



## Medical Microbiology

Diarrheagenic *Escherichia coli*

Tânia A.T. Gomes<sup>a,\*</sup>, Waldir P. Elias<sup>b</sup>, Isabel C.A. Scaletsky<sup>a</sup>, Beatriz E.C. Guth<sup>a</sup>,  
Juliana F. Rodrigues<sup>c</sup>, Roxane M.F. Piazza<sup>b</sup>, Luís C.S. Ferreira<sup>c</sup>, Marina B. Martinez<sup>d</sup>

<sup>a</sup> Universidade Federal de São Paulo, Escola Paulista de Medicina, Departamento de Microbiologia, Imunologia e Parasitologia, São Paulo, SP, Brazil

<sup>b</sup> Instituto Butantan, Laboratório de Bacteriologia, São Paulo, SP, Brazil

<sup>c</sup> Universidade de São Paulo, Instituto de Ciências Biomédicas, Departamento de Microbiologia, São Paulo, SP, Brazil

<sup>d</sup> Universidade de São Paulo, Faculdade de Ciências Farmacêuticas, Departamento de Análises Clínicas e Toxicológicas, São Paulo, SP, Brazil

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## ABSTRACT

Most *Escherichia coli* strains live harmlessly in the intestines and rarely cause disease in healthy individuals. Nonetheless, a number of pathogenic strains can cause diarrhea or extraintestinal diseases both in healthy and immunocompromised individuals. Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children, especially in developing countries. *E. coli* strains that cause diarrhea have evolved by acquiring, through horizontal gene transfer, a particular set of characteristics that have successfully persisted in the host. According to the group of virulence determinants acquired, specific combinations were formed determining the currently known *E. coli* pathotypes, which are collectively known as diarrheagenic *E. coli*. In this review, we have gathered information on current definitions, serotypes, lineages, virulence mechanisms, epidemiology, and diagnosis of the major diarrheagenic *E. coli* pathotypes.

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The genus *Escherichia*, which was named after the German pediatrician Theodor Escherich, consists of facultative anaerobic Gram-negative bacilli that belong to the family Enterobacteriaceae.<sup>1</sup> The genus type species *Escherichia coli* is widely distributed, where it is the major facultative anaerobe inhabiting the large intestine of humans and warm-blooded animals.<sup>2</sup> Although most *E. coli* strains live harmlessly in the colon and seldom cause disease in healthy individuals,

a number of pathogenic strains can cause intestinal and extraintestinal diseases both in healthy and immunocompromised individuals.<sup>3</sup>

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children.<sup>4</sup> Low- and middle-income countries in Africa, Asia and Latin America are the most affected regions with diarrheal diseases occurring more often with lethal outcomes mainly

\* Corresponding author.

E-mail: [tatg.amaral@unifesp.br](mailto:tatg.amaral@unifesp.br) (T.A. Gomes).

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due to poor living conditions (inadequate water supplies, poor environmental hygiene and sanitation, and insufficient education).<sup>5</sup>

*E. coli* strains involved in diarrheal diseases are one of the most important of the various etiological agents of diarrhea, where strains have evolved by the acquisition, through horizontal gene transfer, of a particular set of characteristics that have successfully persisted in the host.<sup>3,5,6</sup> According to the group of virulence determinants acquired, specific combinations were formed determining the currently known *E. coli* pathotypes, which are collectively known as diarrheagenic *E. coli* (DEC).<sup>6</sup> The DEC pathotypes differ regarding their preferential host colonization sites, virulence mechanisms, and the ensuing clinical symptoms and consequences, and are classified as enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC).

Each of these pathotypes represents a group of clones that share specific virulence factors. Nevertheless, it should be pointed out that the plasticity of the *E. coli* genome has hindered the identification of certain *E. coli* isolates as a pathotype, because some isolates combine the main virulence characteristics of different pathotypes and are thus considered potentially more virulent hybrid pathogenic strains.<sup>5</sup>

Another less well-defined pathotype has been described, that is, the diffusely-adherent *E. coli* (DAEC) pathotype, which comprises strains that adhere to epithelial cells in a diffused distribution.<sup>6</sup> Despite their classification as a group distinct from the other pathotypes, the designation of DAEC as a different DEC pathotype requires further epidemiological studies, which have been hampered by the difficulties in its identification and classification.<sup>5</sup> Furthermore, certain *E. coli* strains that have been classified as the adherent invasive *E. coli* (AIEC) pathotype, comprise one of the potential agents for Crohn's disease (CD). CD is an inflammatory bowel disease (IBD), which is thought to be caused by a combination of factors (genetics, the intestinal microbiota, environmental factors, and enteric pathogens).<sup>7,8</sup>

Diarrheal episodes due to DEC infections are an important public health issue among children and adults in developing countries, because of their association with morbidity and mortality of children less than five years of age. It was our aim with this review to gather information on current definitions, serotypes, lineages, virulence mechanisms, epidemiology, and diagnosis of the major DEC pathotypes with emphasis on the studies conducted in Brazil.

## Typical and atypical enteropathogenic *E. coli*

The term enteropathogenic *E. coli* (EPEC) was first used in 1995 by Neter et al.,<sup>9</sup> to describe a number of *E. coli* strains epidemiologically related to a series of outbreaks of infantile diarrhea in the 1940s and 1950s.<sup>10,11</sup> Originally identified by serotype, EPEC are now defined as those *E. coli* strains having the ability to cause diarrhea, to produce a histopathology on the intestinal epithelium known as the attaching and effacing (AE) lesion, and the inability to produce Shiga toxins and heat-labile (LT) or heat-stable (ST) enterotoxins.<sup>6</sup>

Improvements in techniques allowing a better understanding of the genome and virulence mechanisms among EPEC strains over the years have led to the sub-classification of EPEC into typical EPEC (tEPEC) and atypical EPEC (aEPEC).<sup>3,12</sup> Typical EPEC strains causing human infectious diarrhea possess a large virulence plasmid known as the EPEC adherence factor (EAF) plasmid (pEAF), which encodes the type IV fimbriae called the bundle-forming pilus (BFP), while aEPEC do not possess this plasmid.<sup>6,12</sup>

The majority of tEPEC strains fall into well-recognized O serotypes. Classical EPEC O serogroups include O55, O86, O111, O114, O119, O127, and O142. The most common H antigens associated with EPEC are the H6 and H2 antigens.<sup>12-15</sup> A less common EPEC type is H34, and a number of tEPEC strains are classified as non-motile (H-) in conventional tests. Typical EPEC strains belonging to non-classical serotypes have also been reported.<sup>12,16</sup>

Based on multilocus enzyme electrophoresis analysis (MLEE) of allelic differences between housekeeping genes, tEPEC strains have been subtyped into two major lineages, previously designated EPEC1 and EPEC2.<sup>13,14</sup> EPEC1 includes widespread serotypes such as O55:H6 and O119:H6, whereas EPEC2 consists of serotypes with more limited occurrence such as O111:H2 and O114:H2. Based on a whole-genome phylogeny and analysis of type III secretion system (T3SS) effectors, tEPEC strains have been demonstrated to cluster in three main lineages, designated EPEC1, EPEC2, and EPEC4, which probably acquired the locus of enterocyte effacement (LEE) region and pEAF independently.<sup>17</sup>

In turn, aEPEC belong to a large diversity of classical and non-classical serotypes.<sup>12,16,18</sup> Over 20% of strains of non-classical EPEC serotypes are O non-typeable and the O-typeable strains belong to more than 4200 different serotypes, with many non-motile and H non-typeable strains.<sup>12,18</sup> Interestingly, it has been found that 35% of the aEPEC strains also belong to the tEPEC lineages.<sup>17</sup> Thus, it has been hypothesized that at least some aEPEC may have originated from tEPEC strains that lost pEAF in the host or in the environment.<sup>17,19,20</sup>

## Virulence factors, mechanisms and pathogenesis

Typical EPEC strains adhere to HeLa, HEp-2, and other cell lines and to organ cultures *in vitro* in a distinctive pattern of three-dimensional microcolonies, a so-called localized adherence (LA) pattern.<sup>6,21</sup> A similar adherence pattern has been seen in tissue biopsies of EPEC-infected humans.<sup>22</sup>

The LA phenotype is mediated by the BFP,<sup>23</sup> which also contributes to antigenicity, autoaggregation, and biofilm formation.<sup>23-27</sup> An operon of 14 genes contained on the pEAF is necessary for BFP expression, with *bfpA* encoding the major structural subunit (bundlin)<sup>28</sup> and being highly conserved among EPEC1 and EPEC2 strains.

The self-transmissible pEAF pMAR2 is found among strains of the EPEC1 lineage and contains an intact transfer region, unlike pB171, which is more common among EPEC2 strains.<sup>29,30</sup> Besides the *bfp* gene cluster, encoding BFP,<sup>23</sup> the pEAF carries the *per* locus, encoding the transcriptional activator called plasmid-encoded regulator (Per).<sup>29</sup> Between pMAR2 and pB171, the *bfp* and *per* loci share 99% sequence similarity,<sup>30</sup> and both BFP and PerA have been shown to contribute to

virulence in human volunteers.<sup>24</sup> Recent comparative genomics of the EAF plasmids from diverse EPEC phylogenomic lineages demonstrated significant plasmid diversity even among isolates within the same phylogenomic lineage.<sup>31</sup>

Typical EPEC have the ability to form tight, spherical, bacterial autoaggregates when grown in liquid culture.<sup>32</sup> Like LA, autoaggregation requires BFP. Typical EPEC also form biofilms on abiotic surfaces under static conditions, or in a flow through continuous culture system, and a model of EPEC biofilm formation has been proposed.<sup>26</sup> Mutagenesis analysis has identified adhesive structures such as the common type 1 pilus (T1P), antigen 43, BFP and the EspA filament (see below) as participants in bacterial aggregation during biofilm formation on abiotic surfaces.<sup>26</sup>

A hallmark phenotype of both tEPEC and aEPEC is the ability to produce AE lesions.<sup>33</sup> This phenotype is characterized by effacement of intestinal epithelial-cell microvilli and intimate adherence between the bacterium and the epithelial cell membrane. Directly beneath the adherent bacterium, marked cytoskeletal changes are seen in the epithelial cell membrane, particularly the formation of an actin-rich cup-like pedestal at the site of bacterial contact. AE lesions are observed in model EPEC infections with cultured cells and mucosal explants, as well as in intestinal biopsies from EPEC-infected infants or animals.<sup>6</sup>

AE lesions are encoded by LEE, which is a ~35-kb pathogenicity island (PAI)<sup>34</sup> that is organized into five operons (LEE1 to LEE5).<sup>35–37</sup> The LEE1, LEE2, and LEE3 operons encode components of a T3SS, and the global regulator Ler (LEE-encoded regulator).<sup>38</sup> LEE4 encodes the T3SS-secreted proteins EspA, EspB, and EspD (EPEC-secreted protein), which are components of the translocation apparatus by which other effector proteins are translocated into the cell. LEE5 encodes the adhesin intimin and its translocated receptor, Tir.<sup>39</sup>

Intimin is a 94-kDa protein encoded by the *eae* gene and required for intimate adherence of EPEC to host cells at the sites of AE lesions.<sup>6</sup> The N-terminus of intimin is highly conserved, whereas the C-terminus is highly variable.<sup>40</sup> Differences in the C-terminus of intimin have been used as a basis for classification into several distinct subtypes, represented by the Greek letters  $\alpha$  (alpha) through  $\zeta$  (zeta)<sup>41,42</sup>; the  $\alpha$  subtype is expressed by EPEC1 strains while subtype  $\beta$  is associated with human EPEC2 strains. The N-terminus of intimin anchors the protein in the EPEC outer membrane, whereas the C-terminus extends from the EPEC surface and binds to the Tir. Intimin–Tir interaction leads to intimate adherence and pedestal formation beneath adherent bacteria,<sup>39</sup> and inhibits NF- $\kappa$ B activity through tumor necrosis factor alpha (TNF- $\alpha$ ) receptor-associated factors.<sup>43</sup> In addition to Tir, the EPEC genome contains six other LEE-encoded effector proteins that are translocated into the cell (Map, EspF, EspG, EspZ, EspH, and EspB), which interfere with different aspects of the cell's physiology.<sup>13,36,37,44</sup>

In addition to the LEE effectors, various non-LEE (Nle)-encoded effector genes (*cif*, *espl/nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleH*)<sup>36,44</sup> have been described, which are located outside the EPEC LEE region, in at least six chromosomal PAIs, or in prophage elements.<sup>45,46</sup> The Nle proteins have been shown to disrupt the cytoskeleton and tight junctions of the host cell, and to modulate or prevent the host inflammatory

response.<sup>45–47</sup> Although they are not required for AE lesion formation, it is understood that they contribute to increased bacterial virulence.<sup>44</sup>

Intracellular tEPEC have been observed both in tissue culture and in small intestinal biopsies from an EPEC-infected infant.<sup>6</sup> Two studies have reported that O111:NM strains contain plasmid sequences that confer invasiveness upon *E. coli* K12 strains containing the cloned fragments.<sup>48,49</sup> Sequences homologous to these cloned genes are present in only a minority of tEPEC strains (Scaletsky et al., unpublished data).

Typical EPEC strains encode a large surface protein, lymphocyte inhibitory factor (LifA), which inhibits the expression of multiple lymphokines and inhibits lymphocyte proliferation.<sup>50</sup> Two related genes *efa1* and *toxB* have been implicated in adhesion to epithelial cells.<sup>51,52</sup> There is evidence indicating that Efa1/LifA contributes to epithelial cell adherence *in vitro*<sup>53</sup> and is required for intestinal colonization of mice by the related AE pathogen *Citrobacter rodentium*.<sup>54</sup>

Some tEPEC strains possess other fimbriae or pili in addition to BFP. Type 1 fimbriae of EPEC have been found to be antigenic in volunteer studies; however, they do not have a role in adherence to epithelial cells *in vitro*.<sup>6</sup> In addition, some EPEC strains have conserved fimbrial genes encoding homologs of long polar fimbriae (LPF),<sup>55</sup> but a number of polymorphisms within the *lpfA* genes have been identified.<sup>56</sup> Initial studies have indicated that LPF is apparently not necessary for adherence and AE lesion in human biopsies.<sup>55</sup> The *E. coli* common pilus (ECP) has also been shown to act as an accessory adherence factor in EPEC, playing a role during cell adherence and/or in bacterium-bacterium interactions.<sup>57</sup> However, the significance of ECP to EPEC pathogenesis has not been determined. Interestingly, it has been shown that some tEPEC strains may produce a hybrid adherence phenotype in HeLa cells, i.e., LA and aggregative (AA)-like pattern concurrently (LA+/AA-like+).<sup>58</sup> Recently, it was shown that at least some of these LA/AA-like+ strains bear large plasmids, distinct from the pEAF, that encode a so far unknown adhesin.<sup>59</sup> It has been proposed that the ability of such strains in producing AE lesions and an AA-associated biofilm concomitantly could worsen the patient's clinical condition, leading to persistent diarrhea.<sup>59</sup>

Flagella may also be involved in tEPEC adherence to epithelial cells,<sup>60</sup> since certain EPEC mutants are markedly impaired in their ability to adhere and to form microcolonies. Furthermore, in one study, purified EPEC flagella and anti-flagellum antibodies were both effective in blocking the adherence of several EPEC serotypes.<sup>60</sup> However, another study could not confirm the role of flagella in EPEC adherence.<sup>61</sup>

Some tEPEC strains harbor the *astA* gene, which encodes the enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1).<sup>62,63</sup> A recent study reported that 11 of 70 (16%) tEPEC strains tested harbored an intact *astA* gene.<sup>64</sup> Typical EPEC strains of serotype O86:H34 produce cytolethal-distending toxin (CDT).<sup>65</sup> The significance of EAST1 and CDT toxins in EPEC pathogenesis remains unknown.

Autotransporter (AT) proteins, which have been associated with bacterial adherence, aggregation, biofilm formation, invasion, and toxicity<sup>66</sup> in Gram-negative bacteria, have also been described among EPEC strains.<sup>67</sup> One such protein, EspC, which is secreted by the type V secretion system and is

injected by the T3SS in epithelial cells, has an IgA protease-like activity and, once in the host cytoplasm, has various cytopathic effects, including cytoskeletal damage,<sup>68</sup> enhanced lysozyme resistance,<sup>70</sup> hemoglobin degradation,<sup>69</sup> hydrolysis of pepsin, factor V, and spectrin,<sup>70</sup> and fodrin and focal adhesion protein degradation.<sup>71</sup> In addition, oligomerization of EspC gives rise to rope-like structures that serve as a substratum for adherence and biofilm formation as well as protecting bacteria from antimicrobial compounds.<sup>72</sup>

A three-stage model of tEPEC adhesion and pathogenesis, consisting of LA, signal transduction, and intimate attachment with pedestal formation, was proposed.<sup>73</sup> Simultaneously with intimate attachment, a series of bacterial effector proteins are injected into host cells, where they subvert actin polymerization and other host cell processes.<sup>37,44</sup> In the earliest stage and under correct environmental conditions, tEPEC express BFP, intimin, and the T3SS/translocon apparatus. Next, EPEC adhere to the surface of the intestinal epithelium via BFP and EspA filaments, and the T3SS injects the bacterial translocated intimin receptor (Tir) and effector proteins (EspB, EspD, EspF, EspG, and Map) directly into the host cell.<sup>37</sup> The effectors activate cell-signaling pathways, causing alterations in the host cell cytoskeleton and resulting in the depolymerization of actin and the loss of microvilli. Finally, bacteria intimately adhere to host cell by intimin-Tir interactions, causing a cytoskeletal rearrangement that results in pedestal-like structures. Tir promotes cytoskeletal reorganization through interaction with neural WASP (Wiskott-Aldrich syndrome protein) (N-WASP) and subsequent activation of the Arp2/3 complex,<sup>45</sup> leading to the effacement of the microvilli and the production of pedestals.<sup>44,74</sup> The translocated effectors disrupt host cell processes, resulting in loss of tight-junction integrity and mitochondrial function, leading to both electrolyte loss and eventual cell death.<sup>45</sup>

For actin dynamics subversion, tEPEC usually recruits Nck to the adhesion site in a Tir phosphorylated Y474-dependent mechanism. In turn, Tir<sub>EHEC</sub> (enterohemorrhagic *E. coli* [EHEC] O157:H7) is devoid of an Y474 equivalent and employs EspFU/TccP (Tir-cytoskeleton coupling protein), a T3SS-translocated effector protein that binds N-WASP, leading to Nck-independent actin polymerization.<sup>45</sup>

aEPEC are devoid of pEAF and do not produce BFP. It is important to point out that EPEC strains of serotypes O128:H2 and O119:H2 contain a pEAF with defective *bfp* operons, which contain part of the *bfpA* gene but have the rest of the *bfp* gene cluster deleted. Thus, they are classified as aEPEC.<sup>12,75</sup> Most aEPEC produce adherence patterns categorized as LA-like, with loosened microcolonies compared to those of the tEPEC LA pattern.<sup>12,76,77</sup> In addition, some isolates express the aggregative (AA) or diffuse (DA) patterns of adherence, which are characteristics of the EAEC and DAEC pathotypes, respectively,<sup>20,78</sup> or adhere in undefined patterns or are non-adherent.<sup>20,78-80</sup> Remarkably, the epithelial cell adherence phenotype displayed by aEPEC is determined in prolonged assays (6h) of bacteria-cell interaction.<sup>12,76,77</sup> In addition, it has been suggested that lack of the pEAF-encoded Per proteins in the regulatory cascade of the aEPEC virulence genes may promote delayed AE lesion formation, probably making it difficult for such strains to cause disease.<sup>81</sup>

The prevalence of intimin subtypes among aEPEC strains has been reviewed.<sup>18,67</sup> Intimins classified as beta1, epsilon1 and theta appear as the most frequent among aEPEC.<sup>78,82-85</sup> In addition, some aEPEC strains bear adhesive-encoding genes that have been originally described in other DEC pathotypes and/or in extraintestinal pathogenic *E. coli*.<sup>79,80,82,86-88</sup> This observation suggests that aEPEC could employ additional adherence mechanisms besides the Tir-intimin interaction. The only adhesin first characterized in an aEPEC strain (serotype O26:H11) is the locus of diffuse adherence (LDA), which is an afimbrial adhesin that confers the diffuse pattern of adherence on HEp-2 cells, when cloned in *E. coli* K-12 strains.<sup>89</sup> The T3SS-translocon has been also shown to contribute to the adherence efficacy of an aEPEC strain *in vitro*.<sup>90</sup> The prevalence of these different adhesins among aEPEC has been recently reviewed.<sup>18,67,91</sup>

Moreover, it has been recently shown that the flagellar cap protein FliD of an aEPEC strain (serotype O51:H40) binds to unknown receptors on intestinal Caco-2 cell microvilli.<sup>92</sup> Interestingly, an anti-FliD serum and purified FliD reduced adherence of the aEPEC as well as that of tEPEC, EHEC and ETEC prototype strains to the same cell line.<sup>92</sup> Furthermore, it has been suggested that adherence of aEPEC of serotype O26:H11 may be mediated by binding of the flagellin protein FliC (the subunit of the flagella shaft) to cellular fibronectin.<sup>93</sup> However, the role of the flagella in aEPEC *in vivo* colonization has yet to be investigated.

Atypical EPEC strains have also been shown to adhere to abiotic surfaces (polystyrene and glass).<sup>94,95</sup> The non-fimbrial adhesin curli and the T1P have been shown to mediate binding to these surfaces in some aEPEC at different temperatures.<sup>90,96</sup>

The LEE region of some aEPEC strains display a genetic organization similar to that found in the tEPEC prototype E2348/69 strain.<sup>97</sup> Although the T3SS-encoding genes are considerably conserved,<sup>97,98</sup> the effector protein-encoding genes display important differences, and remarkable differences can be detected at the 5' and 3' flanking regions of aEPEC, suggesting the occurrence of different evolutionary events.<sup>99</sup> Atypical EPEC strains may carry two *tccP* variants, *tccP* and/or *tccP2*, suggesting that some aEPEC strains may use both Tir-Nck and Tir-TccP pathways to promote actin polymerization.<sup>100</sup> Interestingly, Rocha and colleagues<sup>101</sup> showed that transformation of a non-adherent aEPEC strain (serotype O88:HNM) with a TccP expressing-plasmid, conferred this strain the ability to adhere to and to induce actin-accumulation in HeLa cells.

The occurrence and prevalence of Nle in aEPEC strains have been recently reviewed.<sup>67</sup> It has been suggested that different isolates can employ distinct strategies to promote damage to the host and cause disease.<sup>45</sup> In addition, the Nle effectors Ibe (invasion of endothelial cells) and EspT have been originally described and characterized in aEPEC strains.<sup>102,103</sup> Ibe appears to regulate Tir phosphorylation and to enhance actin polymerization and pedestal formation,<sup>103</sup> while EspT<sup>104</sup> modulates actin dynamics, leading to membrane ruffling and cell invasion, and induces macrophages to produce interleukins IL-8 and IL-1 $\beta$  and PGE2.<sup>102</sup>

Invasion of epithelial cells *in vitro* in an intimin-dependent pathway has been described in an aEPEC strain,<sup>105</sup> but further studies pointed out that the invasive phenotype is not a common characteristic among aEPEC.<sup>106</sup> Despite their invasive

potential *in vitro*,<sup>107</sup> most aEPEC are considered extracellular pathogens.<sup>5</sup>

It has been shown that apical infection of cultured human mucin-secreting intestinal HT29-MTX cells by some aEPEC strains may induce increased production of secreted MUC2 and MUC5AC mucins and membrane-bound MUC3 and MUC4 mucins.<sup>108</sup> This observation suggests that the apically adhering bacteria could exploit large amounts of mucins to grow more efficiently in the host intestines, characterizing a putative new virulence mechanism in aEPEC.<sup>108</sup>

AT proteins have also been shown to be produced by some aEPEC strains.<sup>67</sup> Abreu and colleagues<sup>109</sup> have shown that the AT protein encoded by the *ehaC* gene, which is involved in biofilm formation in EHEC strains, was the most frequent, with a significantly higher prevalence than in tEPEC. Although the prevalence of the AT protein Pic (protein involved in intestinal colonization), formerly identified in EAEC, is not a common finding in aEPEC strains, it also appears to mediate colonization of mouse intestines, hemagglutination, mucin cleavage, and complement components degradation.<sup>110</sup> More recently, some aEPEC strains were shown to cause cell damage by secreting the AT protein Pet (plasmid encoded toxin) to the extracellular environment.<sup>111</sup>

## Epidemiology

The prevalence of EPEC infections varies between epidemiological studies on the basis of differences in study populations, age distributions, and methods (serotyping, adherence patterns, and presence of the *eae* or conserved LEE genes) used for detection and diagnosis.<sup>112</sup> In addition, differences in geographic regions, periods of time and socioeconomic class may also contribute to differences in the epidemiology of EPEC-induced diarrheal disease.<sup>113</sup> Lack of discrimination between tEPEC and aEPEC in some studies also makes such analysis difficult.

Diarrhea due to tEPEC decreases with age, and infections in adults are rarely reported. This apparent resistance in adults and older children has been attributed to the loss of specific receptors with age or development of immunity.<sup>6</sup>

For many decades, studies conducted worldwide have shown that tEPEC serotypes are strongly associated with diarrhea in children <1 year of age, mainly in poor children in urban centers.<sup>6,12,15</sup> The association with diarrhea was particularly strong in infants less than 6 months of age. Studies in Brazil, Chile, Mexico, and South Africa, showed that 30–40% of infantile diarrhea cases were due to tEPEC serotypes.<sup>15,112,114</sup> However, the epidemiology of EPEC infections has shifted. In numerous developing countries, where the prevalence of EPEC infection had been high until the 1990s, recent studies have not identified a significant association between tEPEC and infantile diarrhea. In Brazil, 92% of EPEC isolates collected from children between 2001 and 2002 were atypical,<sup>115</sup> compared to 38% in a 1998–1999 study.<sup>79</sup> However, other studies still report tEPEC being more prevalent than aEPEC as a cause of diarrhea.<sup>116</sup> In addition, in some less developed areas (Africa and Asia), tEPEC are still some of the most important enteropathogens.<sup>117–122</sup> Based on the recently completed Global Enteric Multicenter Study (GEMS) involving children

less than 5 years of age from seven sites in Africa and Asia, tEPEC was significantly associated with moderate to severe diarrhea in children under 2 years of age in Kenya, whereas aEPEC was not associated with this type of diarrhea.<sup>118</sup>

Transmission of tEPEC follows a fecal-oral process through contaminated surfaces, weaning fluids, and human carriers.<sup>123</sup> Although rare, outbreaks among adults seem to occur through the ingestion of contaminated food and water; however, no specific environmental reservoir has been identified.<sup>6</sup> The infective dose in adult volunteers is high, at  $10^8$  to  $10^{10}$  organisms,<sup>124</sup> while the infective dose that causes disease in children is unknown. EPEC outbreaks have been reported to show a seasonal distribution with peaks during the warm months.<sup>6,125</sup> Humans are the only known reservoir for tEPEC, with symptomatic and asymptomatic children and asymptomatic adults being the most likely source.<sup>6</sup>

In contrast to tEPEC, aEPEC have been found in diarrheic patients of all ages and in adults with HIV-AIDS.<sup>82,126–128</sup> Furthermore, the proportion of aEPEC strains has increased, and aEPEC strains have outnumbered tEPEC strains and have also been associated with childhood diarrhea in some developing and developed countries.<sup>12,18,67,91,112,129</sup> However, the increase in prevalence of aEPEC may also reflect the refined discrimination between tEPEC and aEPEC.<sup>12,18,91</sup>

The role of aEPEC in diarrhea is not clear because of its detection at similar rates in both diarrheic and non-diarrheic patients in various geographical areas.<sup>18,91,128</sup> In studies conducted in the last five years, aEPEC have been found at rates varying from ~0.05 to ~12% in diarrheic versus 0 to ~14% in non-diarrheic patients.<sup>67</sup> Some recent studies have also implicated aEPEC as the cause of persistent and bloody diarrhea.<sup>18,91</sup> Moreover, aEPEC strains have been associated with diarrheal outbreaks in Finland, United States, Japan, China<sup>18,91,112</sup> and Brazil.<sup>85</sup>

In contrast to tEPEC, which are seldom found in animals,<sup>12</sup> many aEPEC strains have been found in both diarrheic and healthy animals.<sup>18,67</sup> Interestingly, animal aEPEC serogroups associated with human diarrhea have been identified (e.g., O26, O103, O119, O128, O142 and O157).<sup>18,130,131</sup> Serotyping and molecular methods such as multilocus sequence typing (MSLT) and pulsed field gel electrophoresis (PFGE) have contributed to demonstrating that domestic and wild animals and the environment are potential sources of aEPEC for human infections in several regions.<sup>18,67,91,131</sup> Therefore, although no direct transmission from animals to humans has been shown so far, it is reasonable to suggest that some aEPEC strains are potentially zoonotic pathogens, with a large variety of animal species serving as important reservoirs.<sup>67,91</sup> In addition, foods including raw meat, pasteurized milk and vegetables and water have also been implicated as vehicles of aEPEC in human infections.<sup>67</sup>

aEPEC strains comprise a very assorted group with various additional virulence mechanisms that altogether can modulate the disease outcome or their occurrence in asymptomatic persons. There have been continuous advances in our knowledge of the genetic background and pathogenicity of aEPEC as well as in the information gathered from epidemiological studies, and may contribute to the discrimination between strains that cause diarrhea and those that cause asymptomatic infections.

## Detection and diagnosis

EPEC can be detected by DNA probes or PCR assays using primers targeting the *eae* and *stx* genes.<sup>132,133</sup> All *eae*-positive and *stx*-negative *E. coli* strains are further tested by PCR for the presence of the *bfpA* gene encoding bundlin<sup>6</sup> and/or the EAF plasmid to differentiate tEPEC from aEPEC.<sup>134,135</sup> However, this may fail to identify all *bfpA*-positive EPEC strains, since multiple alleles of *bfpA* have been identified,<sup>136</sup> suggesting that some current PCR methods may fail to identify all *bfpA*-positive EPEC strains.

However, in routine microbiology laboratories, all *E. coli* colonies obtained from primary isolation plates are traditionally screened by slide agglutination assays using sera against the classical EPEC serogroups O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158.<sup>137</sup> This method is practical and easy to perform, the main advantage of which is the commercial availability of the sera. However, the disadvantage of this method is the heterogeneity of EPEC serogroups that can comprise categories other than EPEC, the inability to distinguish tEPEC from aEPEC within these serogroups, and the occurrence of EPEC strains belonging to serogroups other than the classical EPEC serogroups.<sup>12,18,138,139</sup>

Since EPEC strains are defined based on their virulence properties, a set of proteins, including intimin, BFP and T3SS secreted proteins can be considered targets for diagnosis. BFP expression has been considered the phenotypic marker of tEPEC.<sup>18,78,140</sup> Immunofluorescence and immunoblotting tests using monoclonal or polyclonal antibodies against BFP have been employed.<sup>141,142</sup> These cited authors detected the production of BFP on different media, in which they reported that 91% of the tEPEC strains tested produced BFP in Dulbecco's Modified Eagle Medium (DMEM), 89% in MacConkey, and 83% in EMB agars. These results are particularly interesting, since MacConkey and EMB agars are routinely used for the identification of lactose-fermenting *E. coli* isolated from diarrheal stools. A colony immunoblot assay for tEPEC detection based on BFP expression was also standardized using a rabbit tEPEC anti-BFP polyclonal serum. Standardization was done after growing the bacterial isolates on DMEM agar containing fetal bovine serum or tryptic soy agar containing 5% washed sheep blood (TSAB). This test showed a positivity of 92 and 83% and specificity of 96 and 97%, respectively, when the culture was done in DMEM and TSAB. This method combines the simplicity of an immunoserological assay with the high efficiency of testing a large number of EPEC colonies.<sup>140</sup>

Concerning intimin detection, a rabbit polyclonal sera raised against the conserved region of intimin (Int388-667)<sup>143</sup> was employed in order to detect tEPEC isolates expressing  $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$  and  $\epsilon$  intimin reported an application of immunoblotting with 100% specificity and 97% sensitivity in the detection of *eae* positive *E. coli* strains.<sup>144-146</sup> These authors clearly demonstrated that polyclonal rabbit antisera is suitable for immunoblotting as a diagnostic tool, and showed that protein denaturation and linearization is a critical step for anti-intimin antibody accessibility. Indeed, even employing the recombinant antibody such as single chain fragment variable (scFv-intimin),<sup>147,148</sup> merely by immunofluorescence the scFv-intimin was able to detect tEPEC, aEPEC, and EHEC isolates,

showing that intimin can be a target for EPEC and EHEC diagnosis after bacterial permeabilization.<sup>148</sup>

Regarding secreted proteins, Lu et al.<sup>149</sup> developed a new practical method to identify EPEC by detecting the *E. coli* secreted protein B (EspB) in the culture supernatant by reversed passive latex agglutination (RPLA), after the strains have been cultivated in DMEM. In addition, Nakasone et al.,<sup>150</sup> established a rapid immunochromatographic (IC) test to identify the presence of EspB in EPEC and EHEC isolates. The detection limit of the test has been reported to be 4 ng/mL, and the results showed 96.9% sensitivity and 100% specificity. The IC test for the detection of EspB may be a practical method to define EPEC or EHEC both in clinical laboratories and the field.<sup>150</sup>

In addition, a rapid agglutination test using latex beads coated with anti-EspB mAb was standardized, showing 97% sensitivity, 98% specificity and 97% efficiency, which is required for the diagnosis of enteropathogenic diseases and can be employed in developing countries with poorly equipped laboratories.<sup>151</sup>

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## Enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC)

EHEC/STEC represent a well-known group of foodborne pathogens distributed worldwide. The ability to produce one or more of the Shiga toxin (Stx) family cytotoxins<sup>152</sup> constitutes the main virulence attribute of this pathogroup of *E. coli*. A wide array of infections from mild and almost unapparent diarrhea to more serious manifestations such as hemorrhagic colitis (HC) and the development of a life-threatening syndrome known as hemolytic uremic syndrome (HUS) are caused by EHEC/STEC. Infants and children are the main affected patients, and although the incidence of infection varies in different regions, the impact and importance of EHEC/STEC infections in public health is immense, being the main cause of acute renal failure in children in many countries. The perspective of EHEC/STEC infections has been previously described,<sup>153,154</sup> but a considerable amount of information has been obtained in more recent years related to the epidemiology, ecology and virulence properties of these bacteria.

*E. coli* O157:H7 serotype was the first to be linked to HC and HUS cases in the early 1980s, and has been since then responsible for numerous outbreaks and sporadic cases of severe diseases all over the world, therefore considered to be the prototype of this pathogenic group of bacteria.<sup>155</sup> It is well known that hundreds of other *E. coli* serotypes can harbor the *stx* genes, but epidemiological studies carried out worldwide have proven that only some of them have been responsible for causing human diseases. Some serogroups including O26, O45, O103, O111, O121 and O145 can be highlighted among those most commonly related to human infections.<sup>156</sup> Moreover, in recent years the emergence of some particular clones such as the hybrid O104:H4 enteroaggregative *E. coli* carrying *Stx2* genes, responsible for a severe outbreak of HUS starting in Germany in 2011,<sup>157</sup> the spread of a new O26:H11 clone in Europe,<sup>158</sup> and some other hybrid clones,<sup>159</sup> suggests that

the mobility of genes and certainly the host background are important features implicated in their pathogenic potential.

### **Virulence factors, mechanisms and pathogenesis**

The common feature among EHEC/STEC isolates is the ability to produce Stx. This family of toxins has a conserved AB<sub>5</sub> subunit structure, composed of one active A subunit linked to a pentameric B subunit responsible for the binding of the toxin to specific glycolipid receptors on the surface of target cells. The *stx* operon is usually found within the sequence for an inducible, lysogenic, lambda-like bacteriophage. Stxs inhibit protein synthesis by removing an adenine residue from the 28S rRNA of the 60S ribosome.<sup>152</sup> However, besides this activity, studies have described that Stx also acts on cell signal transduction and immune modulation causing proinflammatory and pro-apoptotic responses.<sup>159</sup> Two major families, Stx1 and Stx2, have been recognized, and on the basis of sequence diversity, each is composed of several variants. The Stx1 family is more homogenous and includes Stx1a, Stx1c and Stx1d; while the heterogenous Stx2 group is composed of Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, and Stx2g.<sup>160</sup> It should be mentioned that the association of some variants such as Stx2a, Stx2c or Stx2d with HC and HUS has been highlighted compared to some others that seemed to be more related to uncomplicated cases of diarrhea such as Stx1 variants or even Stx2e, Stx2f and Stx2g, which are uncommonly found causing human infections so far.<sup>161,162</sup> Indeed, the higher association of Stx2 with severe diseases has been extensively studied by using Vero and endothelial cell lines as well as some animal models.<sup>159</sup> Moreover, knowledge of *stx* phage characteristics and behavior has helped our understanding of how differences in expression of Stx between EHEC/STEC isolates may contribute to pathogenesis and disease.<sup>163</sup>

The ability to adhere to intestinal epithelial cells is another key event in EHEC/STEC pathogenesis. The presence of the chromosomal pathogenicity island LEE,<sup>164</sup> also present in isolates belonging to the EPEC pathotype, is common. Although LEE has been described in the major EHEC/STEC serotypes responsible for a high proportion of HC and HUS cases in several countries, its presence is not a required condition for the occurrence of more serious infections as initially thought, because some LEE-negative strains are also capable of causing outbreaks and sporadic cases of HUS.<sup>165,166</sup>

Therefore, it is clear that EHEC/STEC pathogenesis is a multistep process, and besides the production of Stx toxins and the AE lesion, other factors including different types of toxins and adhesins have been described and found to be involved in virulence.<sup>159</sup>

One should also consider that as a pathogen of the human gastrointestinal tract the ability of EHEC/STEC to monitor nutrients in the gut milieu, and translate this information to sense the host physiological state in order to program the expression of its virulence markers has a pivotal role on the development of infection.<sup>167</sup> In addition, it has been shown that EHEC/STEC can also cross-communicate with the host by exploiting the autoinducer-3 (AI-3)/epinephrine/norepinephrine signaling

system to express two important virulence traits, motility and A/E lesion, required at different time points during intestinal colonization.<sup>168</sup>

The ability to adhere, colonize and form biofilm on food and several types of surfaces may be a way to be an important source and/or vehicle of transmission of EHEC/STEC. In addition, biofilm may also act as bacterial protection against adverse environmental conditions. A study conducted by Biscola et al.,<sup>169</sup> evaluated the capacity of biofilm formation in EHEC/STEC strains isolated from different reservoirs and serotypes. The authors observed that the ability to adhere to abiotic surfaces forming biofilms, under defined culture conditions, occurred in an array of wild-type O157 and non-O157 strains. Biofilm production was identified in several non-O157 STEC serotypes of human, animal, and food origin. On the other hand, among the O157 strains, only those isolated from the animal reservoir and from a water sample produced biofilm. A close correlation between biofilm formation and expression of curli fimbriae and cellulose was observed among O157 strains. However, in addition to curli, the presence of other factors such as type 1 fimbriae and AT proteins may be associated with the ability to form biofilm in non-O157 strains. Matheus-Guimarães et al.,<sup>170</sup> studied O157 and non-O157 EHEC/STEC strains isolated from bovine hides and carcasses and showed that different sets of genes were involved in the interactions of the bacteria with biotic and abiotic surfaces. Moreover, the detection of an O157 strain that was able to form biofilm on both glass and polystyrene and that adhered to and invaded human cells, suggests an important ability of this isolate to persist in the environment and interact with the host. In fact, cell invasion and survival of some EHEC/STEC strains in cultured human intestinal epithelial cells has been previously described.<sup>171</sup> It should be mentioned that this invasive characteristic has been identified in some EHEC/STEC serotypes, many of which are responsible for human infections.<sup>170–173</sup> Therefore, it is conceivable that this virulence strategy may help bacteria to overcome host defense mechanisms and certainly contributes to their persistence in the zoonotic reservoir, ensuring efficient environmental and food transmission.

Another topic of interest has been the analysis and comparison of the virulence profile of EHEC/STEC strains isolated from the animal reservoir and environment with strains recovered from human infections. In general, these studies have shown that despite serotype diversity, the *stx* subtypes and the virulence profile identified among isolates from the animal reservoir and environment are similar to the isolates recovered from patients.<sup>173,178–180</sup> There has been particular interest in some STEC serotypes that have been responsible for causing severe human infections, such as O113:H21, but unlike others, they do not produce adhesins encoded by LEE. By using a PCR microarray, 41 virulence or genetic markers were tested in a panel of 65 O113:H21 strains isolated from clinical infections, environment and food from various countries.<sup>174</sup> The results obtained showed no clear differences in these genetic markers between the pathogens recovered from HUS cases and the environmental strains. Moreover, only *stx* subtypes associated with human infections were identified in all isolates, therefore suggesting that the environmental isolates have the potential to cause human diseases.

## Epidemiology

The incidence of HUS cases in Brazil is low,<sup>175</sup> and although some hypothesis has been proposed to explain this fact, there are limited data on the immune response against Stx. In an attempt to overcome this gap, prevalence of anti-Stx2 antibodies in sera of children diagnosed with HUS and of healthy children was recently determined.<sup>176</sup> The percentage of individuals showing antibodies against Stx2 was higher among HUS patients than controls, and the results also confirmed that STEC strains are circulating in our settings despite the low number of identified HUS cases.

Among the several serotypes associated with human infections, O157:H7 is responsible for more severe cases. Epidemiological investigations of diarrheal outbreaks conducted in four Brazilian states showed that O157:H7 strains were isolated from two hospitalized patients, one with HUS and the other with bloody diarrhea.<sup>177</sup> Besides, O157:H7, EHEC/STEC strains belonging to the top six most important non-O157 serogroups such as O26, O103, O111 and O145 were identified, all of which were recovered from ambulatory patients. In addition, some uncommon serogroups including O1, O24 and O77 among others were also detected, but they were all associated with acute diarrhea. It is interesting to note that the majority of patients from whom STEC was isolated were female (57%), and that patients' ages ranged from 8 months to 80 years, with most being less than five years old (54%).<sup>177</sup>

The distribution of EHEC/STEC in the gastrointestinal tract of a wide variety of animals indicates the zoonotic character of its infections. The role of different animal species as asymptomatic carriers of EHEC/STEC has been extensively studied in the last years in Brazil. Besides cattle, which are their most common natural reservoir,<sup>173,178</sup> the presence of these pathogens has been identified in the feces of dairy buffaloes,<sup>179</sup> sheep,<sup>180,181</sup> pigs,<sup>182,183</sup> birds,<sup>184</sup> and fishes.<sup>185</sup> It is noteworthy that some relevant serotypes linked to human infections such as O103:H2 and O157:H7 have been recovered from the feces of sheep<sup>186</sup> and cattle<sup>173</sup> respectively. Additionally, the high prevalence of O157:H7 EHEC/STEC strains identified in hides of cattle sent to slaughter in a Brazilian processing plant<sup>178</sup> certainly represents a relevant issue that should be considered when thinking about interventions targeting EHEC/STEC related to animal handling, from farm to slaughter, as well as the implementation of food safety throughout production and processing.

The presence of EHEC/STEC in the environment is another issue of concern, since they can survive in the soil, manure, pastures and water, which thus represent important vehicles of transmission. The isolation of STEC strains from drinking water supplies, collected in different municipalities in northern Paraná State, has been recently described, highlighting the importance of drinking water, especially that from untreated water supplies, as a source of STEC strains potentially pathogenic for humans.<sup>187</sup> Taking into account that chicken litter is very useful as an organic soil fertilizer for the production of fruits and vegetables in our settings, the detection of STEC in organic chicken fertilizer used on farms<sup>188</sup> also represents a significant public health safety hazard.

Although data on the detection of EHEC/STEC in foods in Brazil are still scarce, the isolation and identification of O157:H7 serotype from a ground beef sample was described for the first time,<sup>189</sup> while O125:H19 and O149:H8 STEC serotypes were found in refrigerated raw kibbe collected from retail establishments.<sup>190</sup> On the other hand, EHEC/STEC has not been detected in pasteurized cow's milk samples collected in dairies in northwestern Paraná State<sup>191</sup> or in raw milk, pasteurized milk, Minas Frescal cheese and ground beef samples collected in Minas Gerais.<sup>192</sup> One should be aware that despite difficulties in the detection and isolation of EHEC/STEC from foods, the implementation of the most sensitive methods in most laboratories should be the main goal in the near future to help in the analysis of the risk posed by foods as vehicles of STEC transmission to humans.

## Detection and diagnosis

An important concern is how to detect Shiga toxin-producing strains either in stools of infected patients or contaminated food, since selective enrichment is necessary.<sup>193,194</sup> For routine diagnosis, some protocols have already been described.<sup>139</sup> However, the gold standard for Stx detection is still the evaluation of the cytotoxicity of bacterial culture supernatants to eukaryotic cells.<sup>195,196</sup> Thus, multiplex PCR including stx gene and other virulence genes could be useful in screening for STEC using bacterial confluent growth zones or sorbitol fermenting and non-fermenting colonies taken from SMAC.<sup>197</sup>

Numerous assays for the diagnosis of STEC have been developed on the basis of the detection of Stx1 and/or Stx2, which represents the major virulence factors of this *E. coli* category.<sup>198</sup> Sensitivities and specificities vary according to the test format and the manufacturer.<sup>199-205</sup> Nevertheless, the standard by which each manufacturer evaluates its tests also varies; therefore, a direct comparison of performance characteristics of various immunoassays has not been performed.<sup>198,206,207</sup> Moreover, these commercially available tests are not affordable for developing countries. Thus, to outline this, previous works have established different formats of immunoassays, employing either a mixture of rabbit anti-Stx1 and anti-Stx2 sera by indirect ELISA or polyclonal and monoclonal antibodies in a capture ELISA assay for the detection of STEC.<sup>207-209</sup> The standardized methods are reproducible, fast, easy to perform, showing high sensitivity in detecting Stx by capture ELISA, even in low-producing isolates. These assays have not yet been evaluated in terms of industrial quality control and commercial availability, but the estimated cost of the assay is around US\$70 per 96 detections, which is realistically inexpensive for developing countries.

These monoclonal antibodies were rebuilt resulting in single chain fragment variable (scFv) fragments. Stx2-scFv was obtained from a bacteria-induced culture and showed diagnostic ability; the scFv fragment was able to recognize the majority of Stx2-producing strains, with 79.3% sensitivity (confidence interval of 60.3 to 92%), and no reactivity was observed with the non-producing strains, indicating as high as 100% specificity (confidence interval of 86.8-100%).<sup>210</sup> It is worth mentioning that none of the commercially available



immunoenzymatic tests for Stx1/2 toxin detection employ recombinant antibodies produced in bacteria, which indeed will reduce the costs of the diagnostic assays.<sup>198</sup>

## Enteroaggregative *E. coli*

EAEC is the diarrheagenic *E. coli* pathotype defined by showing the characteristic AA pattern on epithelial cells in culture.<sup>211</sup> The AA pattern was defined in 1987 when Nataro et al.<sup>212</sup> distinguished the previously described “diffuse adherence” as the truly diffuse adherence (DA) and the AA pattern. The standard AA was characterized by adherent bacteria in a stacked-brick arrangement on the surface of epithelial cells and also on the coverslip between cells. Strains displaying the AA pattern were then categorized as “enteroadherent-aggregative *E. coli*” but afterwards the category was called enteroaggregative *E. coli* or EAEC, the current nomenclature. The detection of AA *in vitro* is still the gold standard test to define EAEC; however, as described before, the AA pattern may be found in strains of other DEC pathotypes, such as aEPEC. Therefore, an up-to-date definition of EAEC is the diarrheagenic *E. coli* that produce AA in cultured epithelial cells but lack the main genetic markers that define other DEC pathotypes (EPEC, ETEC, EHEC, EIEC). An exception for that is the hybrid EAEC/STEC strain responsible for a massive outbreak of diarrhea and HUS in 2011 in Europe.<sup>213</sup> This strain consists of an EAEC strain that acquired the Stx2-encoding phage. Therefore, this specific O104:H4 strain is a Stx-producing EAEC.

Diarrhea caused by EAEC is watery, often with the presence of mucus, with or without blood and abdominal pain, vomiting and low fever. Acute self-limiting diarrhea is the usual pathology, but some patients may develop protracted diarrhea, i.e., lasting more than 14 days.<sup>214</sup> Prolonged diarrhea occurs depending on the host’s immunity, nutritional status and genetic susceptibility.<sup>215</sup> Genetic susceptibilities associated with EAEC diarrhea were identified in North American travelers to Mexico. Single nucleotide polymorphisms (SNP) in the IL-8 gene promoter and the promoter regions of the genes encoding lactoferrin, CD14 and osteoprotegerin as well were recognized as indicators for symptomatic EAEC infection.<sup>216–219</sup>

A well-described characteristic of EAEC strains is their heterogeneous nature when serotypes, genetic markers of virulence and phylogenetic groups are analyzed.<sup>220–225</sup> This indicates that only EAEC strains carrying specific virulence factors are able to cause diarrhea. While these factors are unknown, some studies have demonstrated the association of specific virulence genes with diarrhea, such as *pet* or *aafA* in Brazil<sup>226</sup> and *sepA* in Mali.<sup>223</sup>

## Virulence factors, mechanisms and pathogenesis

Most of our knowledge about EAEC pathogenesis is based on data accumulated from studies with EAEC strain 042, since its association with human diarrhea in a volunteer study.<sup>227</sup> These putative virulence factors include adhesins, toxins and secreted proteins. However, none of these factors are found in all EAEC strains.

The majority of these virulence factors are plasmid borne, including those mediating AA. Consequently, these high-molecular-weight plasmids are called pAA.<sup>220</sup> Baudry et al.,<sup>228</sup> developed a genetic probe (CVD432) for EAEC diagnosis on the basis of a fragment from pAA1 present in EAEC strain 17-2. In EAEC 042, many putative virulence factors are present in pAA2.<sup>220</sup>

Recently, a division of EAEC strains into typical or atypical subgroups was proposed. This classification is based on the presence or absence of *aggR*, a gene that encodes a global regulator of EAEC virulence genes.<sup>229</sup> Therefore, it has been proposed that typical EAEC have more pathogenic potential by the presence of the AggR regulon and, consequently, pAA virulence factors.<sup>230</sup> However, at least two outbreaks of diarrhea were caused by atypical EAEC,<sup>231,232</sup> and atypical EAEC are commonly isolated from children with diarrhea, in some cases more frequently than typical strains.<sup>233,234</sup>

Numerous adhesins, cytotoxins, enterotoxins and secreted proteins have been characterized in EAEC strains since this pathotype definition.<sup>211,214</sup>

The most studied adhesins are the aggregative adherence fimbria (AAF/I-AAF/V) family, which includes five types.<sup>235–239</sup> They mediate the AA pattern and biofilm formation. Afimbrial adhesins have also been characterized in EAEC strains, including outer membrane proteins between 30 and 58 kDa.<sup>240–242</sup> However, it has been shown that these structures are present in low frequencies in EAEC collections from different settings.<sup>221,226,243–245</sup>

Located in pAA2 of EAEC 042 is the *aap* gene, encoding an antiaggregation protein called dispersin.<sup>246</sup> This protein is secreted and linked to lipopolysaccharide, neutralizing the negative charge of the bacterial surface leading to AAF projection and consequent dispersion along the intestinal mucosa.<sup>247</sup> Although immunogenic, dispersin is found in other *E. coli* pathotypes and in commensal *E. coli*.<sup>248</sup>

Various toxins have been described in EAEC in association with the cytotoxic or enterotoxic effects of culture supernatants *in vitro*. The heat-stable toxin enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST-1) was the first toxin characterized in the EAEC pathotype.<sup>249</sup> EAST-1 activates adenylate cyclase inducing increased cyclic GMP levels, effects observed in a Ussing chamber with rabbit ileum.<sup>250</sup> ShET1 is an A:B type toxin that causes accumulation of fluid in rabbit ileal loops and has secretory response in Ussing chamber assays.<sup>251,252</sup>

The two AT proteins characterized in EAEC 042, Pet and Pic,<sup>253–254</sup> are members of the serine protease autotransporters of Enterobacteriaceae, or SPATE.<sup>255</sup> Pet is a cytotoxin that modifies the cytoskeleton of enterocytes, leading to rounding and cell detachment. The cytotoxic mechanism of Pet arises from the degradation of  $\alpha$ -fodrin, a membrane protein of the enterocytes.<sup>256</sup> Pic is a multitask protein that mediates hemagglutination, mucus cleavage and hypersecretion, intestinal colonization in mice, cleavage of surface glycoproteins involved in leukocyte trafficking and cleavage of key complement molecules.<sup>257,258</sup> The phenotypes identified for Pic suggest its role in promoting colonization of the intestine and immune system evasion. SPATEs are immunogenic proteins, as evidenced by the presence of serum antibodies against Pet and Pic in children recovering from diarrhea caused by EAEC.<sup>259</sup>

In the years that followed the definition of EAEC as a pathotype, research in the field was dedicated to prove the pathogenic capacity of EAEC using different animal models<sup>260-262</sup> and human volunteers receiving oral inoculum of different EAEC strains.<sup>227,235,263</sup> Not all volunteers developed diarrhea after ingestion of different EAEC strains, the first evidence that strains of this pathotype are heterogeneous. Among the strains tested, EAEC O42 (serotype O44:H18) caused diarrhea in three out of five volunteers.<sup>227</sup> Since then, strain O42 has been considered the prototype EAEC strain and is certainly the most studied strain of the pathotype.<sup>264</sup> EAEC O42 was isolated from a case of acute infantile diarrhea in Peru.<sup>265</sup> The clinical data obtained from the volunteers who developed diarrhea suggested that EAEC O42 caused secretory diarrhea, with abundant presence of mucus and absence of blood in the stool.

Studies employing different EAEC strains interacting with intestinal cells from animals or humans have been performed to elucidate the pathogenesis of this pathotype. Data from these *in vitro*, *in vivo* and *ex vivo* experiments strongly indicate that EAEC can bind to jejunal, ileal and colonic epithelium in the characteristic aggregative pattern, forming a strong biofilm in a mucus layer, followed by cytotoxic and proinflammatory effects.<sup>260,266-270</sup> Fragments from terminal ileum and colon excised from pediatric and adult patients were incubated with EAEC strains that were capable to colonize the ileal and colonic mucosa in the typical stacked-brick pattern over an augmented mucus layer.<sup>270</sup>

All these lines of evidence in combination with the identification of several putative virulence factors in prototype EAEC strains allowed the proposal of a three-stage model of EAEC pathogenesis: (a) abundant adherence to the intestinal mucosa, (b) production of cytotoxins and enterotoxins, and (c) induction of mucosal inflammation.<sup>211</sup> In the first stage, the contribution of fimbrial and afimbrial adhesins as well as other adhesive structures is essential. Several colonization factors have been identified in EAEC strains.<sup>271</sup> In this stage, a characteristic increased secretion of mucus on the intestinal mucosa leads to the formation of a strong biofilm where EAEC are embedded.<sup>234,266,272</sup> In the following step, EAEC produce cytotoxic effects on the intestinal mucosa due to the secretion of toxins, inducing microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion.<sup>266,273</sup> EAEC-induced inflammation results from the strong colonization of the intestinal mucosa; however, all bacterial factors that contribute to this condition have not been identified. Inflammatory markers such as IL-8, IL-1 $\beta$ , interferon (INF)- $\gamma$  and lactoferrin have been detected in stools of children and adults colonized by EAEC.<sup>274-276</sup> Although this model summarizes the data so far obtained using *in vivo*, *in vitro* and *ex vivo* approaches it may not be valid for all strains.

A large foodborne outbreak of bloody diarrhea and HUS occurred in 2011 in Europe, affecting more than 4000 patients, most of them from Germany. This outbreak was caused by a Stx2-producing *E. coli* strain belonging to the serotype O104:H4. The genome of that strain was rapidly sequenced, revealing a unique hybrid combination of EAEC and STEC, i.e. the EAEC strain Ec55989 harboring the Shiga toxin 2-encoding prophage.<sup>166,213</sup> Several virulence factors of typical EAEC are present in that strain, including AggR, dispersin, Pic

and ShET-1. Also expressed are two *Shigella* autotransporter proteins called SigA and SepA, implicated in mucosal damage and colonization.<sup>277,278</sup> Interestingly, the EAEC Ec55989 is the prototype strain for AAF/III.<sup>237</sup> Conversely, the outbreak hybrid strain produces AAF/I, showing that the outbreak EAEC/STEC acquired an AAF/I-encoding plasmid.<sup>166,279</sup> It has been proposed that the presence of these virulence factors combined is responsible for the highly virulent attributes of that strain.<sup>166,213</sup>

### Epidemiology

EAEC is an emerging pathogen affecting children and adults worldwide, responsible for cases of acute and persistent diarrhea. Nevertheless, the most important impact in terms of morbidity is among children younger than 5 years living in developing countries.<sup>214</sup> A meta-analysis study of the literature on the epidemiology of diarrhea that included the search of EAEC showed a statistical association of EAEC with acute and persistent diarrhea in developed and developing countries, with diarrhea in HIV-infected patients in developing countries, and adult traveler's diarrhea.<sup>280</sup> In another meta-analysis study EAEC was associated with acute diarrhea in children living in South Asian countries.<sup>281</sup>

It is important to mention that data on the epidemiology of EAEC infection are somewhat inconsistent due to large variation in terms of method of detection, geographical location and patient age and socioeconomic status. Nonetheless, EAEC has been systematically identified as an emerging enteropathogen, strongly associated with acute and persistent diarrhea in children of developing countries. Moreover, in developed countries, EAEC have been frequently isolated from cases of diarrhea in children and adults in the last years.<sup>282,283</sup>

In addition, several foodborne outbreaks of diarrhea caused by EAEC have been reported in Europe, Japan, Mexico and India.<sup>231,232,284-286</sup> One of them affected 2697 school children in Japan, after consumption of school lunches.<sup>232</sup>

Several studies have implicated EAEC as the predominant agent of persistent diarrhea in children.<sup>287-289</sup> EAEC-mediated persistent diarrhea has been linked to malnutrition and decrease in physical and intellectual development in several studies from Brazil.<sup>274,288,290</sup> Notably, asymptomatic patients infected with EAEC also exhibit growth retardation.<sup>274</sup> Since its definition as a pathotype, high rates of asymptomatic young children carrying EAEC have been reported in several studies, involving subjects with low socioeconomic status in developing countries.<sup>214</sup> The persistence of EAEC may induce chronic intestinal inflammation, even in the absence of diarrhea, reducing its absorptive function and leading to malnutrition.<sup>274,291</sup> Growth impairment has also been observed in a mouse model of EAEC oral infection.<sup>292</sup> Considering the high number of asymptomatic EAEC-colonized children in low-income countries, this pathotype has an important impact on public health as one cause of impaired physical and cognitive development.

EAEC is transmitted by the fecal-oral route by food or contaminated water.<sup>232,285,286</sup> EAEC were detected in milk samples from infant feeding bottles that were handled by mothers with low socioeconomic status.<sup>293</sup> Also, viable EAEC were isolated in tabletop sauces from Mexican restaurants.<sup>294</sup> No

relationship has been found between EAEC strains isolated from humans and different animal species, indicating that animals may not represent a reservoir of human pathogenic typical EAEC.<sup>295</sup>

EAEC has also emerged in the last years as an agent of urinary tract infections (UTI). Initially, Abe et al.,<sup>296</sup> described the presence of EAEC virulence markers in strains isolated from UTI, which was subsequently observed by others.<sup>297–300</sup> Also, the presence of uropathogenic *E. coli* (UPEC) markers in EAEC collections has been reported.<sup>301,302</sup> These findings pointed out the potential for some EAEC strains to cause UTI.

A community acquired UTI outbreak caused by an EAEC strain of serotype O78:H10, occurred in Denmark.<sup>303</sup> This multiresistant strain belonged to the multilocus sequence type ST10 and phylogenetic group A. This was the first time that EAEC was implicated as an agent of an outbreak of extraintestinal disease. The uropathogenic properties of this EAEC strain were conferred by specific virulence factors, such as the AAF/I fimbriae.<sup>304</sup> Recently, EAEC was implicated as a causative agent of one case of urosepsis.<sup>300</sup>

### Detection and diagnosis

Among the DEC pathotypes, EAEC is the most difficult to categorize, since it is a very heterogeneous group. The defining characteristic of EAEC is the AA pattern in human epithelial cells or on a glass substrate in a distinctive stacked-brick formation. Thus, the gold standard method for distinguishing EAEC is to culture five *E. coli* colonies per patient in static Luria-broth at 37 °C, and then to infect semi-confluent HEp-2 cells for 3 or 6 h, looking for the typical AA pattern.<sup>212,305</sup> However, this test requires specialized facilities and is time-consuming, restricting its use only to research and certain reference laboratories.

Furthermore, despite that several protein components such as Pic, ShET1, EAST-1, and Pet are involved in the virulence of EAEC, none of them is present in all isolates. The presence of Pet in EAEC isolates was initially detected by immunoblotting assays after a preliminary step of culture supernatant concentration.<sup>256</sup> Vilhena-Costa et al.<sup>306</sup> developed a slot blot immunoassay that avoids the concentration step, allowing the detection of Pet directly from EAEC supernatant, after growing the EAEC bacterial isolate in TSB at 37 °C for 4 h. In this method, it was possible to evaluate Pet expression with specificity and reproducibility, using a rabbit polyclonal anti-Pet serum, which showed no cross-reaction with supernatants of non-Pet-expressing isolates and commensal *E. coli*.

Considering these difficulties, DNA probes were included as a valuable tool for EAEC detection.<sup>307</sup> After sequencing the EcoRI-PstI fragment of pCVD432 (AA or EAEC probe) developed by Baudry et al.,<sup>228</sup> primers complementary to this probe for PCR amplification were designed.<sup>308</sup> This PCR assay was found to be a rapid, simple, and highly sensitive method, and therefore considered to be useful for screening stool specimens for the presence of EAEC strains. Rapid and practical multiplex PCR assays targeting more genes (*aggR*, *aap* and *aatA*, encoding the AggR regulator, dispersin and an ABC secretion system outer membrane protein, respectively) or *aggR*, *pic* and *astA*, encoding AggR, Pic and an EAST-1) have also been employed to

detect EAEC strains.<sup>309–311</sup> Monteiro et al.<sup>248</sup> used PCR to evaluate *aggR*, *aatA* and *aap* in a collection of *E. coli* strains and found that *aggR* and *aatA* were more specific to EAEC than *aap*, suggesting that the simultaneous detection of *aggR*, *aatA*, and *aaiA* (a type VI secretion system protein) could be an improvement in the PCR detection of EAEC.

All these proposed PCR-based protocols detect plasmid genes, which disfavors the detection of atypical EAEC strains.<sup>305,309,312</sup> Others, employing plasmid and chromosome loci, have not reported sensitivity and specificity of the assay.<sup>226,313,314</sup> However, a multiplex PCR based on two genes encoded in the plasmid and two chromosome-borne genes is recommended to increase the ability to detect both typical and atypical EAEC strains. The *aggR* and *aatA* genes<sup>309,313</sup> and *aaiA* and *aaiG* genes<sup>315</sup> incorporated in the assay detecting *aaiA*, *aaiG*, *aggR* and *aatA* demonstrated 94.8% sensitivity and 94.3% specificity, and the assay was able to effectively detect both groups of EAEC among *E. coli* isolated from stool cultures.<sup>316</sup> This method should improve EAEC detection, since this pathotype is responsible for acute and persistent diarrhea in children and adults and is also associated with foodborne diarrheal outbreaks.

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### Enterotoxigenic *E. coli*

EPEC strains are characterized by the production of colonization factors (CFs) and at least one of two enterotoxins: LT and ST. EPEC represents one of the most common causes of diarrhea in children in developing countries and in travelers to these regions. EPEC is also an economic burden to farmers and industry, where it is an important pathogen for broilers, swine, cattle and other farm animals. The group represents a highly diverse pathovar of diarrheogenic *E. coli*, harboring mobile genetic elements such as plasmids and phages. EPEC heterogeneity was first demonstrated by phenotypic traits including the large diversity of lipopolysaccharide (LPS) and flagelin composition and the expression of different CFs and toxin types.<sup>317,318</sup> Serological typing of EPEC strains have relied on the composition of outer membrane proteins and, mainly, in the somatic LPS (O) and flagellar (H) antigens.<sup>318–320</sup> EPEC comprise more than 100 somatic serogroups (O) and at least 34 flagellar types (H), combined in an unpredicted number of O:H serotypes, but only a limited number of serotypes are associated with infectious diseases, such as O8:H9, O6:H16, O78:H12 and O25:H42, and are therefore of major clinical relevance.<sup>318,321</sup>

The genetic diversity of EPEC has also been evaluated by molecular approaches including random amplification of polymorphic DNA (RAPD), MLEE, PFGE, multilocus sequence type (MLST) and whole-genome sequencing.<sup>322–330</sup> More recently, 362 human-derived strains were subjected to next-generation whole-genome sequencing; 21 genotypes could be identified, and EPEC strains could be classified into 5 major phylogroups (A, B1, B2, D and E).<sup>330</sup> Genetic analyses demonstrated that clonally related EPEC lineages sharing the same serotypes and CF and toxin profiles have worldwide distribution.<sup>327,328,330–332</sup> On the other hand, genetically distinct EPEC strains, frequently found among asymptomatic subjects show high antigen heterogeneity with

regard to virulence traits and serotypes.<sup>333</sup> Apparently, these strains have recently acquired the genes encoding virulence-associated traits, and their maintenance is driven by selective pressure.<sup>328,330</sup>

### Virulence factors, mechanisms and pathogenesis

Following the initial discovery of the association of ETEC with diarrheic disease in humans in the 1950s, there was an intense effort to identify ETEC virulence-associated traits that could help to understand the physiology of the pathological process and lead to the development of specific diagnostic methods. ETEC strains characteristically produce adhesins, or CFs, proteinaceous complex that may take the shape of fimbrial, fibrillar or nonfimbrial structures on the bacterial surface. The adhesins expressed by ETEC strains facilitate the adherence of the bacteria to the intestinal mucosa and confer host specificity to the different strains.<sup>317,321</sup>

Approximately 30 antigenically distinct CFs have been identified in clinically relevant ETEC strains, but only a few are usually found among samples collected from diarrheic patients.<sup>317,330</sup> Besides differences regarding biogenesis and structural organization, ETEC CFs show specific antigenic, genetic and biochemical features, which are currently used to cluster them into three main groups: the colonization factor antigen I (CFA/I)-like group, the coli surface antigen 5 (CS5)-like group and the class 1b group.<sup>317,334,335</sup> The CFA/I-like group harbors the first described CF (CFA/I) and some of the most clinically prevalent CFs, including CS1, CS2, CS4, CS14, CS17, CS19 and putative colonization factor O71(PCFO71), while the CS5-like group comprises only CS5 and CS7. The class 1b group includes CS12, CS18, CS20 and the recently described CS26-28 and CS30 types.<sup>317,334,335</sup> Additionally, genetic relationships are also observed between strains expressing CS8 and CS21, CS13 and CS23, as well as between strains expressing CS15 and CS22.<sup>336-338</sup> Other previously characterized CFs, such as CS3, CS6, CS10 and CS11, are not classified into the known CS families.<sup>317</sup> Some CFs, such as CS18 and CS20, are related to swine-derived ETEC fimbriae, which show a lower heterogeneity than those found in strains isolated from humans.<sup>317,321,339</sup> Strains expressing CFA/I, CFA-II (CS1/CS3, CS2/CS3 or CS3), CFA-IV (CS4/CS6, CS5/CS6 or CS6), CS17 and/or CS21 are the most prevalent CFs found in epidemiological studies, whereas other CFs are found in ETEC strains not clearly linked to diarrheal disease.<sup>317,318,331</sup>

After adherence to the intestinal mucosa, ETEC strains produce enterotoxins, which are recognized as the second component associated with diarrheal disease. Two major categories of enterotoxins have been identified among ETEC strains, isolated either from humans or other animal hosts: LT and ST. Both toxin types mediate deregulation of membrane ion channels in the epithelial membrane, leading to the loss of ions and massive amounts of water, the major characteristic of watery diarrhea caused by these bacterial strains.<sup>340</sup>

LT are composed of five identical monomers (11.5 kDa) arranged in a ring shape to form a pentameric B subunit, and a 28-kDa A subunit linked to the B subunit by the helical A2 domain. The B subunit binds to cell surface receptors, particularly to gangliosides, promoting toxin internalization and

retrograde transport up to the endoplasmic reticulum, where the A1 domain is cleaved from the A2 domain and released to the cytoplasm. The A1 domain transfers the ADP-ribose moiety from the NAD<sup>+</sup> cofactor to stimulatory G protein, which becomes active and capable of stimulating adenylate cyclase, leading to an intracellular increase in cyclic adenosine monophosphate (cAMP). Higher cAMP levels in the cell induce protein kinase A activation, which in turn leads to phosphorylation of ion channels, resulting in Cl<sup>-</sup> release as well as decrease in Na<sup>+</sup> uptake and, consequently, massive water release to intestinal lumen, the major characteristic of secretory diarrhea caused by these pathogens.<sup>6,341</sup> ST, a monomeric protein of about 5 kDa, may also induce osmotic deregulation, activating directly the guanylate cyclase C located at the apical membrane of the intestinal cells to produce intracellular cyclic guanosine monophosphate and consequently to generate secretion of Cl<sup>-</sup> ions and water from the intestinal epithelium. However, an ST variant first isolated from pigs shows distinct physiological activity, characterized by the loss of villus epithelial cells and net bicarbonate secretion.<sup>6</sup> The toxins LT and ST, separately or in combination, are able to induce cellular water-electrolyte imbalance, which surely contributes to ETEC pathogenesis.

A hallmark in ETEC biology is the expression of enterotoxins, which also display a significant antigenic heterogeneity. Approximately one-third of the strains isolated from diarrheic patients express only LT or only ST, while another third express both toxin types. In addition, two unrelated ST groups, with different functional and structural features, have been identified: (i) STa, comprising two variants (STh and STp) associated with human disease, and (ii) STb, which is generally found among swine-derived ETEC strains. Similarly, LT are divided into two antigenically distinct groups: LT-I and LT-II.<sup>6</sup> Initially, two LT-I variants, isolated from human or swine-derived ETECs (LTh and LTp, respectively), were described and shown to have high amino acid sequence identity and similar but not equal antigenicity and biochemical and receptor-binding properties.<sup>342,343</sup> The related LT-II variants (LT-IIa, -IIb, -IIc) have been isolated from human beings or other hosts and contaminated food and bind to different receptors.<sup>344-347</sup> The LT-IIa, LT-IIb and LT-IIc share 51, 52 and 49% or 15, 16 and 7% identity with LT-Ih regarding the A and B subunits, respectively.<sup>347,348</sup>

More recently, a pioneer study carried out with ETEC strains isolated in Brazil demonstrated a rather high intraspecific LTh variability between LT-producing ETEC strains.<sup>333,349,350</sup> In a collection of 51 ETEC strains expressing LT and/or ST, 50 genetic polymorphic sites were found in the LT-encoding genes, which revealed 16 natural LT variants according to differences in amino acid sequences. Among these variants, named LT1 to LT16, two (LT1 and LT2) were associated with a limited number of serotypes with a global distribution and mainly isolated from diarrheic patients.<sup>333</sup> In contrast, most of the detected LT variants were observed among LT-producing ETEC strains isolated from asymptomatic subjects.<sup>333</sup> More recently, 12 additional LT types were identified in a larger collection of ETEC strains isolated from different regions of the world.<sup>351</sup> Interestingly, a much reduced genetic variability was found in the LT-encoding genes among ETEC strains isolated from pigs (LTp) and ST-encoding genes.<sup>352,353</sup>

The natural diversity of LT types found among ETEC strains isolated from symptomatic and asymptomatic humans suggests that some LT types can show higher toxicity to eukaryotic cells and can be expressed at different levels compared to other toxin types. Indeed, previous observations indicated that some LT types are endowed with different toxicity, under *in vitro* and *in vivo* conditions.<sup>333,349,350</sup> A natural LT variant, similar to the LT expressed by swine-derived strains, showed reduced toxicity due to an amino acid replacement at a key polymorphic site in the A subunit.<sup>333,350</sup> This amino acid change provided a less flexible A subunit structure, impairing appropriate contact with the cofactor (NAD<sup>+</sup>) at the catalytic site.<sup>350</sup> Other authors observed that natural polymorphisms in the B subunit resulted in decreased receptor binding and therefore reduced toxicity to eukaryotic cells.<sup>352</sup> These results suggest that the presence of ETEC strains expressing different LT variants may correlate with the incidence of symptoms among infected subjects, particularly among infected infants not previously exposed to ETEC infections.

Variable LT expression may also impact the severity of ETEC-associated disease. Previous observations demonstrated that the amounts of LT produced and/or secreted by ETEC are dramatically different among strains and clinical isolates.<sup>332,351,354–356</sup> The presence of single nucleotide changes in the *etx* operon regulatory region may be found and, at least for some of them, are associated with different transcriptional and translational activity among wild ETEC strains.<sup>332, unpublished data</sup> Nonetheless, further studies are required to demonstrate a clear link between transcriptional and post-transcriptional events and the severity of the symptoms associated with ETEC infection.

### Epidemiology

Annually, infections with different ETEC strains cause an astonishing number of diarrheal episodes, greatly exceeding 200 million cases and causing approximately 75,000 deaths, mainly among babies and young children in tropical areas with poor sanitary conditions.<sup>118,357</sup> In Brazil, epidemiological data harvested at different times between 1978 and 2007 have demonstrated that the incidence of ETEC-induced diarrhea ranges from 3.5 to 20.45%.<sup>115,358–361</sup>

### Detection and diagnosis

This pathotype is mainly characterized by the enterotoxins it produces, and diagnosis depends upon identifying either LT and/or ST. One or both toxins may be expressed by ETEC strains.<sup>340,362–364</sup> The diagnosis of ETEC strains should include, in addition to LT and ST detection, complementary PCR assays for the detection of virulence genes such as *clyA*, *eatA*, *tia*, *tibC*, *leoA*, and *east-1*.<sup>340</sup> A sensitive and specific PCR assay with primers targeting the genes *lt* and *st* was reported by Stacy-Phipps et al.,<sup>365</sup> and later by Youmans et al.,<sup>366</sup> using quantitative real-time PCR. Moreover, several multiplex PCR assays were also developed using these two genes.<sup>367–369</sup>

Phenotypical detection of ETEC was initially performed using supernatants obtained from single *E. coli* colonies and by laborious procedures such as rabbit ileal loop test,<sup>370</sup> suckling mouse assay<sup>371</sup> or cytopathic effect studies on CHO or Y1

adrenal cell monolayers, in which the presence of LT in supernatants was indicated by rounding of Y1 cells or elongation of CHO cells after 24 h of incubation.<sup>372,373</sup>

A number of immunoassays have been developed for ST detection, including radioimmunoassay and enzyme-linked immunosorbent assay (ELISA). Both tests correlate well with results obtained with the suckling-mouse assay and require substantially less expertise.<sup>374,375</sup> ELISA assays were then developed using the GM<sub>1</sub> receptor to bind LT obtained from filtered culture supernatants or employing a competitive test for LT, which replaced former procedures.<sup>376</sup>

Immunological assays for LT detection includes the traditional Biken test, latex agglutination, and reliable and easy to perform commercially available tests, such as the reversed passive latex agglutination and the staphylococcal coagglutination test.<sup>321</sup> Several immunological assays where LT is captured either by ganglioside GM1 (its receptor in the host cell) or by antibodies have been described.<sup>139,321,377,378</sup> Assays for ST by indirect ELISA using IgG1 ST-mAb and for LT by capture ELISA employing IgG enriched fraction of a rabbit polyclonal as a capture antibody and IgG2b LT-mAb as a second antibody have been employed as tools for diagnosis. The presence of bile salts and the use of certain antibiotics improved ETEC toxin production/release. Triton X-100, as chemical treatment, proved to be an alternative method for toxin release. Consequently, a common protocol that can increase the production and release of LT and ST could facilitate and enhance the sensitivity of diagnostic tests for ETEC.<sup>355</sup> Afterwards, those monoclonal antibodies were rebuilt resulting in single chain fragment variable (scFv) fragments. The developed recombinant scFvs against LT and ST constitute a promising starting point for simple and cost-effective ETEC diagnosis.<sup>379</sup>

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### Enteroinvasive *E. coli*

Enteroinvasive *E. coli* (EIEC) is a causative agent of dysentery in humans, especially in developing countries.<sup>380</sup> It causes keratoconjunctivitis in experimental guinea pigs<sup>381</sup> and invades human colon cells, causing an infection similar to that caused by *Shigella* sp.<sup>382,383</sup> The first description of EIEC was performed by EWING and GRAWATTI in 1947.<sup>384</sup> The first works emphasizing the particular biochemical characteristics of EIEC samples were presented in 1967 by Trabulsi et al.,<sup>385</sup> in Brazil and by Sakazaki et al.,<sup>386</sup> in Japan. All isolates studied were Serény test positive (guinea pig keratoconjunctivitis) and the strains were lysine decarboxylase negative, late fermenting lactose and generally non-motile, except for samples of the O124 serogroup. The study of the biochemical behavior of 97 samples of EIEC<sup>381</sup> corroborated the results obtained previously. It has been shown that this group of diarrheagenic *E. coli* belonged to well-defined bioserotypes, O28ac:H-, O29:H-, O112ac:H-, O121:H-, O124:H-, O124:H30, O135:H-, O136:H-, O143:H, O144:H-, O152:H-, O159:H-, O164:H-, O167:H- and O173:H-.<sup>381,387–389</sup> In 1964, it was demonstrated that samples of the O32 and O42 serogroups of *E. coli* also had the ability to cause keratoconjunctivitis in guinea pigs.<sup>389</sup> However, the existence of enteroinvasive bioserotypes in O42 serogroup was not confirmed, and O32 bioserotype is actually an aerogenic variant of *S. boydii* 14, as shown by Toledo et al.<sup>390</sup> There

are reports of isolation of EIEC samples belonging to other mobile serotypes, O144H25<sup>391</sup>; however, these are sporadic cases. Recently, the serotype of *E. coli* O96:H19 was described as enteroinvasive *E. coli* in two large outbreaks occurring in Italy and United Kingdom.<sup>392,393</sup> It is worth mentioning that EIEC serotypes considered to be nonmotile produce an unusually large (77 kDa) flagellin that is assembled into functional flagellum filaments that allow the bacteria to swim in modified motility agar (0.2%).<sup>394</sup> Analysis of the *fliC* gene showed that 11 different EIEC serotypes have six molecular profiles of *fliC*. The major EIEC serotypes showed low *fliC* diversity. The dendrogram showed two major clusters, suggesting two different origins for the flagellin gene among these strains. In addition, the presence of the same pattern among strains of the same serotype suggests the existence of a common clone.<sup>395</sup>

### Virulence factors, mechanisms and pathogenesis

Diarrhea due to EIEC and *Shigella* is caused by the invasion and penetration of bacteria in the enterocytes, leading to their destruction. These bacteria bind specifically to the mucosa of the large intestine and invade cells by endocytosis.<sup>396,397</sup> *Shigella flexneri* strains are used as template for most studies of invasion.

The complex process in colonization and EIEC survival in the gastrointestinal barrier depends on the presence of a large plasmid of about 220 kb (*pInu*), very similar to that found in *Shigella*.<sup>397-400</sup> In this process, multiple bacterial genes are involved, both chromosomal and plasmidial. Bacteria without the virulence plasmid do not cause keratoconjunctivitis in guinea pigs, being considered non-virulent.<sup>397,401</sup>

Most of these functions are related to proteins encoded by a 31-kb fragment from *pInu*, containing 38 genes. In this fragment are genes responsible for bacterial invasion and escape, by cell spreading, inhibition of autophagy, regulation of immune response of the host apparatus and type III secretion system (TTSS). Once injected into the host cell, the virulence or effector factors induce or inhibit cell signaling pathways. The changes in host cells induced by bacteria allow intracellular survival of these microorganisms.<sup>402-404</sup>

Due to the great similarity between *Shigella* and EIEC, it can be assumed that the two would share the same ancestor and that at a given moment in evolution there was a division. Why has EIEC retained some *E. coli* properties that have been lost in multiple lineages of *Shigella*? Data obtained by different groups lead to the speculation that EIEC strains are in an intermediate stage and are a potential pre-cursor of "full-blown" *Shigella* strains.<sup>405-409</sup>

Despite the similarities invasion mechanism and symptoms of the disease (dysentery), the infectious dose of EIEC is much higher than that of *Shigella*.<sup>410</sup> Furthermore, the disease caused by EIEC appears to be a milder and self-limiting form.

In the Serény test, it was observed that EIEC induces a milder form of the disease (mild/moderate inflammation), while *Shigella* leads to an exacerbation of proinflammatory response (severe inflammation). Furthermore, keratoconjunctivitis develops more rapidly in guinea pigs inoculated with *Shigella* (two days) than in guinea pigs inoculated with EIEC (4-5 days).<sup>411</sup>

Samples from different serotypes of EIEC have shown polymorphism in some regions of genes involved in invasion. However, the data reveal that there are no changes in genes of the invasion plasmid antigens that could explain the differences in pathogenicity between *Shigella* and EIEC.<sup>400</sup> Moreover, recent studies from our group showed that the genes responsible for cell spreading (*icsA* and *icsB*) and regulation of the immune response of the host (*osp*) did not indicate changes that could explain the difference in pathogenicity between *Shigella* and EIEC (data not shown).

Another important aspect of bacterial colonization is the uptake of iron (Fe) under conditions limited in the host. Iron is an essential element for all living organisms, it is estimated that the microorganisms require iron at concentrations from 10 to 10<sup>-6</sup> M to meet their metabolic needs. It was shown that EIEC has a high adaptability, using, if necessary, the iron capture system that consumes less energy. The ability to capture Fe from different sources can facilitate the development of infectious processes by this bacterium.<sup>412,413</sup>

EIEC, like other enteric pathogens, target M cells (*microfold cells*) present in the intestinal mucosa as a route of entry to deeper tissues of the host.<sup>403,414</sup> Reaching the lamina through the M cells, the bacterial cells are phagocytized by macrophages and dendritic cells. These cells are the first step in the production of the inflammatory response against bacterial invasion. After escape from macrophages and dendritic cells, EIEC are able to invade enterocyte cells from the basolateral side, escaping from the phagosome and replicating in the cytoplasm.<sup>403,414</sup>

Our group first described the phenotypic and genotypic characteristics explaining the lower capacity of EIEC to cause disease when compared with the species of *Shigella*. To this end, use has been made of experimental models that mimic the intestinal microenvironment of the host, such as cultures of intestinal epithelial cells, macrophages and dendritic cells.<sup>411,415,416</sup> Our results showed that the initial ability to invade the intestinal cell is similar between EIEC and *Shigella*, but that the expression of virulence genes (*ipaABCD*, *icsA*, *icsB*, *virF*, *virB*), capacity to escape from the phagosome, intracellular proliferation and dissemination of EIEC, as well as the ability to cause cell damage during the infection, are much lower than with *S. flexneri*.<sup>411</sup> A significantly greater number of EIEC are seen inside macrophages compared to *Shigella* after phagocytosis. Furthermore, *Shigella* shows greater capacity to escape from macrophages as compared to EIEC. The expression of virulence genes, production of proinflammatory cytokines and cell death was found to be less in macrophages infected by EIEC when compared to *Shigella*. It should be noted that the production of antiinflammatory cytokine IL-10 by macrophages is greater in infection by EIEC than *Shigella*.<sup>415</sup>

EIEC interaction with dendritic cells has been evaluated. The data suggest that EIEC induces the production of IL-10, IL-12 and TNF- $\alpha$  by infected dendritic cells, while *S. flexneri* induce TNF- $\alpha$  production. Unlike *Shigella*, infection with EIEC increases the expression of TLR-4 and TLR-5 receptors on dendritic cells and decreases the expression of costimulatory molecules that may cooperate to induce the proliferation of T-lymphocytes, and in addition, there is a greater proliferation of lymphocytes challenged with *S. flexneri* than with EIEC.<sup>416</sup>

## Epidemiology

The EIEC strains have similar biochemical, genetic and pathogenic characteristics as *Shigella* species, which can often make the correct identification of this pathotype difficult.<sup>381,385,386,417</sup> Epidemiological data may be underestimated due to the difficulty in differentiating between *Shigella* and EIEC.

EIEC was responsible for several outbreaks, but there are few reports on routes of transmission and distribution of this bacterium in nature. Water and cheese were described as potential sources,<sup>418–421</sup> as well as the direct transmission through person-to-person contact.<sup>422</sup> In the 1970s, a major outbreak of diarrhea was reported in the United States, which affected 387 patients. The transmission vehicle was an imported cheese, contaminated by O124 serogroup.<sup>419</sup> According to the food and drug control agency of the United States (Food and Drug Administration-FDA), outbreaks caused by EIEC have been associated with milk and milk products and beef; however, any food or water contaminated with human feces of an individual patient can cause disease in other individuals.<sup>423</sup> In Brazil, there is a report of three samples isolated from water.<sup>421</sup> Outbreaks involving two EIEC were recently reported in Europe, one in Italy in 2012 involving 109 cases and another in the United Kingdom in 2014 involving 50 cases.<sup>392,393</sup> In both, vegetables were to blame.

In Calcutta, the prevalence of EIEC in a group of 263 patients hospitalized with diarrhea was high, 16.3% of cases.<sup>424</sup> However, there are reports of a prevalence of 2%.<sup>425</sup> In Thailand, China and other Asian countries, a prevalence of 4 to 7% has been seen.<sup>426–429</sup> In Bolivia, the reports showed a 2% prevalence.<sup>430</sup> Some studies have shown that in Nigeria, Iran and Thailand, the distribution of EIEC is below (less than 0.1%) the rates found in developed countries; in Spain, for example, a prevalence of 0.2% was found.<sup>431–434</sup> The low incidence can be due to difficulties in differentiating EIEC from *Shigella*.

The isolation of EIEC in Brazil has ranged from 0.5 to 15%, depending on the population investigated.<sup>435–440</sup> The data suggest that the presence of EIEC is related to socioeconomic conditions. Toledo and Trabulsi<sup>439</sup> investigated the presence of this microorganism from children under five years of age and non-slum-dwelling children from different areas of the city of São Paulo. This bacterium has been found in 17 of 107 slum-dwelling children with diarrhea (15.9%) and in 16 of 701 non-slum-dwelling children with diarrhea (2.3%). In the first group, EIEC was the enteropathogen most frequently isolated from children over 2 years of age. In non-slum-dwelling children of the same age, it was the fourth most common agent, being more frequent than EPEC, *Salmonella*, *Rotavirus*, and *Yersinia enterocolitica*. Studies performed outside the city of São Paulo showed a low prevalence of these bacteria, 0.5–2.5%.<sup>435,440</sup>

## Detection and diagnosis

Samples of EIEC grow well in culture medium routinely used for isolation of Enterobacteriaceae, such as MacConkey agar, xylose-lysine-deoxycholate (XLD) agar and Hektoen enteric (HE). Highly selective media such as *Salmonella Shigella* agar

(SS) or bismuth sulfite agar may not be as effective for some serotypes.<sup>381</sup>

The identification of *E. coli* species may be carried out using conventional biochemical tests, such as production of indole, fermentation of glucose, sucrose and lactose, gas production from glucose fermentation, pathway glucose fermentation, using citrate as sole carbon source, motility, lysine, arginine and ornithine decarboxylation.<sup>1,441</sup> Fermentation of lactose varies according to the strain; EIEC samples can ferment lactose slowly (72 h), making it difficult to differentiate from *Shigella*.<sup>381</sup> Complementary to the physiological and biochemical characteristics, serotyping may be required for the differentiation, since some serotypes of *S. flexneri* produce indole. In such cases, O antisera of EIEC and *Shigella* should be used.<sup>1,441</sup> Bacterial colonies with this characteristic can be screened for the classical EIEC serogroups O28ac, O29, O112, O124, O136, O143, O144, O152, O159, O164, O169, and O173.<sup>1,441,443</sup> EIEC invasive capacity can be evaluated using the Sereny guinea pig eye test<sup>444</sup> and tissue culture assays,<sup>445</sup> which are more markedly limited to reference laboratories.

To characterize the EIEC pathotype, it is necessary to search for plasmid virulence genes. Currently, the investigation of the *ipaH* gene, a multi-copy gene (4–10) present in EIEC and *Shigella*, by PCR is recommended,<sup>442,446</sup> or studies of other DNA sequences are needed, such as the invasion-associated locus gene (*ial*).<sup>447</sup> The presence of the *iudA* and *lacY* genes can differentiate EIEC from *S. flexneri*.<sup>446</sup> A simple and rapid stool test based on apyrase (ATP-diphosphohydrolase) activity was described for EIEC detection.<sup>448</sup> This is an essential periplasmic enzyme required for unipolar localization of IcsA, which is involved in the pathogen's intracellular and intercellular spread, and is only expressed by EIEC and *Shigella*.<sup>449</sup> The enzyme activity is measured by a colorimetric reaction. The method is robust, requires widely available equipment and affordable reagents, and can be applied for routine use in laboratories with limited resources.<sup>448</sup>

## Conclusions

The genomic plasticity of *E. coli* strains is noteworthy, as can be seen by the variety of strains ranging from commensal residents of the gastrointestinal tract to assorted pathogens that are able to promote intestinal or extraintestinal illnesses with different clinical consequences. It is thus important to note that the continuous evolution of the *E. coli* genome has hindered the classification of certain *E. coli* isolates into a pathotype, because some isolates combine the main virulence characteristics of different pathotypes and are thus considered hybrid pathotypes (reviewed in 5) with the potential of allowing the rise of new and more virulent pathogenic *E. coli* hybrids.

Whole-genome sequencing has provided a great amount of useful information on the genome of pathogenic *E. coli*, which will help improve diagnosis, typing, disease management, epidemiology and outbreak investigations as well as helping to monitor the spread of pathogens.<sup>5</sup> Despite the recent advances in our knowledge of the genetic background and pathogenicity of strains of different DEC pathotypes, various novel genes encoding unknown functions are yet to be

characterized to further our understanding of the interactions of these pathogens with their hosts.

### Conflicts of interest

The authors declare no conflicts of interest.

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