Review Article

The pathogenesis and laboratory diagnosis of infections caused by diarrhoea producing *Escherichia coli*

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ABSTRACT

Escherichia coli strains isolated from patients with diarrhoea are a heterogeneous group displaying several pathogenic mechanisms. Pathogenic *E. coli* cause disease by producing virulence factors, the genes for which are located on chromosomes, plasmids and phage genomes. This article reviews modern microbiological techniques which have enhanced our knowledge of the pathogenesis of infections caused by diarrhoeagenic *E. coli* and the various new diagnostic techniques available.

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INTRODUCTION

E. coli is the most common facultative anaerobe in the human intestine and plays an important role in maintaining intestinal physiology. There are, however, a number of pathogenic strains of *E. coli* which produce extraintestinal and intestinal disease. The main categories of diarrhoeagenic *E. coli* are:

1. Enteropathogenic (EPEC)
2. Enteroadherent (EAEC, including diffusely and aggregatively adherent)
3. Enterotoxigenic (ETEC)
4. Enteroinvasive (EIEC)
5. Enterohaemorrhagic (EHEC)

These categories exhibit distinct clinical syndromes, but still share certain characteristics such as (i) interaction with the intestinal mucosa; (ii) plasmid-mediated virulence; (iii) production of enterotoxin; and (iv) sharing of O and H antigens within a group.[1]

RECOGNITION AND IDENTIFICATION

Bray and Beavan in the early 1940s found that antiserum raised against a strain of *E. coli* isolated from a patient with summer diarrhoea agglutinated 92% of *E. coli* strains isolated from 90 infants with diarrhoea, but only 6% of *E. coli* strains from 180 controls.[2,3] Using the same approach, a series of *E. coli* strains were identified as causing infant diarrhoea. The term enteropathogenic *E. coli* (EPEC) was proposed by Neter to refer to these serotypes.[4]

Mathewson later showed that strains adherent to certain tissue culture cells such as HeLa and HEp-2 were pathogenic in travellers to Mexico.[5] These strains did not elaborate heat-labile enterotoxin (LT), heat-stable enterotoxin (ST), elevated levels of shiga-like toxin, invade epithelial cells, or possess enterocyte-adhesion factor (EAF) plasmids. Based on the patterns of adherence, the strains could be divided into those showing localized (usually EPEC), diffuse and aggregative adherence.[6] Enteroaggregative *E. coli* (EAggEC) have subsequently been shown to be associated with persistent diarrhoea in children.[7] Diffusely adherent *E. coli* (DAEC) do not have the EAF plasmid, but have been shown to be associated with diarrhoea in travellers as well as in Mayan children in Mexico and in Bangladeshi children.[8–10]

In the 1960s and 1970s, ETEC were identified as another category of diarrhoeagenic *E. coli*.[11,12] These are now known to be a major cause of infant diarrhoea in less developed countries and are most commonly responsible for travellers' diarrhoea.[13–15] ETEC were shown to produce cholera toxin-like LT and STs in studies in young animals.[16]

In 1971, certain *E. coli* strains that caused an invasive dysenteric form of diarrhoeal illness in volunteers were described.[17] These EIEC strains were distinct from ETEC and EPEC and closely resembled *Shigella spp.* in their capacity to invade and proliferate within epithelial cells and eventually cause cell death.

EHEC were identified in 1982, when a multistate outbreak of haemorrhagic colitis in the United States drew attention to an unusual clinical syndrome of diarrhoeal disease, and to a new bacterial enteric pathogen, *E. coli* 0157:H7.[18]

PATHOGENESIS OF INFECTIONS

EPEC

Infection with EPEC is associated with the development of attaching/effacing (A/E) lesions characterized by the loss of intestinal cell microvilli and the close association (<20 nm) of bacteria with the cell membrane. Microvilli dissolution is accompanied by the disorganization of the cell cytoskeleton.[19,20] Cytoskeletal alterations involve polymerization and accumulation of actin, actinin, talin and ezrin beneath the adherent microorganisms.[21]

The A/E lesion appears to be associated with the fluid secretion and diarrhoea characteristic of EPEC infection. An *in vitro* test to determine the ability of EPEC strains to produce the A/E lesion has been developed which eliminates the need for identification by electron microscopy. This fluorescence actin staining (FAS) assay uses fluorescein isothiocyanate-labelled phalloidin to detect polymerized actin derived from localized cytoskeletal breakdown in cell cultures to which EPEC organisms have attached.[22]

Following EPEC infection, there may also be oedema, neutro-

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phil infiltration and a disordered arrangement of enterocytes in the intestinal mucosa. With damage to the mucosal surface, there is a marked increase in the release of brush border enzymes into the culture medium from EPEC-infected explants.[23,24] Changes in ion transport take place—sodium absorption is abolished and chloride absorption reversed to secretion.[25]

Cravioto *et al.*[26] showed that 80% of EPEC strains adhere to HEp-2 cells *in vitro* in a mannose-resistant manner. Most EPEC utilize the localized adherence (LA) mechanism in which the bacteria bind to epithelial cells forming microcolonies. These strains have 50–70 MDa plasmids which encode virulence factors known as EPEC adherence factors or EAF.[27] EAF and EPEC have been shown to possess rigid 7 nm long fimbriae demonstrable by ruthenium red staining.[28] The LA is induced 30–60 minutes after transfer of EPEC to HEp-2 monolayers and also in the absence of cells by growing bacteria in a defined tissue culture medium. Growth in the defined medium also resulted in EPEC autoaggregation, suggesting that LA could be due to preformed microcolonies adhering to cells, rather than multiplication of individual EPEC after adherence.[29] Growth on solid media containing sheep blood has also been shown to be associated with cell surface filaments 50–500 nm wide, 15–20 μ long which readily interdigitate with one another. These are called bundle forming pili and are composed of 19.5 kDa subunits which share amino terminal sequence homology with the toxin coregulated pili of *Vibrio cholerae*.[30]

The receptors used by EPEC to bind to susceptible mammalian cells have not been characterized, but LA EPEC have been shown to bind to purified glycolipids all of which possess the disaccharide epitope Gal NAc B1-4Gal.[31] To characterize genes and gene products involved in adherence or formation of A/E lesions, several investigators have used transposon insertion mutagenesis to isolate EPEC strains mutant in these virulence properties. Using Tnpho A mutagenesis and the FAS assay as a measure of A/E activity, the *eaeA* gene was identified.[32] The product of this gene is a 94 kDa outer membrane protein called intimin, the production of which is enhanced by the presence of the EAF plasmid.[33] Two loci on the EAF plasmid are responsible for the enhancement of intimin production; one of these has been sequenced and is designated 'per' (plasmid encoded regulator).[34]

It has been shown that EPEC penetrate HEp-2 cells by an endocytic process and are found in the cells in membrane-bound vesicles in the perinuclear region.[35] The EPEC do not multiply as rapidly as EIEC within epithelial cells. Entry of EPEC into cultured cells could be inhibited by microtubule and microfilament inhibitors.[36] However, the clinical significance of invasion in the pathogenesis of EPEC infection is not clear since EPEC do not cause dysentery or typhoidal syndromes.

The mechanisms by which EPEC mediate lesion formation or cause disease are not known, although it is believed that alteration of host protein phosphorylation patterns may be involved. Two prominent proteins (21 and 29 kDa) show increased phosphorylation in the presence of EPEC. An *E. coli* K-12 strain carrying the EAF plasmid did not cause increased phosphorylation, but all strains causing the A/E lesion did alter it.[37] Protein kinase C (PKC) may be the agent of EPEC induced phosphorylation, since other activators of PKC produce similar patterns. The importance of host protein phosphorylation in the development of lesions and its role in pathogenesis remain to be clarified.

EAggEC

In addition to the localized and diffuse patterns of adherence, an aggregative pattern (earlier referred to as auto-agglutination), where the bacteria are arranged in a 'stacked brick' formation, adhering to glass and tissue culture cells, has been described. This has been found to be associated with persistent diarrhoea in childhood, especially in developing countries.[7] Of the strains showing this pattern, 30% are O typable and the rest are nontypable or rough. Further characterization of the aggregative strains has shown that 40% are hydrophobic, 70% have type 1 fimbriae, and 50% possess mannose-resistant haemagglutinins for human and/or bovine red blood cells. Strains which lack type 1 fimbriae have rigid 6–7 nm fimbriae of 16 kDa, 12 kDa and 18 kDa subunits which do not cross-react.[38] A laboratory strain carrying a cloned 18 MDa fragment which confers aggregative adherence, HEp-2 adherence and haemagglutination of human erythrocytes, was shown to produce long, flexible bundle-forming fimbriae. There is no one plasmid which occurs in all strains, but over 90% have at least one in the 55–65 MDa range, while others have smaller plasmids.[38]

Experiments with rat and rabbit intestinal loops have shown shortening of villi, haemorrhagic necrosis of villus tips, mild inflammatory response with oedema and mononuclear cell infiltration of the submucosa. One strain showed attachment of bacteria with villous effacement and microvillar dissolution at the site of attachment. In adherence experiments with native and formalin-fixed human and animal mucosa, EAggEC were shown to adhere to epithelial cells of the colonic mucosa and those overlying the ileal single mucosal lymph follicles, Peyer's patches and absorptive cells of jejunal and ileal villi.[39]

FAS did not reveal any actin accumulation at the site of attachment in tissue cultures.[22] When the strains were examined for cytopathic effects on 8 cell-lines, no such effect was seen.[38] However, studies on rabbit intestinal epithelium in Ussing chambers revealed the presence of a low molecular weight (2–5 kDa) partially heat-stable, protease-sensitive enterotoxin different from STa which has been named EAST1 (EAggEC heat-stable enterotoxin 1).[40] In addition, a toxin similar to the alpha-haemolysin of *E. coli* has been described,[41] but the role of these toxins in the pathogenesis of diarrhoea needs further study.

Although some studies have implicated DAEC as a causative agent of diarrhoea[9,10] in volunteers, some DAEC isolates have been shown to be non-virulent.[42] It is possible that only a subpopulation of DAEC may have the necessary factors in addition to adherence properties to produce disease, and also that a number of different genetic determinants may specify DA. Two distinct adhesins have been identified, a 100 kDa protein encoded on a plasmid[43] and a fimbrial adhesin encoded on the chromosome.[44] On HeLa cells, DAEC produce dimple formation at the site of bacterial attachment with locking of bacteria by elongated microvilli at the edge of the dimple.[45]

ETEC

ETEC colonize the proximal small intestine and produce watery diarrhoea with nausea, abdominal cramps and low grade fever. A limited number of O:H serotypes occur repeatedly in geographically diverse areas and account for the majority of ETEC strains.[46,47] These serotypes usually elaborate both LT and ST enterotoxins. Human strains of ETEC, unlike porcine or calf strains, produce a variety of antigenically, structurally and genetically distinct surface hair-like fimbriae, pili or colonization-factor antigens found in association with particular O serogroups which help in attachment to the intestinal wall and allow bacteria to overcome the peristaltic defense mechanism of the small intestine.[1]

Evans *et al.*[48] first described a fimbrial colonization factor (CFA I) in a human ETEC strain which produced a particular haemagglutination pattern, the presence of which could be confirmed by using antiserum to CFA I. They later described CFA II, an antigenic factor which produced a different haemagglutination pattern.[49] Further investigation showed that CFA II is composed of 3 distinct antigens, now referred to as CS1, CS2 and CS3. The first two were never found to be expressed simultaneously, while CS3 was found on most strains.[50] Smith *et al.*[16] found that a single plasmid encodes the genes for CS1, CS2 and CS3, and fimbrial antigen expression is a function of the host bacterium related to biotype and serotype.

Mullaney *et al.*[51] described CS1 and CS2 as 6–7 nm in diameter with rigid fimbriae that resemble CFA I morphologically. CS3 was later found to be a thin, wiry, flexible structure, 2–3 nm in diameter, resembling the fibrillar type fimbriae seen in porcine strains.[52,53] Thomas *et al.*[54] described new colonization factor fimbriae in prototype strain E8775 and identified a family of 3 distinct antigens, CS4 and CS5 which were rigid with 6–7 nm fimbriae, while CS6 is not. CS4, CS5 and CS6 have been identified in several ETEC O serogroups for which there were no previously recognized CFAs. Recently other antigens such as CFA III and putative colonization factor 0159:H4 (also called CFA IV) have been identified. CFA IV appears to be a non-haemagglutinating fimbria encoded on a 27 MDa plasmid that also encodes LT and ST.[55]

CFAs have now been identified in all the main O serogroups associated with human ETEC. Of these, CFA I and CS2 can be defined as sialic acid-specific haemagglutinins or lectins.[56] However, cell receptors for these and other CFA structures are yet to be defined in the small bowel mucin and on the enterocyte surface. Smith[57] has shown, in experimental models that expression of CFAs is an essential prerequisite for induction of intestinal colonization and disease production by ETEC.

After colonization of the small intestine, the bacteria elaborate enterotoxins. STa or ST1 is a methanol-soluble small peptide toxin, active in the infant mouse model. It initiates intestinal secretion by stimulating guanylate cyclase and elevating cyclic guanylate monophosphate.[58] The methanol insoluble STb or STii stimulates intestinal secretion by an unknown mechanism probably involving staphylococcal delta-toxin detergent-like destruction of intestinal brush borders.[59]

LT is a heterogeneous family of toxins with two prototypes—human LT (hLT) and porcine LT (pLT)—which are antigenically cross-reactive, but have structural and immunological differences in both the active (A) and the binding (B) subunits. Human LT binds to the GM1 ganglioside and produces diarrhoea by a mechanism similar to that of cholera toxin which also binds to the same receptor.[60] The second LT (LTii) has biological activity very similar to hLT and cholera toxin, but is not neutralized by their antisera and does not bind to the GM1 ganglioside. It is occasionally seen in human ETEC strains.[61]

EIEC

EIEC bioserotypes are distinct from other *E. coli* and closely resemble *Shigella spp.* DNA/DNA hybridization does not distinguish between *Shigella* and EIEC.[62] EIEC also resemble *Shigella* in being non-motile, non-lactose fermenters and do not decarboxylate lysine.[63]

Several plasmids (140 MDa, 180–240 Kb) found in EIEC with equivalents in *Shigella* code for the production of several membrane proteins, induction of phagocytosis by epithelial cells and intracellular multiplication of bacteria.[64,65] The products of these plasmid and chromosomal genes can be classified as:

1. Virulence determinants that directly affect the ability of bacteria to survive intestinal tissues: (i) the aerobactin siderophores (*iuc* ABCD, *iut*), (ii) superoxide dismutase (*sod* B), and (iii) somatic antigen expression (*rfa*, *rfb*).
2. Cytotoxins that contribute to the severity of disease.
3. Regulatory loci that affect expression of plasmid genes: (i) *omp* R-*env* Z (in response to changes in osmolarity), (ii) *vir* R (in response to changes in temperature), (iii) *kcp* A regulates translation of *vir* G, which controls *ics* A expression and intracellular bacterial mobility, (iv) *vir* F controls *vir* G and therefore intracellular spread, (v) *ipa* ABCD (invasion plasmid antigens that may be structural components of the invasion determinants), and (vi) *inv* AKJH (expression of which is necessary for the insertion of invasion plasmid antigens into the outer membrane).

The *p*INV (virulence) plasmid of *Shigella* and EIEC can integrate into specific sites on the host chromosome. Integration reduces the expression of *ipa* and *vir* G (*ics* A) plasmid genes.

The bacteria invade epithelial cells, and multiply and spread to the lamina propria. There is a marked inflammatory reaction with mucosal disruption, necrosis, abscess formation and ulceration seen mainly in the colon.[17] Clinically, the illness is characterized by fever, severe abdominal cramps, malaise, toxaemia, watery diarrhoea followed by gross dysentery consisting of scanty stools with blood and mucus.[1] Simple staining of the faecal mucus reveals sheets of polymorphonuclear lymphocytes. One serogroup, 0143, has been found to produce a Shiga-like toxin.[66] Other factors which may be involved in virulence include production of aerobactin, mannose-resistant haemagglutinin for human red blood cells, fimbriae, the glycocalyx and plasmid-mediated production of Colicin V.[67,68]

Studies on invasion of epithelial cells in tissue culture have shown that the predominant mode of attachment involves close apposition of the bacterial and epithelial surfaces. The bacteria then invade the cell and multiply in membrane-bound vesicles close to the apical surface.[67] A similar mode of attachment is seen with human erythrocytes and it has been found that invasion does not occur with haemagglutination-deficient mutants.[67] Microtubule inhibitors such as colchicine, vincristine and vinblastine have no effect on invasion by EIEC, but microfilament inhibitors such as cytochalasin reduce entry of EIEC into cells.[36]

EHEC

Infection with EHEC is associated with outbreaks of haemorrhagic colitis and the subsequent development of serious complications, including the haemolytic–uraemic syndrome and seizures.[68] EHEC share with EPEC the capacity to cause A/E lesions with pedestal formation and disarrangement of the epithelial cell cytoskeleton in gnotobiotic piglets and infant rabbits.[69–71] Much less is known about EHEC adherence mechanisms than about EPEC. EHEC strains contain large plasmids of approximately 60 MDa which encode structural and/or regulatory genes for fimbriae (composed of 16 kDa subunits) that are important in mediating attachment to Henle 407 human intestinal epithelial cells but not to HEp-2 cells, or human or animal erythrocytes.[72] Attachment to Henle 407 cells is less dense with 2–4 bacteria per cell. Some EHEC strains do show LA on HEp-2 cells after 6 hours of incubation. The 60 MDa plasmid does not mediate bacterial adherence in the caecum and colon of gnotobiotic piglets,[73,74] although in streptomycin-treated

mice, the plasmid cured strain could not co-colonize with a strain containing the plasmid. This suggests that the plasmid may encode some factors important in establishing colonization.[75]

Outer membrane extracts from 0157:H7 have been shown to specifically block adherence of homologous bacteria to HEp-2 cells.[76] Antisera to a 94 kDa outer membrane protein in the extracts effectively blocked EHEC (but not EPEC) binding and active polymerization. These data suggest that EHEC outer membrane proteins may serve as adhesins and are antigenically distinct from outer membrane proteins expressed by EPEC.[77]

The *eae* gene has also been identified in EHEC. It is chromosomally encoded as in EPEC and shares an 83% homology at the amino acid sequence level, suggesting that the product of the EHEC *eae* gene would differ from that encoded by EPEC.[78]

EHEC strains are lysogenized with one or two bacteriophages which encode the structural genes for the Shiga-like toxins.[79] The Shiga-like toxins probably act to exacerbate damage to the colonic epithelium and mesenteric blood vessels, resulting in bloody, oedematous lesions pathognomonic of haemorrhagic colitis.[80] They inhibit eukaryotic protein synthesis, resulting in cell death, hence invasion by EHEC may not have a role in pathogenesis.[77] SLT-I and II are functionally similar protein cytotoxins. They have a bipartite molecular structure which consists of an enzymatically active A subunit that inhibits eukaryotic protein synthesis and an oligomeric B subunit that binds to globo triaosylceramide glycolipid receptors on eukaryotic cells.[79,81]

The A subunit of SLT-I has been shown to contain regions of homology to the ricin A chain and inactivates eukaryotic ribosomes in a similar manner, by catalytically depurinating adenosine in 28S and rRNA.[82] SLT-II has been found to have a number of variants which result from deviations within the primary structure of the B subunit and show differential binding to eukaryotic glycolipids.[83] However, our understanding of the structure and function of the Shiga-like toxins remains incomplete.

LABORATORY IDENTIFICATION

EPEC

The EPEC are usually identified by grouping with polyvalent and monovalent antisera obtained from commercial sources or reference laboratories. The major enteropathogenic serotypes are now classified as:

Class I: 026, 055, 086, 0111, 0119, 0125, 0126, 0127, 0128ab, 0142

Class II: 018, 044, 0112, 0114

Only certain H types are found within each O serogroup, and the EPEC category is divided by some workers into two classes, of which class I exhibit localized adherence to HEp-2 cells and usually possess the EAF plasmid, while Class II EPEC exhibit either diffuse or no adherence and are usually EAF-negative.[84] Possession of a classical EPEC O serogroup does not always make an organism pathogenic.[85] The utility of EPEC serogrouping is now being questioned by many workers, who believe that it is justified mainly in outbreak situations.[86]

Detection methods other than serogrouping such as adhesion assays with tissue culture cells or hybridization with the BFP and EAF DNA probes have the disadvantage that non-adherent EPEC and EAF-EPEC cannot be identified. However, these are the most commonly used methods of identification of EPEC and EAEC in epidemiological studies.[87–89]

The FAS assay detects organisms capable of forming the A/E lesions, but some FAS-positive isolates belong to the non-EPEC serogroups.[90] Hybridization with the *eaeA* gene is specific for EPEC and EHEC and may prove to be a valuable diagnostic test.[32]

EAEC

EAggEC and DAEC are detected by adherence assays with tissue culture cells such as HeLa and HEp-2. Colony hybridization with DNA probes can also be employed but results vary with the probe used. Two DNA probes, the 1 kb probe[91] and 730 bp probe,[92] both derived from the 60 MDa plasmid have been developed for EAggEC. The DAEC probe is derived from the chromosomal adhesin gene.[7]

ETEC

The major O serogroups associated with ETEC are 06, 08, 015, 020, 025, 027, 063, 078, 080, 085, 0115, 0128ac, 0139, 0148, 0153, 0156 and 0167. For years following its introduction in 1953, the ligated intestinal loop model was the only way of identifying the enterotoxins of *E. coli.* In the 1970s, more convenient biological assays were developed. Of these, the Chinese hamster ovary cell[93] and the Y-1 adrenal cell assay[94] were widely used for the detection of LT while the suckling mouse model[95] was used for STa. STb ST is inactive in these tests. ELISA[96,97] and agar diffusion tests[98] replaced the biological assays and are in turn being replaced by DNA hybridization assays.[99–101]

EIEC

EIEC O serogroups are 28ac, 29, 42, 112ac, 124, 136, 143, 144, 152, 164 and 167. Initially, the only test available for diagnosing EIEC infections was the Sereny test,[102] but later it was found that keratoconjuctivitis in rabbits correlated well with the invasion of HeLa and HEp-2 cells in tissue culture.[103,104] More recently, colony hybridization assays,[105] DNA probes[106] and ELISA[107] have been developed as alternative diagnostic procedures.

EHEC

The commonest EHEC serogroup is 0157:H7 which can be detected by serotyping sorbitol-negative colonies on sorbitol MacConkey agar.[108,109] However, other EHEC serogroups are not necessarily sorbitol-negative, and both tests for production of the shiga-like toxins and serological tests are used.[110] DNA probes and colony blot assays are available for identification of strains carrying genes for the production of shiga-like toxins I and II.[111,112] A number of ELISA techniques are also described, and one has recently become commercially available.

The FAS assay is also a sensitive and specific test for EHEC[22] but as it also detects EPEC, serogrouping and tests for shiga-like toxin production are required for identification of these organisms. The *eaeA* gene probe also detects EHEC as well as EPEC.[32]

CONCLUSION

The pathogenetic mechanisms of gastrointestinal infections caused by *Escherichia coli* have become better understood over the past decade helped mainly by the application of molecular biological techniques which have also provided new diagnostic tests.

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