kinases that lead to phosphorylation and alteration of ion channels including the cystic fibrosis transmembrane regulator (CFTR) chloride channel, and inhibition of the Na+/H+ exchanger NHE3 [43], the net effect of which is the accumulation of salt and water in the intestinal lumen leading to watery diarrhea.

ETEC secreting any one of the known toxins rely on chromosomally encoded secretion systems for export. LT is secreted by a type 2 secretion system [44] similar to that responsible for export of CT by *V. cholerae*, while both STh and STp are secreted via the TolC outer membrane efflux protein [45, 46].

Colonization Factors

ETEC express a broad array of plasmid encoded molecules or structures collectively known as colonization factors (CFs) that facilitate intestinal colonization. The first of these, CFA/I, was discovered shortly after the discovery of ETEC as a cause of diarrheal illness [47, 48]. Since that time, more than 20 antigenically distinct CFs have been characterized, and novel CFs continue to emerge as whole genome sequencing (WGS) is applied to strains lacking previously characterized antigens [49]. Although anywhere from 30 to 50% of isolates in prior studies lacked an identifiable CF [50, 51] using CF-specific monoclonal antibodies for antigen detection, WGS analysis of strains previously characterized as lacking a

known CF suggest that most of the corresponding genomes encode novel or previously uncharacterized CFs [52].

Non-canonical Virulence Factors

Although most studies of ETEC pathogenesis to date have centered around the classical plasmid-encoded antigens that were discovered nearly five decades ago, more recent studies suggest that the molecular pathogenesis of these organisms is significantly more complex than had been previously appreciated. Interestingly, enthusiasm for targeting CFs in vaccines was engendered by examination of a plasmid cured strain of ETEC which had lost the CFA/I virulence plasmid, as this isolate, H10407-P, did not cause diarrhea in human volunteer challenge studies while the parent strain isolated from a case of cholera-like illness predictably caused voluminous diarrhea [53].

More recent studies have demonstrated, however, that in addition to CFA/I, this large plasmid carries at least two additional virulence loci, initially discovered by transposon mutagenesis in a search for novel secreted antigens. The *eatA* gene encodes EatA [54], a member of the serine protease autotransporter of the Enterobacteriaceae (SPATE) family, while the *etpBAC locus* encodes the EtpA extracellular adhesin, the EtpB PORTA domain outer membrane transport protein, and the EtpC glycosyltransferase [55].

The secreted EatA passenger domain (~ 110 kD) contains a canonical serine protease motif and is highly immunogenic. Recent data suggests that EatA may enhance ETEC access to intestinal epithelial cells by degrading MUC2 [56], the major mucin secreted by intestinal goblet cells. In addition to degradation of MUC2, EatA also degrades EtpA, potentially preventing the accumulation of the adhesin [57].

EtpA is a large (~ 170 kD) glycoprotein that is secreted by ETEC. Once secreted, EtpA appears to function as a molecular bridge connecting the ends of ETEC flagella [58] with N-acetylgalactosamine (GalNAc) containing glycans expressed on intestinal mucosal surfaces [59]. Although GalNAc is abundant in intestinal mucin [60], the EtpA lectin has the highest affinity for GalNAc presented as the terminal sugar on A blood group glycans. Because these glycans are expressed on intestinal epithelial cells, EtpA-mediated interactions preferentially promote bacterial adhesion to brush border glycans and consequently toxin delivery to small intestinal enterocytes from A blood group individuals. These blood group-dependent interactions may translate to the more severe disease among A blood group experimental human challenge subjects [61] and naturally infected children in endemic regions [7].

While all of the pathovar-specific virulence molecules for ETEC described to date are encoded on plasmids, it is apparent that these act in concert [62] with highly conserved chromosomally encoded features that are part of *E. coli* core genomes. These include type 1 fimbriae [63], the EaeH adhesin [64], surface expressed autotransporter proteins [65] and the YghJ metalloprotease [66]. The coordinate interaction of these core and pathovar-specific features ultimately drive efficient pathogen-host interactions required for optimal delivery of ETEC toxins [67]. ETEC are typically identified by molecular testing for their toxins. Nevertheless, it is clear that the pathogen virulence traits and host features associated with more severe cholera like disease [68] are still being defined [61]. Presently available molecular tools, including whole genome sequencing of pathogens, have also served to illustrate that ETEC is not a static pathovar and that these pathogens are likely part of a dynamic and ongoing admixture of potential virulence genes [69, 70].

Association of ETEC with Post-diarrheal Sequelae

A number of chronic sequelae have been associated with diarrheal illness caused by a variety of enteropathogens including pathogenic *E. coli.* While studies are ongoing which attempt to link specific pathogens to these sequelae [71, 72], in general the pathogenesis of these post infectious phenomena remains very poorly understood.

Post-infectious Irritable Bowel Syndrome

It is estimated that up to 3% of individuals with traveler's diarrhea have protracted symptoms that last more than 1 month [73] and that post-infectious IBS occurs in an estimated 10–30% of patients following acute gastroenteritis [74–78]. One study of more than 500 travelers in Israel documented a 5-fold increase in risk for IBS in those who developed diarrhea relative to those who remained asymptomatic [79]. Given the predominance of enterotoxigenic *E. coli* as a cause of traveler's diarrhea (TD), it is not altogether surprising that at least one study has linked ETEC to the development of IBS. Intriguingly, however, the association was only with LT-producing ETEC, but not with other ETEC [80].

Although it has been suggested that post-infectious IBS generally carries a better prognosis than idiopathic IBS [81, 82], this has not been validated in subsequent studies [83]. IBS associated infectious with diarrheal was associated with a higher frequency of diarrheal illness, however long-term prognosis did not differ significantly from idiopathic IBS in that less than half of patients in either group had resolution of symptoms.

Post-infectious Malabsorption Syndromes, Growth Stunting, and Cognitive Impairment

Tropical Sprue and Environmental Enteric Dysfunction

The first descriptions of individuals with malabsorption syndromes in the tropics date back more than 250 years [84], and

Manson, working in China later referred to similar ailments as "tropical sprue" [85]. Tropical sprue has since been synonymous with post-infectious malabsorption syndromes, potentially caused by a variety of pathogens [86], and is characterized by blunting of the small intestinal villi, persistent diarrhea, steatorrhea, and folate and B12 deficiency [87–89]. Tropical sprue is clearly described as a sequela of diarrheal illness in travelers and expatriates [88, 89]. Interestingly, studies of Peace Corps volunteers traveling to India or Pakistan in the 1970s, all of whom developed diarrhea during an 18–24-month tour (with 90% having had diarrhea ≥ monthly), demonstrated a high incidence of weight loss, as well as abnormal jejunal architecture and malabsorption that reversed on return to the USA [90]. Yet, despite the predominance of ETEC as a major cause of traveler's diarrhea, and the repeated isolation of toxin-producing *E. coli* from small intestinal aspirates of patients with tropical sprue [91–93], most of these studies were done prior the advent of molecular techniques in use today. Although a clinical response to antibiotics in patients with tropical sprue also supports a possible bacterial etiology [86, 94], molecular Koch's postulates [95] clearly establishing a direct link between ETEC and the development of tropical sprue are presently lacking.

Some have speculated that tropical sprue may essentially represent part of a spectrum of illness similar to tropical enteropathy [96, 97] or environmental enteric dysfunction [98, 99] that is frequently associated with diarrheal pathogens among young children in developing countries that results in altered intestinal absorption, growth faltering, poor response to oral vaccines, and cognitive impairment [100–102]. Observations of malnutrition or kwashiorkor following acute diarrheal illness in developing countries are longstanding [103]. Cohort studies of children followed in highly endemic areas of Bangladesh demonstrated that they were more likely to be underweight [104] and/or growth stunted following diarrhea caused by ETEC [7]. Conversely, malnourished children also appear to be at significantly higher risk for diarrheal illness [105], and for development of protracted diarrhea caused by ETEC [104].

ETEC are frequently found in the stools of young children in the developing world without diarrhea, a phenomenon that has significantly confounded [11••] recent estimates of morbidity and mortality attributable to these pathogens [4]. Nevertheless, some studies have suggested that carriage of ETEC and other pathogens may be sufficient to drive changes associated with enteropathy and malnutrition [106••, 107]. Likewise, recent studies suggest that cognitive impairment linked to diarrheal pathogens is associated with enteropathogen carriage even in the absence of diarrheal illness again suggesting perhaps that subclinical infections could contribute to enteropathy [108]. Clearly, the relationship of malnutrition and stunting to infection with a number of enteropathogens is complex and the recent demonstration of small intestinal overgrowth with orpharyngeal microbiota may further complicate attempts to link these sequelae to specific pathogens [109]. Notably, however, these studies also demonstrated an increase in possible enteropathogenic genera defined as *E. coli/Shigella* by 16S rRNA sequencing, suggesting that stunting represents the end result of a number of insults to small intestinal epithelia.

Challenges for ETEC Vaccinology

ETEC vaccines in development have been reviewed extensively [110•, 111, 112]; therefore, here, we focus primarily on the challenges that need to be surmounted in vaccine development. Currently, there are no vaccines for ETEC that are licensed in the USA. Dukoral (Valvena, Sweden AB) is an oral, whole cell killed (WC) *Vibrio cholera* O1 vaccine containing recombinant cholera toxin B subunit (rBS), available in Canada and Sweden for prevention of traveler's diarrhea, but not the USA. In large-scale field trials (involving nearly 50,000 vaccinees) comparing WC to WC-rBS, recipients of the vaccine containing cholera toxin B subunit experienced substantial short-term protection with a protective efficacy (PE) of 86% against severe, life-threatening diarrhea caused by LT ETEC [113]. Although it was anticipated that this vaccine would afford some protection against LT-ETEC due to the considerable homology between cholera toxin and LT, the vaccine also offered short duration protection against ETEC that also produced ST (LT/ST, PE 73%). The short protection afforded by this vaccine led to several smaller studies in travelers that subsequently demonstrated modest PE against ETEC diarrhea ranging from 28 to 50% [114–116].

Clearly, additional effort will be needed to engender the broad-based, long-term protection needed for individuals in developing countries. One major hurdle that ETEC vaccines have had to overcome is the underlying plasticity of *E. coli* genomes [117]. The promiscuous adaptation of colonization factors [50], with more than 20 antigenically distinct antigens described thus far [118], illustrates the need for a multivalent approach that can target the most highly conserved molecules. In an attempt to develop protective vaccines, most formulations to reach clinical trials have embraced this approach by incorporating a combination of the most common CF/CS antigens with mutant versions of LT [119, 120].

Data emerging from genome analyses indicate that some of the more recently described virulence factors including EtpA and EatA are conserved across the ETEC pathovar [121, 122], perhaps providing targets that could complement canonical approaches and broad-based protection. Indeed, recent immunoproteome analysis of human volunteer samples using ETEC protein microarrays indicates that these highly immunogenic molecules are among a relatively small number of pathovar specific antigens recognized during infection [123],

findings that can collectively direct antigen selection in rational vaccine design.

Given that toxoids afford substantial protection against other important toxigenic mucosal pathogens including pertussis [124, 125], combining highly conserved antigens with LT [126] and ST [42••] toxoids in development could accelerate deployment of a broadly protective formulation [127–129]. Mutant versions of LT such as LT(RL211A/L211A) or dmLT appear to be both safe and remarkably effective as mucosal adjuvants, and LT alone was shown previously to afford substantial protection against ETEC that produced only LT [130].

From a feasibility standpoint, a vaccine that targets other enteric pathogens in a combination vaccine, in particular *Shigella* in addition to ETEC, may be required to secure the necessary development resources [13, 14••, 131]. ETEC and *Shigella* along with *Campylobacter* are responsible for the majority of serious bacterial diarrhea in LMIC regions. Therefore, polyvalent combination vaccines encompassing antigens from ETEC and other pathogens could yield a practical multi-pathogen diarrheal disease vaccine [131–134].

Summary

Enterotoxigenic *E. coli* are an important cause of diarrheal illness in LMIC areas of the world where they place a particular burden on the health of young children. Additional efforts to define the contribution of ETEC to non-diarrheal sequelae have increased in importance as deaths from acute diarrheal illness from all causes have declined over the past several decades while both the incidence of infectious diarrhea and morbidity associated with these pathogens continue unabated. Ongoing pathogenesis studies that elucidate the role of recently identified virulence molecules, and reexamine the cellular impact of the established toxins can inform and prioritize approaches to vaccine development for these pathogens of global importance.

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Compliance with Ethical Standards

Conflict of Interest James M. Fleckenstein and F. Matthew Kuhlmann declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors. Informed consent was taken from patients prior to enrollment.

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