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Pathophysiology of Enteropathogenic *Escherichia coli*-induced Diarrhea

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Abstract

Enteropathogenic *Escherichia coli* (EPEC) are important diarrheal pathogens of infants and young children. Since the availability of molecular diagnosis methods, we now have new insights into the incidence and prevalence of these infections. Recent epidemiological studies indicate that atypical EPEC (aEPEC) are seen more frequently than typical EPEC (tEPEC) worldwide, including in both endemic diarrhea and diarrhea outbreaks. Therefore, it is important to further characterize the pathogenicity of these emerging strains. The virulence mechanisms and pathophysiology of the attaching and effacing lesion (A/E) and the type-three-secretion-system (T3SS) are complex but well-studied. A/E strains use their pool of locus of enterocyte effacement (LEE)-encoded and non-LEE-encoded effector proteins to subvert and modulate cellular and barrier properties of the host. However, the exact mechanisms of diarrhea in EPEC infection are not completely understood. From the clinical perspective, there is a need for fast, easy, and inexpensive diagnostic methods to define optimal treatment and prevention for children in endemic areas. In this article, we present a review of the classification of EPEC, epidemiology, pathogenesis of the disease caused by these bacteria, determinants of virulence, alterations in signaling, determinants of colonization vs. those of disease, and the limited information we have on the pathophysiology of EPEC-induced diarrhea. This article combines peer-reviewed evidence from our own studies and the results of an extensive literature search in the databases PubMed, EMBASE, and Scopus.

Keywords

Attaching and effacing lesion (A/E); Epidemiology; Ion transporters; LEE pathogenicity island; Type III secretion system (T3SS); Tight junctions

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Introduction

Escherichia coli (*E. coli*) is the predominant facultative anaerobic species in the intestine. Most strains are non-pathogenic and play an important role in maintaining intestinal physiology.[1,2] This organism was first described by German pediatrician Theobald Escherich in 1885, under the name "*Bacterium coli commune*" as a short rod that had initially been isolated from normal infant feces.[3] The current classification systems of *E. coli* consider many strains (Flowchart 1).[4]

Enteropathogenic *E. coli* (EPEC) is a major cause of infantile diarrhea in developing countries.[5] EPEC strains were epidemiologically associated with outbreaks in 1940s and 1950s and were first described in 1955.[6] These strains currently account for 1.3 million deaths every year.[7] The incidence is now being noted more accurately since the development of molecular diagnostic methods. In this study, we have reviewed the epidemiology of EPEC infections in infants and children and our studies in animal models to understand the pathophysiology of EPEC-associated diarrhea.

Epidemiology of EPEC Infections

Although most strains of *E. coli* are avirulent commensals in the gastrointestinal tract, many can cause diarrhea, urinary tract infection, and sepsis/meningitis. Several *E. coli* pathogens have been implicated in public health problems worldwide.[8] The incidence of EPEC-related disease seems to have decreased over the last several decades. It is unclear if this reduced incidence is due to interventions such as the promotion of breastfeeding, or whether earlier studies based on O:H-serotyping overestimated the relative contribution of these organisms compared to newer molecular methods and/ or adherence assays.[7]

Enteropathogenic *E. coli* was the first strain of *E. coli* identified as the cause of infantile diarrhea in the 1940s and 1950s. These outbreaks of "summer diarrhea" were frequent in developed countries until the 1950s and had high mortality.[6] EPEC strains were first shown to be pathogenic in human volunteer studies carried out by Levine et al.[9] in 1978. They tested classic EPEC strains (O127 and O142) associated with infant diarrhea that had been stored for 7–9 years. These isolated strains did not express LT and ST enterotoxins or show invasiveness. Enteral administration to healthy young adult volunteers caused a notable diarrheal illness.

In a systematic review of 266 studies published between 1990 and 2002, EPEC was identified with a median prevalence of 8.8% (inter-quartile range, IQR of 6.6–13.2) in the community setting, 9.1% (IQR 4.5–19.4) in the outpatient setting, and 15.6% (IQR 8.3–27.5) in the inpatient setting. Enteropathogenic *E. coli* may be the second most frequently seen cause of diarrhea after rotavirus (25.4%) in the inpatient setting. However, there are important regional and temporal variations.[10] Investigators from Peru combined data on six different diarrheagenic strains of *E. coli* from eight different studies of children <3 years of age. Multiplex real-time PCR showed that the average EPEC prevalence in diarrheal stool samples (n = 4,243) was 8.5% (95% CI: 7.6–9.3), second only to enteroaggregative *E. coli* (EAEC; 9.9%). Enteropathogenic *E. coli* prevalence increased with age; these strains were found in 3% of diarrheal samples in children <6 months, in 11% of children 6–12

months, and in 16% of children 13–24 months. In these cohorts, exclusive breastfeeding was more frequent than in other studies (>80% for infants younger than 6 months), and hence young infants may have been protected from symptomatic infection. Among asymptomatic controls (n = 3,760), EPEC was detected in 10.9% (95% CI: 9.4–11.4).[11–13] Similarly, in a recent study in India, EPECs were identified in 3.2% of 648 children <5 years of age who were hospitalized for diarrhea.[14] In another study, EPEC has been noted to be the most prevalent pathotype with an average prevalence of 10.9% (95% CI: 9.4–11.4), followed by EAEC (10.4%).[7] A study reported that more than 20% of all episodes of persistent diarrhea in the pediatric population; aged >14 days are mainly caused by diarrheagenic *E. coli* such as aEPEC. Another study identified specific *E. coli* strains from patients of infantile gastroenteritis. This study reported that serogroups O111 and/or O55 were more putative in causing diarrhea in recipients, and disease outcome in terms of severity of symptoms was largely dependent on the size of the dose.[15–17]

Enteropathogenic *E. coli* is known to be an important cause of infantile diarrhea in Brazil, Chile, Peru, and Iran.[18] Studies in Brazil, Mexico, South Africa, and Bangladesh have shown that EPEC infections cause 30–40% of infant diarrhea with high mortality rates.[19–23] In several studies conducted in Latin America, tEPEC was found to be the main cause of endemic diarrhea in children < 1 year of age. The frequency of tEPEC infection drops with an increase in age group, and adults rarely experience tEPEC episodes.[18] This may be due to development of immunity or the loss of receptors interacting with some specific adhesins. Although tEPEC were major agents of acute diarrhea in infants until the 1990s, a clear decline in many of these countries was seen in the global enteric multicenter study, a population-based case–control study including seven countries in Africa and Asia with the goal to identify the etiology, burden, and mortality of acute moderate-to-severe pediatric diarrhea.[24,25] The reasons for the decline are unclear but may be linked to improved public health with active interventions, therapy, sanitary conditions, and control of hospital infections.[24,26] However, tEPEC infections remain associated with a 2.8-fold higher risk of death among infants aged 0–11 months.[25]

Atypical EPEC continues to be frequently detected in various parts of the world.[7] Thirteen studies from peri-equatorial/tropical countries showed aEPEC isolates in 78% (131/169) of all EPEC cases in children.[18] Wheeler et al.[27,28] reported the identification of 142 aEPEC strains with only one tEPEC in 2774 samples from symptomatic children from the UK. A study from Australia identified 61 EPEC strains from a stool samples of symptomatic patients and highlighted the higher frequency of aEPEC at 95.1% (58/61).[29] In 2009, the aEPEC strain O76 was reportedly responsible for a nursery outbreak in Finland.[30]

In another study, Sakkejha et al.[31] detected 109 EPEC isolates in England from 2010 to 2012, with 93% of the patients with diarrhea; aEPEC were seen more frequently than tEPEC. Overall, according to 266 studies published between 1990 and 2002, EPEC remains major pediatric pathogen.[7] As such, in 2014 a European, multicenter, prospective quarterly point-prevalence study of community-acquired diarrhea (EUCODI) showed a high frequency of EPEC.[32]

For unknown reasons, EPEC disease is becoming less frequent in infants in developed countries in developed/temperate climate zones of the world. However, day care centers and pediatric wards of hospitals are still prime breeding grounds for EPEC outbreaks.[33–35] Globally, EPEC is responsible for infantile diarrhea in underdeveloped nations with nearly 30% mortality.[36]

Even though EPEC is strongly associated with infant diarrhea, many studies have also found EPEC, particularly aEPEC, in asymptomatic controls.[18] There may be multiple possible reasons for this apparent anomaly: (a) host susceptibility.[37] There may be genetic variability in specific mucosal receptors, including proteins and carbohydrate moieties; (b) individual variability in non-specific host barriers such as the gut microbiome, mucus layer, and epithelium. The variability in the strength of these barriers may influence bacterial overgrowth and susceptibility to disease.[38] (c) Immune status of the host, which may limit bacterial flora to colonization but not cross numerical thresholds needed to cause disease.[39] In addition, secretory immunoglobulin A (sIgA) in the intestine and in human milk can limit/prevent enterocyte colonization/mucosal invasion by enteropathogens.[40] Human milk also contains other non-specific defense factors such as lactoferrin and enterotoxin-binding oligosaccharides. In endemic areas, colostrum contains specific sIgA against EPEC.[41] In addition, children may acquire natural immunity with age. Opintan et al.[42] showed that EPEC carriage, not disease, is frequently seen in healthy children in endemic areas after 2 years of age. Bacterial factors are also important in asymptomatic carriage of EPEC. Some strains are more likely to not cause symptoms, such as those with the phylogenetic marker gene *yhaA*. Children without diarrhea frequently carried aEPEC strains that were OI-122 *efa1*/*lifA*-negative and *yhaA*-positive. There is considerable variability in the severity of disease between individual strains.[43]

The variability in diagnostic tests also needs attention. In this regard, the bacterial load is an important consideration. Barletta et al.[44] compared children with diarrhea *vs.* asymptomatic controls. When a quantitative real-time PCR assay was used, the bacterial load was significantly higher in the symptomatic infants than in age-matched controls. Other factors may also need consideration. For instance, the collection of control samples and sample size are pivotal factors.[45] The transmission of EPEC from controls to other patients needs further consideration. Finally, environmental factors such as poor hygiene and fecal contamination may also increase the bacterial load in control groups.[7]

EPEC Definition and Classification

Escherichia coli serotypes were first classified based on the Kauffmann system in the 1940s. The three antigen systems included the somatic O, flagellar H, and the capsular K surface antigens.[46,47] In 1955, the term EPEC was coined to describe strains that were primary intestinal pathogens but were rarely encountered in the feces of healthy individuals and in infections other than diarrheal diseases.[4] Formally, 187 O serotypes were documented, but currently 176 are considered as true O serotypes. Six (O31, O47, O67, O72, O94, and O122) are no longer considered as O serotypes, some being duplicate names for an O antigen and others were in organisms that were reclassified into other genera and three (O34, O89, and O144) strains are also removed from this classification which are incapable of producing O

antigens and are removed from these O serotypes. For O serotypes, the most variable cell component is O antigen because of existence of variations in sugar moieties and the linkages present within as well as between O units. Due to the existence of these variations, there is diversity of various clones in the species. Each expresses different surface antigen on the cell surface which offers selective advantages in varied environments. The O antigen is one of main virulence factors and its loss can severely impair the pathogenicity and virulence. O antigens play vital roles, including protection against phagocytosis and clearance via neutrophils and monocytes, as well as have inhibitory effects on the bactericidal activity of lysozyme, a key player in host innate immunity.[48] The major O serogroups, including O55, O86, O111, O119, O125, O126, O127, O128ab, and O142, are considered to contain EPEC serotypes.[49,50] The variability in O surface antigen provides basis for typing of the bacterial species for taxonomical as well as epidemiological purposes. It is most widely utilized to signify the presence of enteropathogens and considered as a basic tool for bacterial outbreak investigations and surveillance.[48] O55 serotype is most rarely found in healthy individuals. However, varied pathogenicity levels are exhibited within O serotypes as all serotypes are not equally pathogenic, and only a limited number of H serotypes are incriminated within O serotypes.[49] Another antigen, H (flagellar) is also expressed by EPEC strains. H2 and H6 are predominantly expressed flagellar antigens, and the least frequent ones include H7, H8, H9, H12, H21, H27, H25, and H34. However, some EPEC strains lack H flagellar antigens and are, therefore, classified as H-negative. These strains are non-motile.[51]

Several EPEC serogroups may share characteristics with the Shiga toxin-producing *E. coli* (STEC).[52,53] Both can induce attaching-and-effacing (A/E) lesions on intestinal epithelial cells (IECs), and the bacteria attach to IECs and efface the microvilli on the cell surface (Fig. 1).[54] There is a need to identify specific virulence genes to distinguish between the two bacterial genera as these differ in pathogenicity. EPEC pathotypes do not produce the Shiga toxin (*stx*$^{-}$), but some aEPEC strains such as the O55:H7 resemble the LEE-positive Shiga toxin-producing *E. coli* such as the STEC O157:H7 in their genetic and virulence characteristics.[4] Most tEPEC and aEPEC strains may differ in adherence patterns; tEPEC strains show localized adherence (LA) patterns, but the aEPEC can produce a localized-like adherence, a diffuse adherence (DA), or an aggregative adherence (AA) pattern.[24]

Enteropathogenic *E. coli* binds IECs by an outer membrane protein called intimin, which is encoded by the gene *eae*. The genetic elements needed to produce the A/E lesions are encoded on a genomic pathogenicity island, the locus of enterocyte effacement (LEE).[55,56] Another pathogenicity factor is the plasmid *E. coli* adherence factor (pEAF).[4,57] Enteropathogenic *E. coli* is classified as typical or atypical based on the presence of pEAF, which contains two important operons.[24] These include a type IV bundle-forming pilus *(bfp*) and a plasmid-encoded regulator (*per*). The *bfp* promotes bacterial adherence and formation of compact microcolonies. *Bfp* and *per* are important transcriptional activators for LEE pathogenicity island.[4,58]

The plasmid pEAF imparts important characteristics to EPECs. Three subgroups can be seen:

1. Typical EPECs (tEPECs) are *eae*$^{+}$ *bfpA*$^{+}$ *stx*$^{-}$. Most belong to classical O:H serotypes and express *bfp* to show the localized adherence (LA) phenotype.[59] The expression of EPEC virulence genes on classical EPEC serogroups is not universal. However, tEPEC strains are more homogeneous in their virulence traits than aEPEC. Most of the typical strains produce the virulence factors encoded by the LEE region and EAF plasmid.[24]

2. Atypical *E. coli* (aEPEC) strains lack the EAF plasmid and hence are bfpA negative and are defined as *eae*$^{+}$ *bfpA*$^{-}$ *stx*$^{-}$. The lack of Bfp, makes atypical EPEC strains exhibit localized-like (LAL) pattern, which is mainly characterized by the presence of bacterial microcolonies. LAL is the most common pattern, but atypical EPEC strains also exhibit diffuse (DA) or aggregative adherence (AA) patterns.[58,60] LAL^{+} aEPECs show pili and other known adhesins. Some aEPECs express the enteroaggregative heat-stable toxin (EAST1) and other potential virulence factors not encoded in the LEE, such as a hemolysin.[60–62]

3. Non-typeable EPECs, which are identified among aEPECs and do not belong to classical EPEC serogroups. There are >200 of these strains.[63,64]

Virulence Factors and Signaling

For successful infection and formation of an A/E lesion, two major virulence factors are needed, the type IV bundle-forming pilus (BFP) and LEE.

Type IV Bundle-forming Pilus (BFP)

Type IV BFP is a dynamic fibrillar organelle responsible for the initiation of initial non-intimate attachment of EPEC to the host IECs. Further, BFP recruits individual EPEC together as aggregates and leads to the formation of microcolony on the host cell membranes, typically known as a localized adherence (LA) phenotype. The ~80 kb plasmid (pEAF) encodes 14 genes, which are required for the biogenesis of BFP and consequently in the formation of the EPEC adherence factor (EAF). The strains lacking pEAF are incapable of forming typical LA phenotype.[57,65,66] Activation of BfpA is mediated by the plasmid-encoded regulator A (PerA). The activated form is a major pilus subunit and is called pre-bundlin. Further, pre-bundlin is acted upon by the prepilin peptidase, BfpP, and is then converted to the mature forms.[67,68]

Two nucleotide-binding proteins, BfpD and BfpF, further mediate the extension of the pilus and retraction, respectively. Aggregation of EPEC is promoted by BfpD, whereas BfpF facilitates the separation of EPEC from cellular aggregates that are maintaining a constant supply of bacterial cells for further infectious steps. BfpF-mediated dissociation of bacterial cell aggregates permits the intimate attachment of individual EPEC to the gut epithelium, resulting in efficient activation of T3SS and successful translocation of effector molecules into the host cells (Fig. 2).[65] In addition to filamentous actin, cytoskeletal proteins such as α-actinin, talin, ezrin, myosin-light chain, vasodilator-stimulated phosphoprotein (VASP), the Wiskott–Aldrich syndrome protein (WASP), and the actin-related protein 2/3 (Arp2/3) complex are also observed in EPEC-induced A/E lesions. Additionally, many proteins involved in focal adhesion such as α-actinin and vinculin were found to be recruited

to sites of A/E lesions.[69–73] After EPEC attachment to the host surface, kinases encoded by Ab1/Arg, Src, and Tec families lead to phosphorylation of tyrosine residues in the cytoplasmic domain of translocated intimin receptor (Tir). Phosphorylated Tir interacts with two adaptor proteins (Nck1 and Nck2). This interaction results in the recruitment of actin nucleation-promoting factor, N-WASP, which further activates the Arp2/3 complex that assembles actin beneath EPEC (Fig. 2). These signaling events lead to the formation of actin-rich pedestals on host cell luminal membrane, along with inflammatory response and diarrhea.[65]

Locus of Enterocyte Effacement

Once the bacterial aggregates dissociate from the host cell membranes via BfpF, EPEC expresses the LEE for further intimate attachment to intestinal epithelial cells (Fig. 2). Enteropathogenic *Escherichia coli* contains a 35,624 base pair LEE pathogenicity island (LPI), which contains 41 open reading frames (ORFs) of more than 50 amino acids arranged in five major polycistronic operons (LEE1 to LEE5).[74,75] Locus of enterocyte effacement pathogenicity island encodes for the majority of EPEC effector proteins. Locus of enterocyte effacement encodes for the T3SS machinery (Esc and Sep proteins), outer membrane adhesin (intimin), translocators (EspA, EspB, and EspD), chaperones (Ces proteins), effector proteins (EspF, EspG, EspH, Map, and EspZ), translocated intimin receptor (Tir), regulatory proteins Ler (LEE-encoded regulator), repressors including GrlR (global regulator of LEE proteins), and activators such as GrlA (global regulator of LEE proteins).[76] Various factors influence the regulation of LEE, including Ler, GrlR, and GrlA; and *E. coli* global regulators such as the H^+NS, IHF, and FIS.[77,78] These genes are separated into three functional domains – a region encoding intimate adherence (Tir and intimin), a region encoding the EPEC-secreted proteins (including espA, espB, espD, and espF) and their putative chaperones, and the region encoding a type III secretion system.[79]

LEE1, LEE2, and LEE3 encode for the genes involved in the production, assembly, and regulation of T3SS. Locus of enterocyte effacement-encoded structures are comprised of three vital components: (a) outer membrane needle complex (EscC, EscD, EscF, EscI, and EscJ); (b) inner membrane, which contains an export apparatus (EscRST, EscU, and EscV); and (c) a cytoplasmic sorting platform (EscA, EscK, EscL, EscN, and EscQ). The gene of translocation apparatus, the extracellularly secreted proteins of T3SS are encoded via LEE4 genes (EspA, EspB, and EspD). The role of EspB is implicated in the effacement of microvilli on the intestinal surface. The EspABD translocon apparatus of T3SS is responsible for the translocation of six LEE-encoded effectors (Tir, Map, EspF, EspG, EspZ, and EspH). These effectors are involved in the sequential events during EPEC infection which include disruption of tight junctions, mitochondrial dysfunction, and formation of filopodia in host intestinal epithelial cells. The genes for adhesin (intimin), 94 kDa outer membrane protein of EPEC, and its translocation receptor (Tir) are encoded via LEE5.[65] The gene encoding for intimin, *eae* (*E. coli* attaching-and-effacing), is comprised of four distinct intimin subtypes (α, β, γ, and δ).[80] Different intimin subtypes are expressed in different tissues; the small intestinal mucosal layer expresses intimin-α clones, and the Peyer's patches exhibit expression of intimin-γ.[81] Different intimin types could bind to the host cell protein nucleolin, which then colocalizes with adherent bacteria.[82] Chaperone

proteins have also been discovered in T3SS in EPEC and are essential for secretion of espD, espA, and espB.[76]

Effectors encoded outside the LEE pathogenicity island have been described in all A/E-producing pathogens.[24] Scattered across the whole genome, six pathogenicity islands harbor the clusters of non-LEE-encoded (Nle) effectors.[75,83] These Nle effectors include NleA-H, EspG2/Orf3, Cif, EspJ, and EspL. NleA (also called EspI) suppresses protein secretion; EspJ inhibits phagocytosis; and NleE and NleH activate innate immune responses. A/E *E. coli* strains utilize both LEE-encoded and non-LEE-encoded effector proteins to subvert and modulate cellular and barrier properties of the host for successful infection in a well-controlled manner.[7]

Pathogenesis

Enteropathogenic *E. coli* is generally considered to be a noninvasive pathogen but can cause subclinical to fatal diarrhea.[4] Studies with adult volunteers reported that 12–24 hours post infection with tEPEC (10^9–10^{10} of bacterial inoculum) can induce diarrhea.[9] As discussed before, EPEC strains attach to IECs in two different patterns – localized adherence (LA) in which bacteria adhere in discrete microcolonies and diffuse adherence (DA) in which bacteria adhere uniformly over the cell surface. Localized adherence was highly correlated with specific EPEC serogroups in strains isolated from patients with diarrhea.[66] The BFP is usually seen as the initial EPEC attachment factor.[84] The major pilin subunit of BFP is identified as the *bfp*A. Bundle-forming pilus is encoded by a cluster of 14 genes on the EAF plasmid and mediates LA phenotype, which is further responsible for antigenicity, biofilm formation, autoaggregation, and compact microcolony formation.[51,57,85] Genes external to the *bfp* gene cluster were also necessary for full expression of BFP. This included the global regulator element of EPEC pathogenesis *per*ABC (*bfp*TVW) and the chromosomal *dsb*A gene encoding for a disulfide isomerase.[86]

The BFP-mediated interbacterial interactions may allow the dispersal of individual bacteria from autoaggregates and colonization to other epithelial sites, contributing to the spread of infection within the gut. In addition to BFP, additional fimbrial structures have also been characterized and could have roles in EPEC-host cell adhesion. There may be rod-like fimbriae and fibrillae, suggesting that the bacterial–host cell interaction is a multifactorial process. More recently, flagella have been implicated in EPEC adherence to IECs.[87] However, there is some uncertainty because a flagellated strain that lacked BFP, intimin and EspA failed to adhere to IECs in *ex vivo* studies. The term EPEC adherence factor (EAF) refers to the plasmid-mediated adhesion. *Escherichia coli* strains isolated from outbreaks of infantile gastroenteritis almost invariably possess the EAF plasmid.[88] EPEC adherence factor plasmid generally promotes non-intimate cell adhesion. For A/E lesion formation, chromosomally encoded factors were required for the A/E phenotype, and the genes on the plasmid may play a secondary role.[89] Localized adherence (LA) pattern is exhibited by various EPEC serogroups including O55, O86, O111ab, O119, O125, O128ab, and O142.[90] The existence of 60 MDa plasmid (denoted as pMAR2) is responsible for localized adherence pattern exhibited by EPEC strain E2348/69 (O127:H6).[91]

Mucosal adhesion by EPEC may involve two distinct stages: (a) initial attachment of EPEC promoted by plasmid-encoded adhesins; and (b) effacement of brush border microvilli leading to intimate EPEC attachment. Although the second stage could occur without the first, the presence of plasmid-encoded adhesin enhanced mucosal colonization.[89] A/E lesions exhibit association of bacterial cells to IECs followed by extensive disruption, loss of brush borders and microvilli, alterations in F actin rearrangements, and ultimately cup and pedestal formations.[7] These structures may provide a strong attachment of EPEC to the cell surface, preventing dislodgement in the ensuing diarrheal response. Many affected bowel segments show depletion of glycocalyx. Some areas show a mucous pseudomembrane coating on the mucosal surface. There are characteristic cytoskeletal alterations with disruption of the brush border cytoskeleton and proliferation of filamentous actin beneath the foci where bacteria adhered to the host cell surface. There are at least three prominent changes: (a) adherence to IECs; (b) delivery of 25–50 virulence factors into the host cell using a type III secretion system (T3SS)[51]; and finally, (c) firm adherence to the cell surface with the formation of pedestals (Fig. 2).[86] The T3SS is one of the five most important secretion systems utilized by Gram-negative bacteria, besides the T4SS, T5SS, T6SS, and T7SS, to inject effector proteins into the host cells to promote colonization and virulence. It is important because it is exclusively involved in virulence.[92,93]

The T3SS, intimin, and the translocated intimin receptor (tir) are all essential virulence determinants of the intimate adherence, a process that requires the T3SS to inject tir into the host cell. Tir acts as a receptor for bacterial binding via tir–intimin interaction. These trigger many signaling cascades such as phosphorylation of a host phospholipase and recruitment of cytoskeletal proteins beneath the adherent bacteria. Intimin can also subvert cellular processes independently of tir.[86] Mitochondrial-associated protein (Map) targets host cell mitochondria and also contributes to the disruption of the epithelial barrier.[94]

The hallmark of EPEC infection is A/E lesion which marks the intimate attachment of the bacteria to the host enterocytes and results in the effacement of the microvilli. The IEC membrane in these foci can also be raised locally in a characteristic pedestal shape that may extend up to 10 μm outwards from the cell to form pseudopod-like structures.[95] This near-complete destruction and extensive loss of intestinal epithelial surface with villus atrophy and thinning of the mucosal layer is frequently seen during severe EPEC infections.[96] The extensive loss of microvilli on the infected IECs alters the expression and function of ion transporters, channels, and tight junctions. The pathogenesis of microvillus effacement is seen as a 2-step process that requires synergistic action of three effectors (Map, EspF, and Tir) on intimin, and retention of the detached microvillar material. Other studies have focused on the type III secretion system and its effectors including tir, map, espF, and espG.[65] Enteropathogenic *E. coli* also rapidly inactivates the sodium-D-glucose cotransporter (SGLT-1) by multiple mechanisms. SGLT-1 plays a crucial role in the daily uptake of fluids from the intestinal lumen.[97] Calcium signaling may also be important; it may activate actin-severing proteins, resulting in cytoskeletal rearrangement and brush border effacement. However, all these possibilities need further confirmation.[98,99] Some aEPEC is strongly associated with acute disease, whereas others have been noted in persistent diarrhea.[100] Clinically, aEPEC outbreaks may cause mild but prolonged non-

dehydrating, non-inflammatory diarrhea. There is usually no fever, vomiting, or abdominal pain.

Pathophysiology of Diarrhea

We now understand EPEC pathogenesis at cellular and genetic levels, but the pathophysiology of the resulting diarrhea remains elusive. The extensive loss of microvillus and subsequent reduction in absorptive surface certainly contributes to diarrhea. However, the rapid onset of diarrhea remains unexplained and appears to be multifactorial in nature. Enteropathogenic *E. coli* can alter epithelial permeability by activating signaling cascades that phosphorylate Ser/Thr residues on the myosin light chains.[101] This might contribute to diarrhea through increased permeability and disruption of tight junction integrity (Fig. 3). Recently, another EPEC effector molecule, the espF, was shown to be translocated by the T3SS into host cells, where it disrupts host IEC tight junctions and could contribute to diarrhea.[102] Enteropathogenic *E. coli* can also activate NF-κB in host cells and induce host inflammatory responses, which, in turn, could increase paracellular permeability and cause tissue damage.[103] The stimulatory effects of EPEC infection have been implicated on NF-κB activation and downstream enhancement of Cl secretion and fluid accumulation in the colon.[104,105]

Prolonged EPEC infection leads to inflammation and disruption of structure and barrier function of tight junctions (Fig. 3).[5,106,107] Enhanced paracellular permeability, inflammation, and disruption of tight junctions have been implicated in EPEC-mediated chronic diarrhea.[108] Studies have highlighted the downstream effects of prolonged inflammation in terms of increased influx of neutrophils, resulting in the release of 5-AMP that is further converted into secretagogue adenosine.[109,110]

Enteropathogens such as EPEC likely cause diarrhea by altering electrolyte transport.[111] Impairment of ion and solute transport may directly or indirectly influence the fluid transport processes and barrier integrity in gut epithelial cells.[112] Recent advances indicate that EPEC infection can directly influence ion transport mechanisms involving $Cl^-/HCO_3^-/OH^-$ exchange, Na^+/H^+ exchange, serotonin transporter, and short-chain fatty acids transporters. The following section will review the potential mechanism(s) involved in the regulation/alteration of ion and nutrient transporters on the gut epithelial cells during EPEC-induced diarrhea.

(a) Effect of EPEC Infection on Na^+/H^+ Exchanger Type 3 (NHE3)—Diarrhea caused by enteric pathogens may involve decreased NaCl absorption, enhanced Cl^- secretion, or both.[5] In early onset diarrhea, decreased intestinal NaCl may be pathophysiologically more important than the rise in Cl^- secretion.[113,114] The effector proteins of EPEC namely, NleA and Map, interact with Na^+/H^+ exchanger regulatory factor 2 (NHERF2) and alter its function and ultimately leading to decreased Na^+ uptake.[115] In intestinal epithelial cells, the expression of NHE2 and NHE3 is restricted to the apical surface, whereas NHE1 is expressed on the basolateral membranes. Our group has shown that EPEC infection in *in vitro* models activated NHE2 but inhibited the NHE3, the key Na^+ absorbing transporter (Fig. 4).[116] In *in vitro* models, EPEC infection leads to inhibition of

the $Cl^-/HCO_3^-/OH^-$/exchange activity critical for intestinal chloride absorption.[116] Also, as stated above the activity of NHE3, which is a major Na^+ absorbing isoform, is inhibited.[114] These findings may be a source of uncertainty in the relative pathophysiological importance of NHE2 vs. NHE3; NHE3 here could very well be the more important of these two as a regulator of Na^+ absorption and determinant of the onset of diarrhea.[114] Also, prolonged EPEC infection contributes to inflammation and disruption of the structure and barrier function integrity of tight junctions and could contribute to diarrhea.[117]

(b) Effect of EPEC Infection on Downregulated in Adenoma (DRA/SLC26A3)—Intestinal epithelial cells express an integral membrane Cl^-/HCO_3^- transporter, the downregulated in adenoma (DRA/SLC26A3).[115] EPEC suppresses the function and apical expression of DRA/ SLC26A3, and may thus contribute to the pathophysiology of diarrhea. Studies from our group demonstrated an increased endocytosis and decreased apical expression of DRA/SLC26A3 in EPEC-infected cells (Fig. 4).[118] Other studies suggest that reduced exocytosis may also play a role. The virulence factors EspG1 and EspG2 may alter DRA/SLC26A3 expression on epithelial cells via mechanisms involving microtubule disruption.[118]

(c) Effect of EPEC Infection on Absorption of Short-chain Fatty Acids (SCFAs)—Short-chain fatty acids play a significant role in sustaining colonocyte health and metabolism, integrity of epithelial lining, and in the maintenance of colonic fluid and electrolyte balance. Butyrate, a key SCFA, has been shown to play an important role in fluid balance by enhancing electroneutral NaCl absorption[119] and reducing Cl^- secretion.[120] Our group has shown that EPEC infection can significantly reduce butyrate uptake by intestinal epithelial cell lines (Fig. 4).[117] EPEC infection reduced the expression of monocarboxylate transporter 1 (MCT1), the primary SCFA transporter in gut epithelial cells. Butyrate also plays an anti-inflammatory role,[121] and decreased availability of butyrate has been noted both in acute and chronic inflammatory conditions.[122]

(d) Effect of EPEC Infection on Apical Sodium-dependent Bile Acid Transporter (ASBT)—Apical sodium-dependent bile acid transporter (ASBT) is a putative transporter responsible for stimulating the intestinal absorption of bile acids. Reduced ASBT expression/function has been implicated in the pathogenesis of diarrhea. Annaba et al.[123] have shown the negative impact of EPEC infection on ileal ASBT expression/function in various *in vitro* models.

(e) Effect of EPEC Infection on the Serotonin Transporter (SERT)—Serotonin transporter is a key regulator of the extracellular availability of serotonin (5-HT), and its function was inhibited in response to EPEC infection in intestinal epithelial cells. Serotonin transporter activity is reduced via activation of the Src-homology-2 (SH2) domain containing protein tyrosine phosphatase (PTPase). In the absence of SERT, 5-HT circulates in the extracellular *milieu* resulting in the activation/sensitization of its cognate receptors.[124] In this study, SHP2 is associated with SERT during EPEC infection due to dephosphorylation at tyrosine residues and thereby inhibiting its function and activity. High

luminal serotonin levels (due to inhibition of SERT) have been linked to fluid accumulation in the gut lumen.

(f) Effect of EPEC Infection on Sodium D-glucose Transporter (SGLT-1)—In addition to SERT and other transporters outlined above, EPEC has also been shown to inhibit the function of the sodium-D-glucose transporter (SGLT1), which is a major contributor of fluid uptake in the small intestine[112] and hence could contribute to diarrhea.

Effect of EPEC Infection on Tight Junctions

Enteropathogenic *E. coli*-mediated disruption of the gut epithelial barrier also contributes to the onset of diarrhea. Epithelial cells are normally bound together by a network of tight junctions. The membrane barrier is selectively permeable for the passage of ions and solutes across the paracellular space. It also serves as a boundary that prevents the coalescence of apical and basal plasma membrane proteins to maintain the polarity of the epithelial cells and prevents the backflow of fluids into the lumen.[115] The cell–cell adhesion is maintained by the transmembrane proteins which are associated with the cytoskeleton via the adaptor proteins. The members of claudin family and the transmembrane proteins of the marvel-domain containing protein families, such as occludin, tricellulin/marvelD2, and marvelD3, are key regulators of paracellular permeability. Tight junction-associated exchange factors for Rho GTPases also modulate the actin cytoskeleton and membrane permeability.[115,125–127] During EPEC-induced diarrhea, leakages are observed in the tight junctions; studies suggest the potential role(s) of effector proteins EspF, Map, EspG1/G2, and NleA in disrupting the host cell tight junctions.[128–130] The *N*-terminus of EspF contains mitochondrial- and nucleolus-targeting sequences that can alter the function of these organelles. The *C*-terminus of EspF contains three proline-rich repeats that interact with the eukaryotic sorting nexin 9 (SNX9) and neuronal Wiskott–Aldrich syndrome protein (N-WASP), and are ultimately involved in the activation of the Arp2/3 complex and regulation of actin polymerization.[128–132] EspF may recruit zonula occludens (ZO-1 and ZO-2) into actin pedestals.[133] In murine models, EspF can disrupt tight junctions via internalization of claudin-1, 3, and 5.[115]

Another EPEC effector protein, Map, interacts with EspF and is involved in the disruption of tight junctions. Similar to EspF, Map is recruited to mitochondria where it modulates the mitochondrial processes and functions. Map acts as a guanine-nucleotide exchange factor (GEF) for Cdc42 GTPase and promotes its activation leading to the formation of transient filopodia. A Thr-Arg-Leu motif is present at the C-terminus of Map, which interacts with the Na^+/H^+ exchanger regulatory factor 1 (NHERF1). This complex links with ezrin and then promotes the interaction between Map and actin cytoskeleton.[128,134,135] The tight junction proteins, the zonula occludens-1 and occludin, are disrupted by NleA leading to increased paracellular permeability.[130]

conclusions

Studies show aEPEC to be more prevalent than tEPEC worldwide. Therefore, it is important to further characterize the pathogenicity of these strains, virulence mechanisms, and the pathophysiology of these infections. While there is strong evidence showing that EPEC-

induced diarrhea is multifactorial in nature and involves compromised gut barrier integrity and decreased absorption of fluid, which is contributed by decreased NaCl and solute absorption. However, the exact mechanisms of diarrhea in EPEC infection are still evolving. From the clinical perspective, there is a need for fast, easy, and inexpensive diagnostic methods to define optimal treatment and prevention for children in endemic areas.

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Key Points

- Enteropathogenic *Escherichia coli* (EPEC) is a leading cause of infantile diarrhea worldwide and particularly in developing countries.
- Global prevalence of atypical EPEC (aEPEC) is higher than typical EPEC (tEPEC).
- Enteropathogenic *E. coli* strains adhere to intestinal epithelial cells (IECs) in two patterns; the first one is of localized adherence (LA), where bacteria adhere in discrete microcolonies, and the second one is of diffuse adherence in which bacteria adhere uniformly over the cell surface.
- Enteropathogenic *E. coli* employs its type III secretion system and effector proteins to modulate cellular and barrier properties of the host intestinal milieu.
- Enteropathogenic *E. coli* infection leads to extensive disruption of microvilli on IECs and consequent loss of absorptive surfaces and altered electrolyte transport that may be secondary to both altered expression of ion/solute transporters and the loss of mucosal surface area.

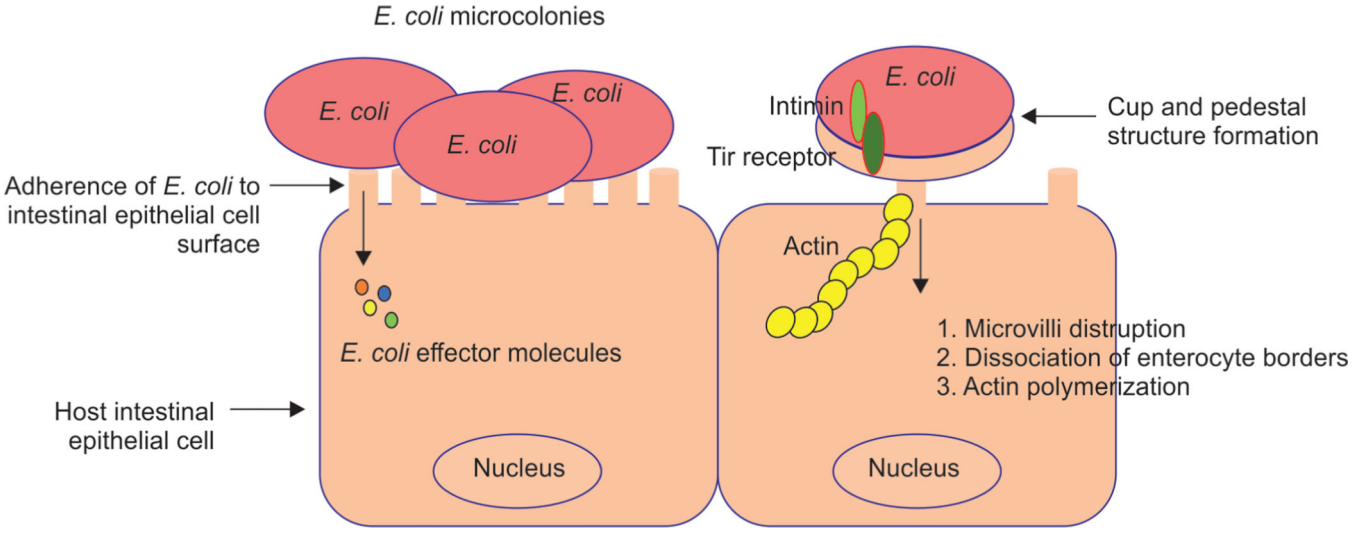


Fig. 1:

Attachment of EPEC to the host epithelial cells results in the formation of cup and pedestal structures in the A/E lesion. A/E lesions exhibit intimate bacterial adherence to intestinal epithelial cells, extensive disruption of microvilli and enterocyte borders, and alterations in F-actin arrangement with accumulation of cytoskeletal proteins beneath adherent microcolonies resulting in the formation of a typical cup and pedestal-like structure

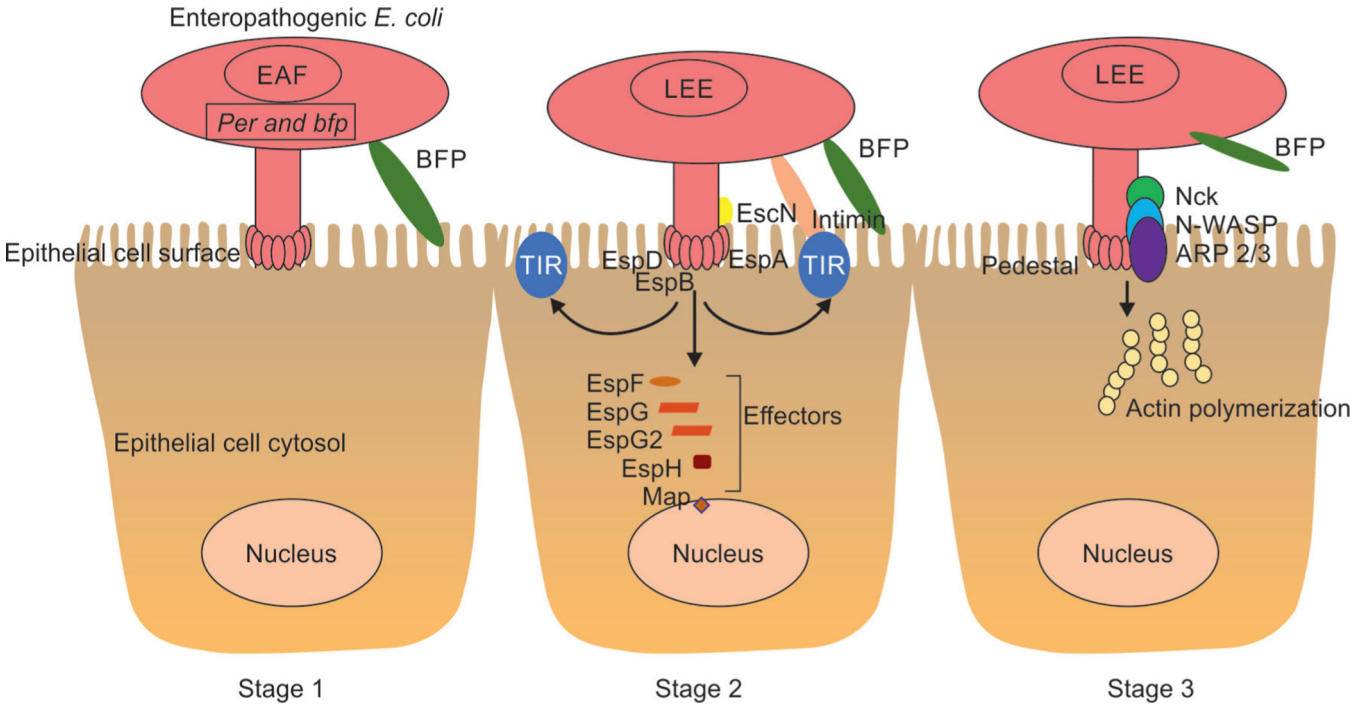


Fig. 2:

Schematic representation of localization of virulence factors type IV BFP and LEE of EPEC on the small intestine and other interacting proteins involved during A/E lesion formation: Stage 1: Initial adherence and microcolony formation of EPEC on intestinal epithelial cells induced via type IV BFP and its activator Per. Stage 2: Effacement of microvilli mediated by activation of LEE operons via EspABD complex and translocation of T3SS effector proteins into intestinal epithelial cells. EPEC utilizes a type III secretion system (T3SS) to inject bacterial virulence factors directly into host cells. The T3SS apparatus is composed of several key protein components, including EspA, EspB, and EspD. EspA forms a needle-like channel and EspB and EspD cap this structure to form a pore that allows direct translocation of secreted effector molecules known as *E. coli* secreted proteins, EspF, EspG, EspH, Tir, and Map into the host cytosol. Translocated intimin receptor (Tir) is inserted into the plasma membrane, where it serves as a receptor for intimin, with Tir-intimin interaction triggering signaling events leading to pedestal formation. Stage 3: Intimate attachment of EPEC on the surface of host epithelial cells mediated by the interaction of adhesin intimin with Tir. This is followed by phosphorylation of Tir and recruitment of host cellular proteins and other adaptor proteins (Nck, N-WASP, and Arp2/3 complex) resulting in induction of actin polymerization beneath attached EPEC (BFP, bundle forming pilus; LEE, locus of enterocyte effacement; Tir, translocated receptor; EPEC, Enteropathogenic *Escherichia coli*; EAF, EPEC adherence factor; PER, plasmid-encoded regulator; A/E, attaching and effacing; Nck, Non-catalytic tyrosine kinase; WASP, Wiskott–Aldrich syndrome protein and Arp2/3, actin-related protein 2/3 complex)

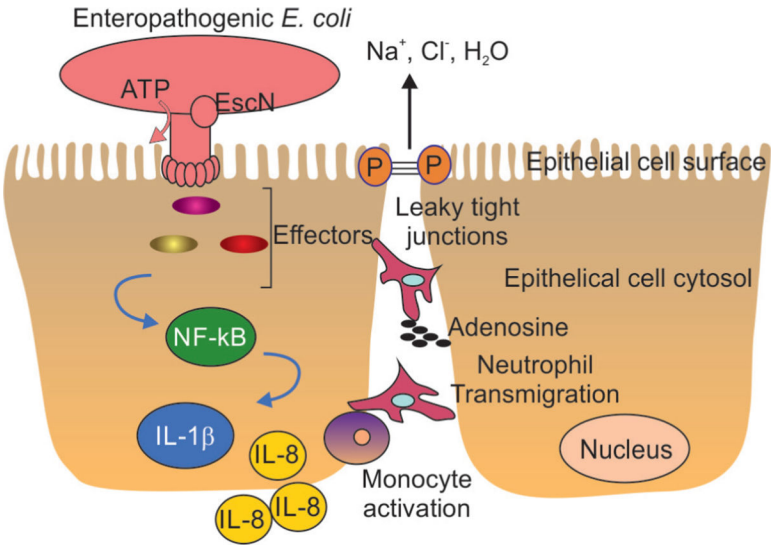


Fig. 3:

EPEC infection induces inflammation and disrupts the epithelial barrier resulting in leaky tight junctions. Bacterial overgrowth, cytokine expression, biofilms, and leukocyte infiltration all create positive-feedback loops of inflammatory changes. Cytokines such as interleukin (IL)-1β and chemokines such as IL-8 activate regulatory factors such as the nuclear factor-κB and progressively enhance the inflammatory changes and dysfunction of the epithelial barrier

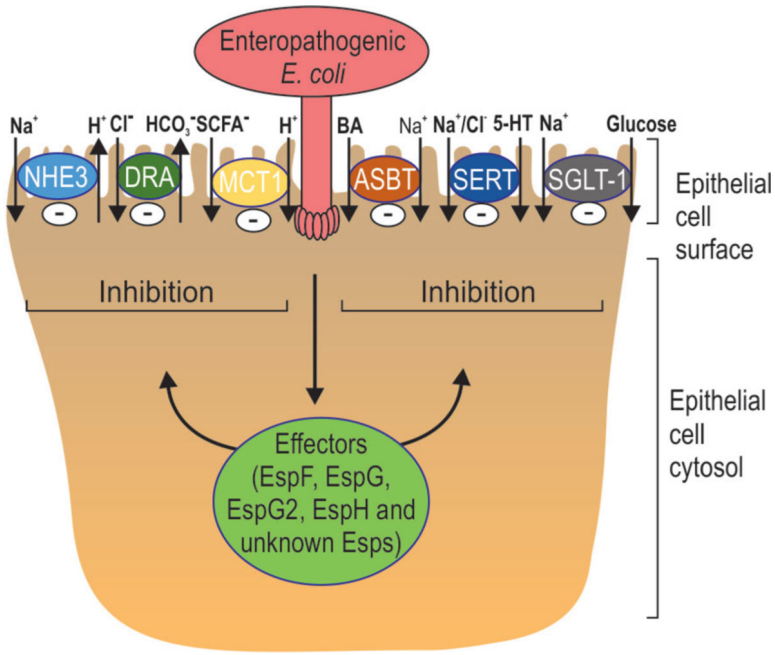
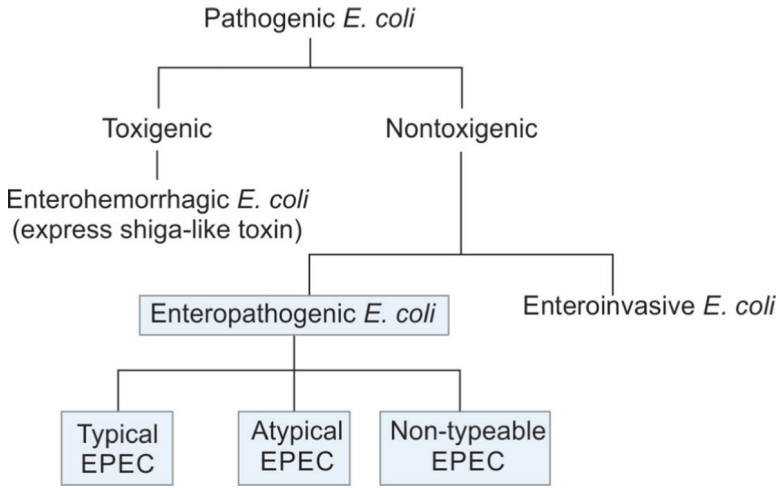


Fig. 4:

Schematic representation of transporters affected during EPEC infection. EPEC infection affects intestinal epithelial barrier and leads to reduced expression/function of ion and solute transporters and results in the development of diarrhea. Type III secretion system of EPEC is responsible for the release of *E. coli*-secreted proteins (Esps) into the infected host cells. EspF exhibits inhibitory impact on Na^+/H^+ exchange isoform 3 and EspG disrupts the microtubules, which further leads to decreased apical expression of DRA resulting in reduction of apical Cl^-/ OH^- (HCO_3^-) exchange activity and inhibition of electroneutral NaCl absorption in the intestinal milieu. EPEC inhibits butyrate absorption by reducing the plasma membrane expression of monocarboxylate transporter 1 (MCT-1). EPEC also inhibits the function of serotonin transporter (SERT) and increases 5-HT availability by activating protein tyrosine phosphatases (PTPases), which can further modulate the ion absorption and contribute to the onset of diarrhea. EPEC-induced inhibition of SGLT-1 also promotes fluid accumulation with similar effects. (NHE3, Na^+/H^+ exchanger type 3; DRA, downregulated in adenoma; MCT-1, Monocarboxylate transporter 1; ASBT, Apical sodium-dependent bile acid transporter; BA, Bile acid; SERT, Serotonin transporter; SGLT-1, D-glucose transporter)



Flowchart 1:
Categorization of pathogenic *E. coli*