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The Biology and the Evolutionary Dynamics of Diarrheagenic *Escherichia coli* Pathotypes

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Abstract

Escherichia coli is a commensal of the gastrointestinal tract of humans and animals, and a leading cause of gastroenteritis, bloodstream, and urinary tract infection, among others. Pathogenic *E. coli* causing diarrhea is delineated into six different types (pathotypes) based on the type of infection they cause. While these pathotypes have similar mechanisms to colonize the intestinal epithelial layers and cause diseases, they differ in their capacity to acquire virulence, resistance determinants, and other accessory genes essential for niche adaptation. The advent of whole-genome sequencing technologies has greatly enhanced our understanding of the physiology, emergence, and global spread of multidrug-resistant and pathogenic clones of *E. coli*. In this chapter, we provided a snapshot of the resistome and virulome, as well as their contributions to the ecological adaptation, evolution, and dissemination of *E. coli* pathotypes.

Keywords: *E. coli*, pathotypes, antimicrobial resistance, genetic lineages, pathogenicity, genomics

1. Introduction

Escherichia coli inhabits and adapts to different hosts, a quest that resulted in the acquisition and loss of genes, which further drive diversity in this bacterium and contribute to the evolution of harmless strains to pathogenic lifestyles [1]. While *E. coli* is an integral part of the microbiota of different hosts, it can also cause severe infections in humans and animals [2, 3]. A subgroup of *E. coli* that are pathogenic can cause a broad range of human diseases due to evolution that resulted in the development of patho-features enabling it to adapt and survive in different environments. These environments range from the gastrointestinal tract to extraintestinal sites such as the urinary tract, or meninges, [4] in addition to fecal contamination of food that could cause enteric infection resulting from food poisoning or contamination [5]. Based on the type of infection they cause, pathogenic *E. coli* are divided into intestinal or diarrheagenic *E. coli* (DEC) that cause diarrheal illness and extraintestinal *E. coli* (ExPEC) that are implicated in infections such as urinary tract infections [3]. Diarrheal illness constitutes a public health burden and

is a leading cause of mortality worldwide, causing >300 million illnesses and about 200,000 deaths annually, particularly in children in developing countries, including sub-Saharan and Southeast Asian countries (**Figure 1**) [6, 7].

The treatment of *E. coli* associated illness is toppled by its growing resistance to antibiotics, culminated by either the acquisition of resistance determinants or mutations that encodes for low uptake and tolerance to a higher concentration of the antimicrobials. Hence, *E. coli* could serve as a major reservoir of resistance genes not only for other *E. coli* strains but also for Enterobacteriaceae [8]. In addition, virulence determinants and genes that are associated with stringent response in nutrient low environments could also be acquired, thereby contributing to the survival and persistence of this bacterium in its environment [9]. Transmission of these antibiotic-resistant or pathogenic *E. coli* strains between different hosts, particularly in animals and humans could be through several routes such as direct contact with fecal-contaminated samples or other secretions from animals, or via the consumption of contaminated food [5].

Assessing the antimicrobial resistance, virulence, and transmission dynamics of *E. coli* requires characterization of this bacterium. A widely accepted classic method for characterizing *E. coli* is the serotyping technique that is based on the Kauffman classification scheme, where the O (somatic) polysaccharides and H (flagellar) surface antigens are determined [10, 11]. Other methods of typing and assessing the genetic relatedness and detecting outbreaks of *E. coli* strains are pulsed-field gel electrophoresis (PFGE) [12], multilocus enzyme electrophoresis (MLEE) [13], multilocus variable-number tandem repeat analysis (MVLA) [14], or multilocus sequence typing (MLST) [15]. These methods have proven to be effective in the epidemiological investigation of pathogenic *E. coli* [16] and the assessment of the emergence and dissemination of multidrug-resistant clones. However, none of these methods can accurately define the evolutionary relationships between *E. coli* strains, hence the need for a tool with a higher resolution. The advent of whole-genome sequencing (WGS) technologies has greatly enhanced not only the epidemiological investigation of outbreaks and the global spread of multidrug resistant and pathogenic clones of *E. coli* [17], but also our understanding of the physiology and evolutionary history of how some pathogenic strains evolve from commensal *E.coli* strains.

Figure 1. Global mortality rate from diarrhea in children under 5 years in 2016. Data represent the analysis of diarrhea burden in 195 countries in 1990–2016, showing the regions most affected by the illness. Reprinted from Troeger et al. [6] which was published under Creative Commons License.

Based on clinical manifestation, presence of specific virulence determinants and phylogenetic profiles, diarrheagenic *E. coli* are categorized into six main pathotypes namely, enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), entero-invasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) [3, 7]. This chapter provides a snapshot of the biology of *E. coli* by focusing on the resistome, virulome, as well as the population structure of diarrheagenic *E. coli* pathotypes.

2. Enterotoxigenic *E. coli* (ETEC)

2.1 An overview of ETEC

Enterotoxigenic *E. coli* (ETEC) is a major cause of travelers' diarrhea, with a high prevalence in developing countries and responsible for about 200 million cases of diarrheal illnesses and 100,000 deaths each year [18, 19]. The incidence of ETEC could be recovered from symptomatic and asymptomatic carriers and is most common in younger children with a high mortality rate in this group. In regions such as Africa, South America, and Southeast Asia the incidence of ETEC-related infection is estimated to be at least one to two episodes per year [3] further reinforcing the significance of this pathotype in that region. While ETEC is not limited to humans, it is also a common cause of edema and post-weaning diarrheal diseases in food production animals such as cattle, pigs, and sheep [20]. ETEC are transmitted through the fecal-oral route by contaminated food as well as surface and groundwater in developing countries with limited access to clean water (Figure 2) [3, 6].

Figure 2. Dissemination and transmission routes of pathogenic *Escherichia coli* pathotypes. Solid black arrows represent a direct transmission while gray arrows depict an indirect transmission of pathogenic *E. coli* pathotypes. ETEC: Enterotoxigenic *E. coli*; STEC: Shiga toxin-producing *E. coli*; EPEC: Enteropathogenic *E. coli*; EAEC: Enteroaggregative *E. coli*; EIEC: Entero-invasive *E. coli*; DAEC: Diffusely adherent *E. coli*.

There is a great genetic diversity in ETEC as more than 100 different O antigens have been reported to be associated with clinical isolates. Among these, the O6 serogroup is most common, and geographically diverse among all ETEC serogroups, and has been implicated in multiple outbreaks in different countries [21]. Additionally, at least 34 H antigens are also associated with this pathotype. Among the serotypes as determined by the combination of O and H antigens, O6:H16 (heat-stable (ST) or heat-labile (LT) toxin), O148:H28 (ST), O167:H5, O153:H45 (ST), O169: H41 (ST only) are frequently isolated from humans, animals, environmental matrices, and from outbreaks in developing countries [22–24]. ETEC produces one or more colonization factors that facilitate its attachment to specific receptors on the mucosal layer of the small intestine of humans and animals, and secretes enterotoxins that cause electrolyte imbalance in the intestinal lumen resulting in dehydration, metabolic acidosis, and diarrheal [3, 18]. The ST toxin is a nonimmunogenic small protein molecule, but the LT toxin is structurally homologous and exhibits a similar mechanism of action to cholera toxin produced by *Vibrio cholerae* [23, 25].

2.2 Virulome of ETEC

ETEC employs an array of genetic factors that are either chromosomal or plasmid-borne that mediate colonization and adherence to the intestinal epithelium, proliferation within the host, and evasion of host defense mechanisms (**Table 1**) [26].

2.2.1 Colonization and adhesion

Colonization and adhesion are the primary and essential steps in the pathogenesis of pathogens. ETEC is not an exception as the colonization of the host intestinal epithelium by this pathotype is mediated by plasmid-borne genes that encode adhesins and one or more colonization factors (CFs) namely, pilus or pilus-related adhesins [23]. Pili are hair-like appendages on the cell surface of bacteria where they mediate the attachment of bacteria to surfaces.

They are composed of protein subunits (pilins) that are structurally polymeric and are almost exclusively plasmid-borne (**Figure 3**) [27]. ETEC CFs are designated as CS (coli surface antigens) followed by a number, except for CFA/I and PCFO71 [27]. Presently, at least 30 CFs have been reported in ETEC of human origin. It is estimated that about 50% of strains in this pathotype carry one or more CFs that are not detectable, suggesting that there could be more CFs that are yet to be discovered and characterized [18, 23]. The co-expression of one or more CFs with toxin-encoding genes has been described. For example, CFA/I + LT, CS7 with LT, CS5 + CS6 with LT + ST, CS2 + CS3 with LT + STh, among others [28, 29]. In the prototypical ETEC strain H10407, the production of CFA/I is mediated by *cfaABCE* operon that is tightly regulated by CfaD, a transcriptional regulator that triggers its expression.

Other plasmid-encoded genetic factors that have been reported to play a significant role in the pathogenesis of ETEC include a class I SPATE (serine protease autotransporters of the Enterobacteriaceae) EatA that digests EtpA secreted by ETEC, thereby promoting the adhesion of flagella to the host receptor [30, 31]. ETEC can invade the host cell with two chromosomally encoded genes *tia* and *tibA*. The former (*tia* gene) is borne on a 46-kb pathogenicity island (PAI). The expression of these genes was reported to be associated with adhesion and invasion of ETEC in host cells [27]. Likewise, a *leoA* gene encoding GTPase is reported to be associated with virulence in ETEC (**Table 1**) [32].

<i>E. coli</i> pathotypes	Main reservoir	Clinical presentations	Virulence factors
ETEC	Humans and animals	Watery noninflammatory diarrhea, adherence to small intestinal epithelium, nutrient malabsorption	Colonization factors (CFs), EatA, Tia, TibA, LeoA, ST, LT, EAST1
STEC	Animals	Watery bloody diarrhea, hemorrhagic colitis, Hemorrhagic Uremic syndrome, attaching-effacing lesions in the large intestine	LEE, Saa, Paa, EhaA, LpfA, OI-7, ST, EhxA, ToxB, EspP, KatP
EPEC	Humans and animals	Shigella-like toxin, watery and/or bloody diarrhea, noninflammatory diarrhea, attaching-effacing lesions in the colon, nutrient malabsorption	LEE, pEAF, Bfp, OI-22, EAST1
EAEC	Humans and animals	Clump intestinal cells; mucoid watery diarrhea with persistent inflammation, nutrient malabsorption, postinfectious irritable bowel syndrome	AA, Afp, CapU, Air, Shf, AatA, Pic, EAST1, ShET1, Pet, SigA, SepA, pAA, HlyE
EIEC	Humans and animals	Bacillary dysentery, watery diarrhea with or without blood and leukocytes, inflammation of the large intestinal epithelium	pINV, SepA, SigA, Sat, ShET2
DAEC	Humans	Watery diarrhea, persistent diarrhea, chronic inflammatory colon disease	Afa/Dr. Adhesins, Sat, Pet, SenB, HlyE

Legend: ETEC: Enterotoxigenic E. coli; STEC: Shiga toxin-producing E. coli; EPEC: Enteropathogenic E. coli; EAEC: Enteroaggregative E. coli; EIEC: Entero-invasive E. coli; DAEC: Diffusely adherent E. coli.

Table 1.
Summary of the clinical characteristics and virulence factors of Escherichia coli pathotypes.

Figure 3.
Colonization and adherence patterns of diarrheagenic Escherichia coli to the host epithelium. Enterotoxigenic E. coli (ETEC) uses colonization factors (CFs) to attach to host intestinal mucosa. Shiga toxin-producing E. coli (STEC) and Enteropathogenic E. coli (EPEC) attach to the intestinal epithelial cells and efface microvilli, forming characteristic A/E lesions. EPEC also forms microcolonies using bundle-forming pili (Bfp) resulting in a localized adherence pattern. Enteroaggregative E. coli (EAEC) forms a biofilm matrix on the intestinal mucosa that promotes the formation of a “stacked brick” adherence pattern. Enteroinvasive E. coli (EIEC)/Shigella are intracellular pathogens that penetrate the intestinal epithelium through M cells to gain access to the submucosa. Diffusely adherent E. coli (DAEC) is scattered over the surfaces of intestinal cells, resulting in a diffuse adherence pattern.

2.2.2 Enterotoxin secretion

One of the salient features that define ETEC is its ability to produce two types of enterotoxins, ST or LT [23]. STs are non-antigenic small enterotoxins that are frequent in human diseases, found in about 80% of ETEC either singly or in

combination with LT [18, 33]. STs are classified into two different classes (STa and STb) based on their structure and function. STa is soluble in methanol and protease-resistant. It is frequent in human diseases and encoded by *estA* genes, whereas STb is insoluble in methanol and sensitive to protease, and causes disease only in animals and is encoded by *estB* gene [34]. Based on host specificity, STa is further designated into two genetic variants namely STp and STh. The former (STp) is 18 amino acids in length and produced by ETEC strains of porcine, bovine, and human origin, while STh is 19 amino acids long and exclusive in ETEC strains of human origin [29]. Recently, six genetic variants of STa encoding gene (*estA*) have been reported, where *estA1*, *estA5*, and *estA6* are common in ETEC strains of porcine origin and *estA2*, *estA3/4* and *estA7* are frequent in isolates of human origin (STh), while *estA5* gene is described to be frequent in ETEC strains causing disease both in animals and humans, especially traveler's diarrhea in adults [29, 34]. Secretion of STh and STp in the intestinal epithelium of the host requires the efflux protein TolC [35].

Unlike, STs, LTs are hexameric and strongly immunogenic that are encoded by the *eltAB* operon [29]. LTs have two subtypes: LT-I and LT-II, both of which have been reported in ETEC strains causing diarrhea in humans and in different species of post-weaned animals. LT-Is are plasmid-borne and highly similar to cholera toxin produced by *V. cholera* [3, 36]. Conversely, LT-II is chromosomal and has been hypothesized to be prophage encoded [3, 36]. LT-II is classified into LT-IIa, LT-II, and LT-IIc, with LT-IIc being the more frequent in LT-II ETEC strains [36].

LTs promote the adherence of ETEC to host intestinal epithelial cells and evade the host defense mechanisms by inhibiting the expression of antimicrobial peptides produced by the hosts, in addition to the activation of host signaling pathways [3]. Another virulence factor encoding enterotoxin in ETEC strains is enteroaggregative heat-stable toxin (EAST1). EAST1 toxin is heat-stable and 38 amino acids long encoded by *astA* gene that is commonly plasmid-borne [37]. ETEC strains producing EAST1 toxin have been recovered from humans and animals. This toxin was reported to have originated from EAEC but it is prevalent in ETEC [38, 39]. While the role of EAST1 toxin in enteric infection is not clear, there has been evidence and direct associational studies linking this toxin to diarrheal illness [38]. EAST1 toxin is functionally and structurally similar to STa, sharing 50% identity in their functional regions [38]. Overall, enterotoxins secreted by ETEC strains have a similar mechanism of causing diarrheal diseases in the host. ETEC enterotoxins increase cyclic AMP or cyclic GMP levels in the intestinal epithelium of the host. This results in excessive secretion of chloride and reduction in the adsorption of sodium chloride in the intestinal epithelium thereby resulting in electrolyte imbalance, fluid loss and dehydration [29, 40].

2.3 Antibiotic resistance in ETEC

Since the first isolation of ETEC in Kolkata about five decades ago [41], the emergence and increase in multidrug-resistant strains have been reported. A homogenous and high antibiotic susceptibility pattern was observed for ETEC strains at a time but the treatment of travelers' diarrhea with different classes of antimicrobials such as macrolides (erythromycin and azithromycin), fluoroquinolones (norfloxacin, ofloxacin, ciprofloxacin), tetracycline (doxycycline), rifamycin and sulfamethoxazole-trimethoprim that are used to treat other types of infections [22] may have also contributed to the emergence of antimicrobial resistance in this pathotype [22]. Another contributor could be the indiscriminate use of antibiotics for the treatment of diarrheas caused by viral agents that are sometimes misdiagnosed because they present similar symptoms [3].

There are several studies from different countries assessing the antibiotic resistance profile and distribution of resistance determinants in ETEC. In a study, the antimicrobial resistance profile among patients with recent travel history to ETEC endemic regions between 2001 and 2004 reported that up to 60% of the ETEC isolates were resistant to sulfamethoxazole-trimethoprim, tetracycline, and/or ampicillin [42]. Ciprofloxacin resistance was reported to markedly increase from 1% to 8% within 10 years (1994–2004) in patients [42] which clearly suggests a rapid emergence of resistance with time in this pathotype. In a recent study on the WGS analyses of eight strains representing the major ETEC lineages that are causing diarrheal diseases in humans around the globe, all the strains showed resistance and carried resistance determinants to at least two of the 14 antibiotics tested, with resistance to penicillin, norfloxacin and chloramphenicol being the most common. In this study, two plasmids designated (pAvM_E1441_17 and pAvM_E2980_15) carried resistance determinants to mercury (*mer* operon) and multiple antibiotics including streptomycin (*aadA1-like*, *strA*, and *strB*) and ampicillin (*bla*TEM-1b, *ampC*) [43].

ETEC in animals, however, may be slightly different. In a study of 112 ETEC isolates recovered from pigs in Canada over a two-decade period (1978–2000), *tetB* gene that encodes resistance to tetracycline was the most common and found in 80% of the collection [44]. Another interesting observation from this study was the increase in the determinants encoding resistance to gentamicin (*aac(3)-IV*), kanamycin (*aph(3')-Ia*) and trimethoprim (*dhfrV*), while others appear to be either consistent or decrease over time [44].

2.4 Population structure of ETEC

ETEC strains are epidemiologically and phenotypically diverse and exhibit high genetic diversity. In addition to being polyphyletic, the distribution of ETEC lineages is not restricted by geography [45]. Several reports on the phylogenetic analyses of strains from the human origin using MLEE and MLST, and well as CF-toxin-based phylogeny showed that this pathotype might have evolved multiple times through clonal expansion and probably due to lack of common clonal lineage [46, 47]. In spite of the genomic diversity among strains in this pathotype, Turner and colleagues [48] reported ETEC to be associated with sequence type 10 (ST10). In a broader evolutionary study of a large collection of 1019 ETEC isolates from humans in 13 countries using MLST, 42 clonal groups were observed with evidence for horizontal gene exchange of plasmid-encoded CF genes between the lineages [46]. Since the advent of next-generation sequencing technologies, the study of the population structure of ETEC has improved the understanding of the genetic diversity and evolution of the pathotype [24, 49, 50].

A global collection of ETEC isolates from humans collected over a period of three decades (1980–2011) in 20 countries and representing four continents was assessed for genetic relatedness using WGS-based single nucleotide polymorphism (SNP) [49]. Indeed, ETEC strains are genetically diverse as they were reported to be distributed across different *E. coli* phylogenetic groups (A, B1, B2, D, and E) (**Figure 4**), an observation that is also in accordance with the structure defined by MLST [46, 48, 51]. An interesting finding from the study that could be attributed to the higher resolution of WGS was the identification of ETEC-specific clusters (L1-L14) that clustered geographically diverse strains that were phylogenetic related and associated with specific plasmid-encoded virulence determinants. The L1 and L2 clustered the commonly found ETEC strains expressing O6 antigen and carried similar profiles for CF and LT and ST enterotoxins, suggesting that these plasmid-encoded virulence determinants could be important to understand the evolutionary histories of these clusters [24, 49].

Figure 4. Single nucleotide polymorphism based Phylogenetic tree of Escherichia coli. Draft genomes of *E. coli* pathotypes were downloaded from NCBI and core genomes were defined and aligned against *E. coli* K12. SNPs from the core genome alignment were called using Snippy (<https://github.com/tseemann/snippy>). Concatenated SNPs alignment cured of recombination (<https://github.com/sanger-pathogens/gubbins>) was used to construct a phylogenetic tree and visualized using iTOL (<http://itol.embl.de/>). Different colors and shapes depict *E. coli* phylogroups and pathotypes, respectively.

Similarly, in a local study on the phylogenomic diversity of 94 ETEC isolates from Bangladesh [24], a polyphyletic scenario and a direct correlation between lineages and virulence profiles and CFs were noted. Using comparative genomic tools, the authors identified six novel CF variants. However, the experimental validation of these CFs would be important to decipher their association with other virulence determinants as well as their interaction with the host cells.

3. Shiga toxin-producing *E. coli* (STEC)

3.1 An overview of STEC

Shiga toxin-producing *E. coli* (STEC), also known as verocytotoxin-producing *E. coli* (VTEC) was first identified as a pathogen in 1982 and is defined by the presence of Shiga toxin genes (*stx1* and *stx2*) that are known to be encoded on lambdoid prophages [52]. STEC had since emerged as a major enteric foodborne and zoonotic pathogen causing gastroenteritis and enterocolitis. STEC-related infection could progress to hemolytic-uremic syndrome (HUS) that in some cases could be fatal or result in renal failure in children (**Table 1**) [3]. Enterohemorrhagic *E. coli* (EHEC) is associated with hemorrhagic colitis which makes it a subset of STEC pathotype [3, 53]. STEC causes more than one million cases annually with about 30% of such

cases found in the United States [54]. According to Blanco et al. [55], 435 STEC serotypes strains have been identified but only a few are frequently associated with human infection among which are O26, O45, O103, O111, O121, O145, and O157 [56, 57]. STEC O157:H7 has been widely studied most likely because it is frequently implicated in STEC foodborne outbreaks. Although a series of outbreaks that are mostly caused by STEC O157:H7 have been reported globally in developed and developing countries, some are believed to go undetected or underreported [54, 58].

The main reservoir for STEC strains causing infection in humans are known to be ruminant food production animals including cattle, sheep, and goats. These animals are asymptomatic carriers and shedders as the vascular receptor that facilitate the transportation of the Shiga toxins to organs are absent. This *E. coli* pathotype is also common in the gastrointestinal tracts of poultry, pigs as well as some companion animals such as dogs and cats [59]. Other STEC asymptomatic carriers have been reported in other ruminant and monogastric animals, as well as in insects, suggesting their roles in the food contamination, dissemination, and transmission of different strains of this pathotype to humans (**Figure 2**) [59, 60]. Since STEC is part of the intestinal flora of food-production animals and is readily shed through feces, direct contact with contaminated environmental matrices and/or the consumption of contaminated products including undercooked meat, unpasteurized dairy products, vegetables, and/or water are a potential route of transmission of different strains of this pathotype to humans [61, 62]. STEC transmission due to direct contact with infected person or animals or their environments or products has been documented [63].

3.2 Virulome of STEC

STEC carries genes encoding adhesins and enterotoxins that are either chromosomal, on PAIs, or plasmid-borne. These determinants mediate colonization, attachments, and invasion of host cells (**Table 1**) [64].

3.2.1 Colonization and adherence

Ingestion of contaminated food or direct contact with contaminated environmental matrices or infected persons or animals precedes STEC-related diseases. Colonization and attachment/adherence of STEC to intestinal epithelium is mediated by several genetic factors some of which are carried by the locus of enterocyte effacement (LEE) PAI [65]. LEE locus which is also often present in EPEC strains [66] encodes a type III secretion system (T3SS) that plays a role in the secretion and translocation of virulence-associated genetic factors into host cells [67]. One of these genetic factors is *eae* gene that encodes intimin, an adhesin that is essential for the attachment of STEC to the host intestinal mucous membrane and facilitates the production of attaching-and-effacing (A/E) lesion (**Figure 3**) [65]. The injection of its translocated intimin receptor (Tir) into the host cells and the interaction between this protein (Tir), proteins that form the needle component of the T3SS (EspADB), and intimin facilitates the induction of lesions [65, 68]. Intimin has at least 30 reported subtypes [69] and some of these variants are associated with specific serotypes. Oftentimes, intimin encoding gene (*eae*) subtype $\gamma 1$ is associated with STEC O157:H7 and O145:H28 [69, 70], while subtypes $\beta 1$, ϵ , and θ are frequently found in O26:H11, O103:H2 and O111:H8 STEC strains, respectively [69, 70]. Intimin is reported to be common in clinical strains of STEC with a prevalence that could range from 70 to 90% [71–73].

LEE is unarguably important for the pathogenesis of STEC strains, but STEC LEE-negative strains/serotypes (e.g. O103:H21) have been implicated in infections [74].

This implies that there are several other virulence-associated factors that are carried on PAIs or mobile genetic elements mediating colonization and adherence to host cells in these strains [74]. For example, a gene (*saa*) encoding autoagglutinating adhesin was isolated from a large plasmid of a LEE-negative STEC O113:H21 implicated in an outbreak. The expression of this gene is described to enhance adherence of STEC strains to HEp-2 cells [75]. Other protein-encoding genes reported to promote the colonization and adherence of STEC strains to mucosal membrane include *paa* that encodes attachment of bacterial cells to enterocytes in pigs [76], *ehaA* a STEC auto-transporter [77], *lpfA*, long polar fimbriae [78] that are both involved in the attachment of STEC to surfaces. Additionally, “O” islands (OI) that encode macrophage toxin and ClpB-like chaperone (OI-7), urease clusters (OI-43 and OI-48), two toxins and PagC-like virulence factor (OI-122) among others have been reported to be linked with virulence in STEC [57, 79].

3.2.2 Cytotoxic toxin production

STEC-related infections in humans are reported to be associated with the presence and expression of several virulence determinants, with the phage-encoded Shiga toxin genes *Stx1* (*Stx1a*) and *Stx2* (*Stx2a*) being the main virulence factors [64, 80]. *Stx1* consists of 293 amino acids while the *Stx2a* is longer by only four amino acids. At least, 16 subtypes of these two toxins have been described based on amino acid differences and the level of cytotoxicity. *Stx1* contains four variants encoded by *stx1a*, *stx1c*, *stx1d* and *stx1e*, whereas *Stx2* comprised 12 subtypes encoded by *stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, *stx2g*, *stx2h*, *stx2i*, *stx2j*, *stx2k* and *stx2l* [80]. While *Stx1a* has been implicated in human infection, *Stx2a*, *Stx2c*, and *Stx2d* are the major subtypes that are frequently associated with hemorrhagic colitis and HUS [64]. However, *Stx2a* and *Stx2d* subtypes are described to exhibit higher cytotoxicity relative to *Stx2b* and *Stx2c* in a mouse model [64, 81]. The interaction of Stxs with the host cell receptor is very complex and is based on characteristics of the environment of the receptor in the plasma membrane [64]. Stxs bind to the globotriaosylceramide Gb3, an insoluble molecule that has multiple binding sites and comprised a lipid component. The interaction between these two molecules (Stx and Gb3) is described to be important in the uptake of the toxin (**Figure 3**). Stxs are ribotoxins that disrupt protein synthesis within the host cell and provoke apoptosis [64, 81].

STEC strains can carry *stx1* or *stx2* genes or both [2]. In a study of 351 STEC strains from bovine feces, the great majority of the strains (82%) carried *stx2* while 18% carried *stx1*. Both genes were found only in ~3% of the collection [2]. In another study involving 220 STEC strains from humans and animals, *stx1* and *stx2* were found in 15% and 53%, respectively, while both genes were found in 32% of the isolates [82]. Stxs subtypes are heterogeneously distributed in the population, but specific variants have been reported to be host-specific. For example, *Stx2e* is less cytotoxic and sporadic in human diseases, and is commonly associated with edema diseases in weaned pigs [83]. Likewise, *Stx1c* is reported to be associated with STEC of ovine origin [84]. Indeed, the severity of STEC infection has been noted to be directly proportional to a number of Stx types or subtypes carried by the infecting strains [85]. While the production of only Stxs has been described to cause HUS, the infection is however exacerbated when associated with other virulence determinants including the LEE [21]. In addition to Stx, other toxins or hemolysins have been reported to be associated with STEC virulence. These include the hemolysin, encoded by the *ehxA* or *hlyA* gene, that are usually found on megaplasmid pO113 and/or pO157 and linked to cytotoxic effects on endothelial cells that may also promote the development of HUS [86, 87]. Other virulence

determinants carried on these plasmids include *toxB* that is essential for adherence of STEC to host cells [88], *espP* that encodes an extracellular protease and *katP* that is associated with catalase-peroxidase production important for oxidative stress response [89].

3.3 Antibiotic resistance in STEC

Treatment of STEC infections with antibiotics is not encouraged as this might exacerbate the disease by activating the lytic cycle of the phage carrying Shiga toxin that could aggravate tissue damage in infected individuals. Antibiotics such as rifaximin, fosfomycin, azithromycin, and meropenem that do not encourage the release of Shiga toxin have been used for the treatment of early onset of STEC infection to prevent the progression of the diseases to HUS [90].

Several studies on the prevalence of antibiotic resistance in STEC from different countries and host or environments have reported that resistance to beta-lactams, sulfonamide, tetracycline, and trimethoprim are common STEC, while multidrug-resistance is more frequent in non-O157 than O157:H7 serotypes [91]. For STEC O157, resistance to ampicillin and cephalothin is common in strains of human origin, whereas tetracycline and sulphamethoxazole resistances are frequent in strains of animal origin [92]. In a study involving 54 STEC strains recovered from cattle and pigs, genetic determinants that encode resistance to trimethoprim (*dfrA1*), tetracycline (*tetA* and *tetB*), beta-lactam (*bla_{TEM-1}*), and aminoglycoside (*aac(6)-Ib*) were found in the great majority ($\geq 81\%$) of the isolates, while chloramphenicol resistance gene (*cat1*) was also carried in more than 50% of the collection [93]. Likewise, in a 15-year surveillance study of STEC in Sweden [94], 70 antibiotic resistance determinants that were associated with 10 different classes of antibiotics were found in 184 STEC isolates, where 50% of these genes were present in all isolates. Six resistance determinants to fluoroquinolone (*crp*, *hns*, *acrB*, *marA*, *mdtM*, and *emrA*) were found to be frequent. Equally, *emrE* that encodes resistance to multiple antibiotics was associated with STEC O157:H7, whereas *fosA7*, *sat-1*, and *bla_{TEM-150}* and *dfrA5* were associated with non-STEC O157 serotype.

3.4 Population structure of STEC

Genetic relatedness of STEC isolates from different hosts and countries have been studied using different molecular tools that ranged from serotyping, PFGE, conventional MLST, and WGS. Evidence for transmission and dissemination of different STEC serogroups and clones have also been documented. Unlike ETEC that evolved multiple times through clonal expansion, STEC appears to have evolved by parallel evolution. Indeed, phylogenetic analyses of STEC strains have shown that isolates form multiple distinct clonal lineages, where strains with the same serotype and virulence content were nested together in the cluster [95]. STEC strains are spread across *E. coli* phylogroups and the great majority belonged to phylogroup B1 (**Figure 4**) [96]. STEC O157:H7 are further delineated into three lineages, I, I/II, and II [97, 98] that are disseminated globally. Lineage I is predominant among clinical isolates of human origin while lineage II is more prevalent in animals [99]. Intra-lineage diversity is apparent as lineages varied in the adherence and virulence determinant expression, Stx-encoding bacteriophage (Stx ϕ) insertion sites, *stx2* expression, and stress resistance [97]. This intra-clonal diversity is hypothesized to have been a consequence of the global spread of a single clone and geographic expansion [97]. Interestingly, a time-dependent clonal replacement and geographical-dependent clonal expansion of lineages and sub-lineages of STEC O157:H7 have been reported [97, 100]. The STEC O157:H7 lineage I/II that was predominant in human infection in the 1980s in the UK declined

and was replaced by sub-lineage Ic in the 1990s. Also, in the past few years, this region has reported the replacement of the dominant sub-lineage (Ic) by sub-lineage IIb, a phenomenon they reported to have been a consequence of the acquisition of prophage encoding *stx2a* [97, 100].

In a recent study using WGS to understand the population dynamics of 757 STEC O157:H7 isolates from humans and animals from four continents, seven clades were reported and designated as A-G [101]. The most recent common ancestor of the isolates in this study was reported to have originated in the Netherlands in the late 19th century (1890) and then spread to other parts of the world. Although isolates were clustered on a geographical basis, there was an admixture of strains from different hosts suggesting transmission events between them [101]. The pangenome analyses of these isolates also showed that STEC O157:H7 from humans and animals differed in phage-related protein content. The molecular epidemiology of non-STEC O157:H7 is equally important especially considering their roles in outbreaks. From 1995 to 2017, a total of 674 outbreaks by non-O157 STEC strains were reported worldwide, where O26:H11 was predominant during this period [102]. Other serogroups implicated in these outbreaks include O26:H11, O45, O103:H25, O104:H4, O111:H8, O121, and O145:NM [102]. MLST-based phylogenetic analysis of 894 non-STEC isolates from patients over a period of 18 years (2001–2018) in Michigan revealed that the great majority of the isolates (95%) belonged to one clade [103]. Although the information on the evolutionary dynamics of STEC is inexhaustible, studies focusing on identifying new genetic factors associated with ecological adaptation of different lineages are elusive. Further studies should focus on this area.

4. Enteropathogenic *E. coli* (EPEC)

4.1 An overview of EPEC

Enteropathogenic *E. coli* (EPEC) is a pathotype that causes infrequent diarrheal diseases in adults and has also been implicated in gastroenteritis outbreaks in children in health care settings [104, 105]. EPEC was the first pathotype described in 1955 to refer to *E. coli* causing infantile diarrheal and implicated in a few outbreaks between the 1940s and 1950s [106]. Infection from this pathotype is frequent in children under two years living in low- and middle-income countries, and is the second leading cause of death among this age group, amounting to about 1.5 million deaths annually [105]. Like other DEC, the onset of EPEC-related diarrheal is characterized by acute watery stool which if it persists could result in loss of electrolytes and malabsorption of nutrients in children [3, 107]. Infection caused by EPEC strains is not limited to humans as they have also been implicated as a causative agent of diarrheal illness in young calves (**Figure 2**) [108].

EPEC strains are previously classified solely based on the combination of the three immunogenic structures O, H, and K antigens but the diversity observed for these antigens rendered serotyping unreliable rapid diagnostic tool for this pathotype [17, 107]. However, as recommended in 1987 by World Health Organization, 12 serogroups; O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 belonged to EPEC pathotype. In addition to six others; O39, O88, O103, O145, O157, and O158 have been classified and belonged to this pathotype although some of these serogroups consist of *E. coli* strains from different serotypes [108, 109]. EPEC strains are classified as either motile (H⁺) or non-motile (H⁻). Among EPEC strains with flagellar associated antigens, H2 and H6 are the most frequent, whereas others that are less common include H7, H8, H9, H12, H21, H27, H25, and H34 [3, 107].

EPEC pathotype is defined based on the carriage of LEE locus that mediates the induction of A/E localized lesions [110], a feature that is shared with some STEC strains. However, the inability to produce Shiga toxins or other enterotoxins differentiate EPEC pathotype from EHEC/STEC strains [3, 10]. Additionally, based on the presence or absence of *E. coli* adherence factor plasmid (pEAF), EPEC pathotype is sub-grouped into two subtypes; typical (tEPEC) and atypical (aEPEC) [111]. Relative to tEPEC that is regarded to be more virulent, aEPEC group is reported to be highly diverse and more prevalent in diarrheal illness in children [111, 112]. Several O and/or H antigens of aEPEC strains are nontypeable. Of the typeable serogroups belonging to this subtype, O51 is the most frequent followed by five others (O145, O26, O55, O111, and O119) [3]. aEPEC O55:H7 is closely related with STEC O157:H7 and from the evolutionary perspective, the latter is believed to have evolved and diverged 400 years ago from the ancestor of the former [113]. EPEC like other diarrheagenic *E. coli* pathotypes is transmitted through the fecal-oral route as well as contact with contaminated surfaces or secretions. While humans are believed to be the major reservoir for tEPEC strains, aEPEC strains are present both in healthy individuals and in animals (**Figure 2**) [111, 112, 114].

4.2 Virulome of EPEC

Since EPEC does not produce Shiga toxin or other enterotoxins, the major feature the pathogenic strains in this pathotype employ is their ability to attach tightly to the host mucosal membrane, destroy microvilli, and induce the formation of lesions (**Table 1**) [115]. In addition, EPEC carries other genes encoding proteins that have been linked to colonization and adherence to host cells [116, 117].

4.2.1 Colonization and adherence

The defining characteristic of EPEC is the carriage of LEE locus that is essential for inducing A/E lesions, causing localized lesions by attaching closely to the surface of the intestinal epithelial cells. Like some STEC strains, all the EPEC strains carry *eae* and *tir* genes as well as T3SS that is able to inject a large number of effector proteins into the host cell [3, 111]. Studies have shown that the presence of LEE locus in EPEC strains is enough to cause infection in the host even in aEPEC-related infection scenario [118].

The ~80 kb pEAF plasmid that defines tEPEC carries *per* and *bfp* operons (**Table 1**) [119, 120]. The *per* operon (*perABC*) is plasmid-borne and contains *perA* that encodes a regulator that activates the transcription of *bfp* operon that encodes the type IV pili called bundle-forming pilus (BFP) (**Figure 3**) [119, 120]. The *bfpA* gene which encodes the bundling of the major structure of BFP and 13 other genes are carried on the pEAF plasmid [116]. The carriage of this plasmid has been described to be essential in the localized adherence of EPEC to intestinal epithelium in the host [118]. tEPEC strains carry *lifA* gene that encodes lymphocyte inhibitory factor, a large surface protein that is described to promote the intestinal colonization of mice by *Citrobacter rodentium* [3]. Although pEAF plasmid is absent in aEPEC strains, they often carry virulence determinants typical of STEC strains most likely because they share a common ancestor [113]. Afset et al. [117] identified 12 genes that were statistically associated with aEPEC-related diarrhea in children. Of note are *efa1/lifA* genes that are located on O1-122, as well as *lpfA* gene previously reported in STEC [121]. Likewise, *astA* gene that encodes EAST1, an ST-like toxin that is present in ETEC is also carried by EPEC, being more prevalent in aEPEC than in tEPEC strains implicated in diarrhea [122, 123].

4.3 Antibiotics resistance in EPEC

Although EPEC-related infection could resolve itself or simply by oral rehydration therapy that replenishes the lost fluid, the persistence of this infection may necessitate the use of antibiotics. In this case, especially in adults, the recommended antimicrobial is trimethoprim/sulfamethoxazole, norfloxacin, or ciprofloxacin [124]. However, studies on antibiotic resistance of EPEC strains from different sources and countries have shown high resistance of this pathotype to ampicillin, cefpodoxime, nalidixic acid, trimethoprim, and tetracycline [125, 126]. While resistance to the great majority of these antibiotics is reported to be frequent in tEPEC, trimethoprim resistance is more common in aEPEC strains [127].

In a global study of 185 aEPEC isolates collected from healthy and diarrheal children living in seven sites in sub-Saharan Africa and South Asia, at least 55% of the isolates showed phenotypic resistance to ampicillin, trimethoprim, trimethoprim/sulphamethoxazole, and tetracycline, while streptomycin resistance was reported in 43% of the isolates. Shockingly, more than 50% of the isolates were resistant to three or more of the tested antibiotics [128]. The study also reported point mutations in genes that are associated with resistance to quinolone (*gyrA*, *parC*) and nitrofurantoin (*nfsA*) in addition to over forty different antibiotics resistance genes reported. Equally, more than 50% of the isolates carried at least four resistance determinants that include *bla*_{TEM} (ampicillin), *strA* and *strB* (streptomycin), *sul2* (sulphonamides), and *dfr* genes (trimethoprim/sulfamethoxazole). These resistance determinants were found singly or co-localized on plasmids (pCERC1, pCERC2) or in transposons (Tn6029).

4.4 Population structure of EPEC

The acquisition of LEE and pEAF has been the defining evolutionary phenomenon for EPEC pathotypes [3, 129]. While tEPEC that carries pEAF plasmid is believed to be less diverse, aEPEC is greatly heterogeneous. The loss of pEAF plasmid in aEPEC and its close relatedness with LEE-positive STEC in serotypes, genetic characteristics, virulence properties, and reservoirs make serotype-based lineage definition unreliable [111, 130]. Based on the conventional MLEE and MLST, EPEC strains belong to six clonal lineages (EPEC1–EPEC6) that were represented among the EPEC strains worldwide [129, 131]. The whole genome-based phylogeny reported nine more EPEC lineages designated as EPEC7–EPEC15 [104, 132]. These phylogenomic EPEC lineages belonged to four *E. coli* phylogroups (A, E, B1, and B2) (**Figure 4**), where the great majority were found in B1 and B2 [104, 133], suggesting a clear genetic heterogeneity within this pathotype.

The close relatedness of aEPEC to other pathotypes could play a significant role in the diversity within this pathotype. This EPEC subtype can also include tEPEC that have lost the pEAF plasmid and LEE-positive STEC strains that have lost the Stx encoding bacteriophage during transmission events between hosts, within-host evolution, interaction with the host microbiota, or selective pressure in the environment [104, 130]. This could be a possible explanation why some aEPEC strains would cluster with other pathotypes. Indeed, phylogenomic analyses of 106 Brazilian and 221 global aEPEC genomes showed that isolates were clustered into the previously reported phylogroups for this pathotype and phylogroup D. Additionally, 42.5% of the isolates belonged to the four previously defined EPEC lineages [129, 131], while the remaining isolates were found in EPEC11–EPEC14 phylogenomic lineages, suggesting a gradual and continuous clonal expansion of this pathotype [132]. Of note, dissemination of the phylogenomic lineages of EPEC pathotype is not restricted by geography. Conversely, in a multicentre study

involving seven sites in developing countries, EPEC isolates from sub-Saharan countries (The Gambia and Kenya) were clustered into two EPEC lineages (EPEC5 and EPEC10) in phylogroup A [104]. Overall, EPEC represents a pathotype that is still undergoing clonal expansion due to the occurrence of novel phylogenomic lineages with distinct accessory gene content and their pathogenic potential.

5. Enteroaggregative *E. coli* (EAEC)

5.1 An overview of EAEC

Enteroaggregative *E. coli* (EAEC) is implicated in epidemic diarrheal illnesses, being a causative agent of traveler's diarrhea, persistent diarrhea in children in EAEC endemic areas, and in immunocompromised patients, particularly in human immunodeficiency virus (HIV) patients [3, 134]. EAEC was first described in 1987 by comparing adherence patterns of *E. coli* isolates to HEp-2 cells, where it showed a stacked-brick aggregative phenotype. EAEC strains are able to infect the colon and/or small bowel of their host where they disrupt the intestinal epithelium and result in loss of electrolytes, watery diarrhea with or without blood and mucus, vomiting, among other symptoms [134]. Persistent EAEC-related diarrhea could result in chronic intestinal inflammation that induces the production of fecal lactoferrin and interleukin (IL-8), and malabsorption of nutrients [135]. Studies on the development of postinfectious irritable bowel disease syndrome in acute EAEC-related diarrhea have been documented [135] but the role of EAEC is not fully understood.

EAEC strains are identified using a molecular probe AA that hybridizes with a region of pAA plasmid encoding an ATP binding cassette transporter apparatus which translocates dispersion across the bacterial cell membrane [136]. Isolates that carry the *aggR* gene that encodes autoagglutination that are associated with persistent diarrhea in patients are able to hybridize with the AA probe. In addition, EAEC has a different adherence pattern, and not all HEp-2 adherent EAEC strains isolated from humans with diarrhea carried *aggR*. Hence, EAEC was classified into two subtypes: typical (*aggR* positive) and atypical (*aggR* negative) subtype [134, 137]. Humans are the reservoir for typical EAEC (tEAEC), whereas atypical EAEC (aEAEC) strains have been isolated from young calves, piglets, and horses as well as companion animals, suggesting the role of animals as a reservoir for this subtype (Figure 2) [3].

Although some serotypes including O126:H27, O111:H21, O125, O44:H18 are frequently isolated from EAEC strains, the autoagglutinating phenotype by some EAEC strains complicates the serotyping of this pathotype [3, 138]. In several studies, EAEC strains are often described as nontypeable or as "O?" or O-rough. In a study of EAEC strains from children in Germany, 14 out of 16 isolates that were typeable belonged to different serotypes [139]. Likewise, in a study in the UK, 97 out of 143 EAEC strains that were typeable belonged to more than 40 different O-types [140]. While serotyping is no longer a dependable diagnostic tool for EAEC strains causing diarrheal illness [3, 138], a specific Shiga toxin producing EAEC serotype O104:H4 is associated with a series of outbreaks worldwide [141, 142].

5.2 Virulome of EAEC

EAEC strains that are implicated in diarrheal illness employ several virulence factors that initiate colonization, promote persistence through adherence to mucosal layers of the intestine, and enterotoxin and cytotoxin secretion (Table 1) [134].

5.2.1 Colonization and adherence

EAEC colonizes the intestinal epithelium of the host using aggregative adhesion fimbriae (AAFs) that also activate the host inflammatory responses and afimbrial adhesins [143]. So far, five AAF variants have been described and are encoded by *aggA* (AAF/I), *aafA* (AAF/II), *agg3A* (AAF/III), *agg4A* (AAF/IV), and *agg5A* (AAF/V) that are regulated by the transcriptional activator *aggR*, borne on EAEC plasmid pAA [143]. *AggR* also regulates the expression of a type VI Secretion System (T6SS) and a chromosomal PAI encoded by *aaiA*-*aaiP* operon [144] as well all other virulence genes involved in the aggregation and toxin production in pathogenic strains of EAEC (**Figure 3**) [143, 144]. However, in EAEC strains where AAF is absent, an aggregate-forming pili (AFP), a type VI pilus that is encoded by *afp* operon was reported to be responsible for the establishment of a similar aggregative adhesion pattern (**Figure 3**) [144]. Other virulence determinants associated with colonization and adherence of EAEC include *air* gene that codifies for an enteroaggregative immunoglobulin repeat protein and *capU* that encodes a hexosyltransferase homolog, as well as *shf* and *aatA* that have been linked to biofilm formation [3, 144].

5.2.2 Enterotoxin and cytotoxin secretion

EAEC produces enterotoxins and cytotoxins including EAST1 and colonization factors encoded by *astA* and *pic* genes, respectively [37, 145]. The latter (*pic*) is often associated with *set1A* and *set1B* encoding two subunits of *Shigella* enterotoxin 1 (ShET1) that are linked to the induction intestinal secretion during infection [145]. This pathotype also carries genes encoding class I cytotoxic SPATE protein family that includes autotransporter proteases encoded by *sigA* and *sepA* and plasmid-borne toxin encoded by *pet* [144, 146]. Also, EAEC strains produce dispersin, an anti-aggregation protein that is encoded by *aatPABCD* located on plasmid pAA, and promotes the dispersion of bacteria in the mucosal layer of the intestine [147]. A gene *hlyE* encoding a hemolytic pore-forming toxin that has a cytotoxic effect on cultured cells has also been reported to be present in some EAEC strains, although the role of this gene in the pathogenicity of EAEC is still unclear [3, 148]. Some EAEC strains carry Stx2a phage-encoding Shiga toxin that is associated with HUS in STEC-related infection [141, 146].

The prevalence of the virulence determinants varies with studies and EAEC subtypes [144, 146]. For example, in a study, *pic* gene was reported to be the most prevalent, present in only 47%, while *sepA* and *sigA* were present in less than 15% of the studied isolates [149]. In the study, the authors also noted that *pet* and *pic* genes were associated with tEAEC, whereas *sepA* was associated with aEAEC.

5.3 Antibiotic resistance in EAEC

EAEC-related diseases such as travelers' diarrhea where antimicrobial therapy is proposed, fluoroquinolones, azithromycin, and rifaximin are often recommended. In immunocompromised patients that require chemoprophylaxis, fluoroquinolones are also considered [150]. For Shiga toxin producing EAEC O104: H4 related infections, azithromycin which has been shown to inhibit *stx* expression in *in-vitro* assay is seldomly used [3, 150].

Although a highly successful treatment rate is achieved with these antibiotics, EAEC strains that are resistant to multiple antibiotics have emerged in different regions [150]. Studies on the resistance of EAEC strains from Southeast Asia,

India, Africa, and Latin America with travelers' diarrhea showed that more than 50% of the isolates were resistant to ampicillin, sulphamethoxazole, and tetracycline [150, 151]. In a similar study in Iran, 78% and 60% of the extended-spectrum beta-lactamase (ESBL) producing EAEC strains carried the transposable *bla*_{TEM} and *bla*_{CTX-M} genes, respectively [152]. Also, plasmid-mediated quinolone resistance (PMQR) genes (*qnr*) that encode resistance to quinolone have been identified in EAEC in different studies [153, 154]. In England, among the 155 EAEC strains from diarrhea patients in 2015–2016 [155] showing antibiotic-resistant phenotypes, 43 genetic determinants that encode resistance to seven different classes of antibiotics were identified, with *bla*_{TEM-1} being the most common (40%) followed by *sul2* (37%) and *strA-strB* (32%). Undoubtedly, the rise in antibiotic resistance in this pathotype should be a concern for public health.

5.4 Population structure of EAEC

EAEC subtypes are defined based on the presence of virulence plasmid pAA that carries *aggR* gene that regulates the expression of other virulence determinants located on the plasmid. This and the high serotype diversity, as well as other accessory genes contribute to the high heterogeneity noted for this pathotype. An earlier study on the phylogenetic analysis of EAEC revealed that isolates belonged to multiple lineages [156]. A similar observation was noted with MLST where 150 Nigerian EAEC strains were clustered into 96 STs [157]. Indeed, EAEC strains are known to belong and spread across four *E. coli* phylogroups (A, B1, B2, and D) (**Figure 4**) with diverse serotypes [144, 156].

In a recent study [146], of the 97 EAEC strains analyzed using MLST, 42% were reported to belong to phylogroup B1, while the majority of the few strains that belonged to phylogroup A lack the AAF-associated genes. Although serotype diversity is high in this pathotype, this study also noted that EAEC strains that belonged to phylogroup D were clustered into three serotype-specific lineages (lineage 1–3). All strains in lineage 1 were O166:H15 and belonged to ST349, lineage 2 consisted of serogroups O44, O73, and O17/O77 in combination with either H18 or H34 and ST130, while lineage 3 carried O153:H30 serotype and ST38 [146]. Contrarily, in India, EAEC strains implicated in diarrhea were more prevalent in phylogroup D [158] suggesting that the diversity in the pathotype is not limited by geography. While EAEC subtypes are believed to differ in their virulence determinants content, this hypothesis and comparative phylogenetic analysis of aEAEC and tEAEC are underexplored. Also, large-scale phylogenomic and phylogeographic analyses of this pathotype are scarce. Further studies should focus on this.

6. Enteroinvasive *E. coli* (EIEC)

6.1 An overview of EIEC

Enteroinvasive *E. coli* (EIEC) pathotype causes bacillary dysentery in humans worldwide characterized by abdominal cramps, bloody and mucous diarrhea [159]. The incidence of EIEC-related diseases varies by geographic region but is highly frequent in developing countries. In developed countries, EIEC-related infections are mainly travelers' diarrheal cases in people with recent travel history to endemic regions [160]. The first EIEC strain belonging to serotype O124 was reported in 1947 [161]. In the later years, some of the bacterial species implicated in dysentery that were previously classified as *Shigella* were renamed as EIEC [162]. EIEC and *Shigella* spp. share several serogroups, phenotypic and other genotypic

characteristics, which often makes it challenging to discriminate between the two genera in clinical samples. Like some *Shigella* spp., most EIEC strains are non-motile and lack the ability to decarboxylate lysine or ferment lactose [3]. EIEC invades the human intestinal epithelial layer where it induces dysentery syndrome that is characterized by watery stool containing blood, mucus, and leukocytes, symptoms that are similar to those presented by *Shigella* spp. associated infection (**Figures 2 and 3**) [159].

At least, 20 serotypes have been assigned to this pathotype [159] among which some of the EIEC-associated O antigens including O28, O112ac, O121, O124, O143, O144, O152, and O167, are identical to O antigens present in *Shigella* spp. [3, 159]. Humans are the major reservoir of EIEC strains and transmission occurs through the fecal-oral route from the ingestion of contaminated foods or water and person-to-person contact [159, 160]. The incidence rate and morbidity for EIEC are less or underreported but it appears to follow a similar trend as *Shigella* [3, 159]. Although EIEC strains cause sporadic cases of infection, they are also implicated in outbreaks. Of note are outbreaks of EIEC linked to O96:H19 strain that was traced to cooked vegetables and salads that were contaminated by asymptomatic food handlers in 2012 and 2014 in Italy [163] and the United Kingdom [164], respectively. Recently, the first case of EIEC outbreak in the US in about half a century was caused by EIEC serotype O8:H19 [160].

6.2 Virulome of EIEC

EIEC strains cause infection in humans by their ability to invade the colon mucosa layer with the expression of essential virulence determinants that mediate colonization, adherence, and invasion of the intestinal epithelial cells of the host (**Table 1**). These genes that are also shared with *Shigella* are located on the chromosome or virulence plasmid.

6.2.1 Colonization and adherence

The colonization, adherence, and invasion of intestinal epithelial cells by EIEC are mediated by genetic factors encoded by genes on a plasmid, pINV. pINV is a virulence plasmid found in EIEC that encodes the type III secretion system necessary for attachment, invasion of the host cell, and intercellular spread. This plasmid is structurally and functionally similar to those in *Shigella* strains [159], and with the replication (*rep*) and conjugation (*tra*) regions in IncFIIA plasmids. pINV had large deletions in the *tra* region which makes it incapable of self-transfer by conjugation but can be mobilized by other conjugative plasmids. Among the numerous functional insertion sequences present in this plasmid is the IS1111 family, but only defective copies of the IS family are found in *Shigella* pINV plasmids [159]. pINV carries a PAI-structure that is composed of gene clusters encoding a T3SS apparatus (Mxi and Spa), its effector proteins (IpaB, IpaC, and IpaD) with their chaperons (IpgA, IpgC, IpgE, and Spa15), and two global transcriptional regulators (VirB and MxiE) that activate and regulate the expression of most of the virulence genes [3, 159]. All T3SS effectors are carried on the pINV except for a few effector proteins of the IpaH family that are chromosomal (**Figure 3**).

Also carried on pINV are genes that encode IcsA, a protein that facilitates the bacterial movement inside the cytoplasm, VirA, a GTPase-activating protein, and RnaG, a small RNA that negatively control the expression of *icsA* gene [159, 165], as well as the gene encoding OspG and OspF proteins which facilitate the evasion of the host innate immune response. All EIEC isolates are reported to carry this plasmid as it is essential for the pathogenesis and pathoadaptation

of this pathotype. However, loss of this genetic element has been reported in some EIEC strains [159, 165].

6.2.2 Cytotoxins production

EIEC strains also carry a plasmid-borne gene, *sen* that mediates a novel 63-kDa enterotoxin (ShET2) [166]. A mutation in this gene was reported to cause a substantial loss in the enterotoxic ability of EIEC strain. Although the role of this gene in the pathogenesis of EIEC is not fully understood, toxins are known to be important in the induction of watery diarrhea during *E. coli* infection. Additionally, plasmid encoding enterotoxigenic and cytotoxigenic factors namely *pic*, *sepA*, *sigA*, and *sat* that belong to SPATEs family and that are reported to contribute to intestinal fluid accumulation in an animal model are carried by EIEC strains. Nonetheless, these genes are not carried by all EIEC strains. Two different studies on the prevalence of the virulence genes among EIEC reported that *sen*, *sigA*, and *pic* were found in at least 70%, 64%, and 27% of the isolates, respectively [167, 168], whereas *sat* gene was found only in 15% of the collection [167].

6.3 Antibiotic resistance in EIEC

EIEC-related infection is self-limiting that could be managed with rehydration to replenish the loss electrolyte. Zinc supplementation and nutritional therapy with iron-rich green plantain have also been shown to reduce the severity and the duration of diarrheal illness. However, in rare cases of severe symptoms antimicrobial treatment therapy has been reported to be effective [3, 10]. Since *Shigella* and EIEC present similar symptoms and are often misdiagnosed, similar antimicrobials include azithromycin (macrolide), ceftriaxone, (cephalosporin), and ciprofloxacin (fluoroquinolone) are recommended [3, 159].

Like other *E. coli* pathotypes and *Shigella*, there is an emergence of multidrug-resistant EIEC strains. In a study of EIEC isolates from adults with enteric infection in Cameroon, high resistance to ampicillin and sulfamethoxazole-trimethoprim was noted in 57.14% and 71.43%, respectively. Resistance determinants to ampicillin (*bla*_{TEM} and *bla*_{Oxa}) were found in 28.57% of the isolates. Additionally, *cat1* and *cat2* genes were noted in chloramphenicol resistant strains while *tetA*, *tetB* encoding resistance to tetracycline, *dfr12*, *dfr7*, *dfr1a* to sulphamethoxazole-trimethoprim, and *sul1* gene to sulfonamide were present in more than 85% of the EIEC isolates [169]. In a large-scale study of *Shigella* and EIEC isolates from eight countries in four continents between 1971 and 1999, 48% of EIEC isolates were resistant to tetracycline [170]. In another study, an EIEC O164 strain isolated from a traveler with diarrhea in Japan was found to be resistant to streptomycin, spectinomycin, co-trimoxazole, and ampicillin, with reduced susceptibility to ciprofloxacin [171]. In this strain, resistance determinant for trimethoprim (*dfrXII*), streptomycin and spectinomycin (*aadA2*), and an ORF of unknown function was carried on a class 1 integron located on a transferable plasmid. While ampicillin resistance gene *bla*_{TEM} was detected, the reduced susceptibility to ciprofloxacin was reported to be due to a single mutation P158-to-S in *parC*.

6.4 Population structure of EIEC

EIEC pathotype is diverse and highly specialized due to the carriage of large virulence plasmid, a genetic element that is shared with *Shigella*. The pINV plasmid that is the hallmark of EIEC lacks the ability for autonomous horizontal transfer. Although there is no consensus on the evolution of pINV in *Shigella*/

EIEC, it has been hypothesized that this genetic element was probably acquired in an ancestral *E. coli* prior to the diversification of the two bacterial species and the emergence of different *Shigella*/EIEC lineages. Conversely, *Shigella*/EIEC strains could have evolved from different *E. coli* strains that had acquired the pINV independently from other pINV-carrying *Shigella*/EIEC or from an unknown donor [172].

A phylogenetic analysis of 32 EIEC strains based on four housekeeping genes (*trpA*, *trpB*, *pabB*, and *putP*) revealed four clusters (clusters 4–7) where most of the O antigens were found in a single cluster [173]. EIEC cluster 4 comprised strains with serotype O28, O29, O124, O136, and O164. Serotypes O124, O135, O152, and O164 belonged to cluster 5, and O143 and O167 to cluster 6 while cluster 7 had only O144 [173]. This is similar to a SNP-based phylogeny described in another study [174]. WGS alignment-based phylogeny of 20 EIEC isolates revealed that the great majority of the strains belonged to three distinct EIEC lineages (lineage 1–3) that belonged to three different *E. coli* phylogroups (A, B1, and E) (**Figure 4**). All the EIEC strains in lineage 1 (phylogroup E) were all serotype O143:H26, whereas, in other lineages the serotypes were diverse. EIEC lineage 3 (phylogroup B1) was reported to be globally disseminated as the strains from six different countries were clustered together in this lineage [175].

Insertion sequences were recently reported to contribute to the population structure of EIEC. A recent study [176] on the evolutionary dynamics of *Shigella* and EIEC lineages identified the genetic factors driving strain-to-strain variation within each population and contributing to functional gene loss within and between species. In the study, the author found that all *Shigella* and EIEC lineages had higher IS copy numbers relative to other *E. coli* pathotypes indicative of IS expansion in these lineages. The authors also found that *Shigella* and EIEC lineages carried the same five ISs (IS1, IS2, IS4, IS911, and IS600) indicative of a parallel expansion of these IS types, although at a high degree in *Shigella*. The data also suggests that *Shigella* and EIEC lineages underwent an expansion of their native IS1 alleles and that pINV is a potential source for the introduction of other ISs (IS2, IS4, IS600, and IS911) that are rare in *E. coli* into *Shigella* and EIEC lineages [176].

In a comparative pangenome analysis of EIEC with *Shigella* and other *E. coli* pathotypes, seven gene clusters were identified to be enriched in EIEC strains but absent in all other *E. coli* pathotypes and *Shigella* strains. These included genes that encode a putative pyruvate kinase, a periplasmic protein, and some uncharacterized proteins. However, when *Shigella* isolates were excluded, the authors identified 96 gene clusters that were present in more than half of the EIEC strains. A total of 87 gene clusters were reported when EIEC and *Shigella* genomes combined were compared to other *E. coli* pathotypes. Among these were plasmid-associated genes encoding a hypothetical toxin-antitoxin system and putative proteins hypothesized to be involved in conjugal transfer [175].

The EIEC lineages were reported to have distinct phenotypic and genotypic features. Lan et al. [173] reported that EIEC strains belonging to cluster 4 lack mucate fermentation ability, whereas strains in cluster 6 were able to utilize acetate and ferment mucate [173]. Likewise, Hazen et al. [175] identified up to 155 gene clusters that were exclusive in EIEC strains belonging to one phylogroup. Additionally, 12–155 gene clusters were also reported to be lineage-specific in the EIEC pathotype [175]. Protein-encoding genes that are linked to transcriptional regulation, metabolism, and transport, and a colicin were exclusive in EIEC lineage 1, whereas genes that encode membrane protein, the aerobactin siderophore receptor, and hypothetical proteins were exclusive for EIEC lineage 2. Genes encoding several transcriptional regulators and hypothetical proteins were limited to EIEC lineage 3 [175].

7. Diffusely adherent *E. coli* (DAEC)

7.1 An overview of DAEC

Diffusely adherent *E. coli* (DAEC) pathotype is a group of *E. coli* causing diarrhea that can attach to host cells but not in a localized or A/E adherence pattern [3]. *E. coli* strains belonging to this pathotype binds to the entire surface of the epithelial HEP-2 cells in a scattered pattern termed diffuse adherence. Although the adherence pattern is unique, this pathotype is difficult to classify or identify, a possible reason for the scarce epidemiological studies on this group [177].

DAEC is widespread and associated with diarrhea in both developing and industrialized countries around the world [3]. DAEC strains are associated with watery diarrhea in children under 5 years and can persist resulting in an increase in severity of disease in this age group [177]. DAEC has also been implicated in extraintestinal infections such as UTI, pregnancy complications [3, 177]. It has been speculated that the asymptomatic carriage of DAEC by this age group and adults can lead to chronic inflammatory colon disease such as Crohn's disease [3, 177]. Meanwhile, there is no universal detection method for this pathotype but based on DNA hybridization of fimbrial encoding *daaC* gene probe and adherence pattern to HEP-2 cells of 221 diarrheagenic *E. coli* from different age-groups in Brazil [178], DAEC was identified and shown to be associated with diarrhea in children under 12 months of age in this region. The authors also noted that the presence of DAEC in younger children was not associated with diarrhea, suggesting that the association would probably be based on geographic regions [178]. The asymptomatic carriage by different age groups and the lack of epidemiological data from different regions undermines the development of a universal identification method for this pathotype.

While it is unclear how DAEC is transmitted or its reservoir, the fact that there are asymptomatic carriers could suggest humans as the main reservoir and the fecal-oral route as the primary means of transmission (**Figure 2**). Information regarding serotypes associated with DAEC is scarce. A study of 112 DAEC isolates from diarrheal and asymptomatic individuals in Brazil reported 45 different serotypes, of which 19 were exclusive in patients with diarrhea [179], whereas in another study [180] the serotypes were nontypeable.

7.2 Virulome of DAEC

DAEC strains infect the intestinal epithelium of the host by expressing surface-exposed adhesins that mediate colonization and attachment which allow them to resist host clearance mechanisms [177]. There are several virulence factors that mediate this process, and they include fimbria or afimbrial structures, adhesins, and secretion of cytotoxic toxins that promote the invasion of the host cells (**Table 1**) [177].

7.2.1 Colonization and adherence

DAEC pathotype carries genes that encode Afa/Dr adhesins [3, 177]. Afa/Dr family includes Afa, Dr, and F1845 adhesins that are both afimbrial (such as AfaE-I and AfaE-III), and fimbrial (such as F1845 and Dr) adhesive structures on the bacterial surface encoded by the *afa*, *dra*, and *daa* operons, respectively [177, 181]. These adhesins have been found not only in DAEC but also in UPEC (Uropathogenic *E. coli*), indicating that strains that produce Afa/Dr adhesins may cause both intestinal and extraintestinal infections. Afa/Dr adhesins bind to human

decay-accelerating factor (hDAF) and carcinoembryonic antigen-related cell adhesion molecules that induce receptor clustering resulting in a partial internalization of bacterial cells (**Figure 3**). These adhesins can induce the production of cytokine IL-8 and result in intestinal inflammation, loss of microvilli structure, and watery diarrhea [177]. There are two different subclasses of atypical DAEC; a subclass that contains all the adhesins typical of the Afa/Dr family of adhesins in another *E. coli* pathotype such as diffusely adherent EPEC, while the other subclass does not bind hDAF and expresses a different array of adhesins on its surface, including AfaE-VII, AfaE-VIII, AAF-I, AAF-II, and AAF-III and still able to induce proinflammation [177].

The prevalence of the genes constituting the operons that encode the Afa/Dr adhesins have been reported to vary in DAEC pathotype. In a study, the prevalence of the Afa/Dr adhesin family encoding genes *afaE-1*, *afaE-2*, *afaE-3*, *afaE-5* and *daaE* were reported in 64.3%, 14.2%, 28.6%, 21.5% and 21.5% of DAEC isolates, respectively [182], while in another study [183], the prevalence of these genes were 44%, 10%, 2%, 2% and 6%, respectively.

7.2.2 Secretion of cytotoxins

DAEC secretes a class I SPATE toxin that is called secreted autotransporter toxin (Sat), encoded by *sat* gene. Sat is reported to have enterotoxic activity in an animal model, and mediate the induction of fluid accumulation, loss of microvilli, inflammation, and polymorphonuclear lymphocytes (PMNL) infiltration, like the LT effect of ETEC (**Figure 3**) [184]. Although *sat* gene is reported to be equally expressed in DAEC strains from diarrheal and asymptomatic adults, this gene is significantly associated with DAEC-related diarrhea in children [183]. For example, in two studies, *sat* gene was identified in 44–63% of DAEC strains collected from children with diarrhea while it was found in 0–20% of DAEC strains from asymptomatic children [183–185]. Noteworthy, *sat* gene is not exclusive for DAEC pathotype. In fact, it is prevalent in other *E. coli* pathotypes including EAEC and UPEC [186]. Other virulence factors that have been reported in DAEC include *pet* [187], *astA* [187], and *senB* [188] genes that encode enterotoxins and *hlyE* gene that encodes alpha-hemolysin [186]. However, the role of these genes in the pathogenesis of DAEC still remains unclear.

7.3 Antibiotic resistance in DAEC

Oral rehydration solution therapy is the only recommended treatment for DAEC-related watery diarrhea. However, there are reports of antibiotic resistance in this pathotype. In a study of 112 DAEC strains isolated from children with watery diarrhea in Brazil [179], all DAEC isolates were susceptible to five antibiotics including gentamicin, ofloxacin, and nalidixic acid while 70% were resistant to three or more antibiotics and 50% showed resistance to either ampicillin, co-trimoxazole, streptomycin, sulfonamide, or tetracycline. Additionally, 20% of the strains were resistant to chloramphenicol [179]. A similar observation was noted in a study from Iran where 75–100% of DAEC strains from pediatric diarrhea were resistant to ampicillin, cefotaxime, and trimethoprim-sulfamethoxazole [182].

7.4 Population structure of DAEC

DAEC is a heterogeneous group that has also been implicated in extraintestinal infections such as UTI. Despite its implication in diarrhea in children, studies on its population structure are limited. The few studies available on the phylogenetic

analysis of DAEC strains using MLEE reported that they are distributed among all of the phylogroups [47, 156, 189]. Conversely, a study of 31 DAEC strains from diarrhea and asymptomatic carriers in Peru reported that 87% of the isolates belonged to phylogroup D [190]. A large-scale genomic analysis of DAEC strains would be important to understand the population structure, determine dissemination and transmission dynamics of genetic lineages of this pathotype, as well as identify novel virulence determinants and other genetic factors that contribute to its patho-adaptation in the intestinal epithelium.

8. Conclusion

Much progress has been made on the biology and the genomic epidemiology of diarrheagenic *E. coli* pathotypes since the development of WGS technologies. In particular, there is a notable advancement in the timely detection of outbreaks and the understanding of the population structure of some of the DEC pathotypes. However, the distinct phenotypes underlying the genomic signatures that drive the evolution of pathogenic *E. coli* are not fully understood. Large-scale genomic analyses of different *E. coli* pathotypes are scarce, hence, the genetic factors that define each pathovar and specific lineages are still underexplored. These should form the direction for future studies to better understand the evolutionary dynamics of *E. coli* pathotypes.

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