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Updates on defining and detecting diarrheagenic *Escherichia coli* pathotypes

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Abstract

Purpose of review—Several types of *Escherichia coli* cause acute diarrhea in humans and are responsible for a large burden of disease globally. The purpose of this review is to summarize diarrheagenic *Escherichia coli* (DEC) pathotype definitions and discuss existing and emerging molecular, genomic, and gut microbiome methods to detect, define, and study DEC pathotypes.

Recent findings—DEC pathotypes are currently diagnosed by molecular detection of unique virulence genes. However, some pathotypes have defied coherent molecular definitions because of imperfect gene targets, and pathotype categories are complicated by hybrid strains and isolation of pathotypes from asymptomatic individuals. Recent progress toward more efficient, sensitive, and multiplex DEC pathotype detection has been made using emerging PCR-based technologies. Genomics and gut microbiome detection methods continue to advance rapidly and are contributing to a better understanding of DEC pathotype diversity and functional potential.

Summary—DEC pathotype categorizations and detection methods are useful but imperfect. The implementation of molecular and sequence-based methods and well-designed epidemiological studies will continue to advance understanding of DEC pathotypes. Additional emphasis is needed on sequencing DEC genomes from regions of the world where they cause the most disease and from the pathotypes that cause the greatest burden of disease globally.

Keywords

Escherichia coli; diarrhea; pathotypes; genomics; molecular detection; gut microbiome

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INTRODUCTION

Escherichia coli is one of the most important and widely studied etiologic agents of diarrhea worldwide [1-4]. Though usually a benign member of the commensal gut microbiota [5], some *E. coli* strains have horizontally acquired virulence characteristics that enable them to cause diarrheagenic and extraintestinal illness in humans and other animals [6]. Human diarrheagenic *E. coli* (DEC) with specific combinations of virulence traits are grouped into pathotypes, each with unique host preferences, global prevalence, disease burdens, and

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CONFLICTS OF INTEREST
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modes of transmission [7]. DEC pathotypes include the enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), *Shiga* toxin producing *E. coli* (STEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

Precise definitions for each pathotype are important to effectively diagnose and treat DEC infections, design vaccines, and understand pathotype-specific disease burdens. However, *E. coli* strains within each pathotype are genetically heterogenous, and some have defied coherent molecular definitions of pathogenicity [8-10]. Pathotype definitions are complicated by the frequent isolation of pathogenic *E. coli* from individuals who are asymptomatic for acute diarrhea [11], and the existence of hybrid strains [12]. It is critical that DEC detection methods are as sensitive and specific as possible, which requires accurate and comprehensive characterization of each pathotype. Several excellent reviews have summarized research efforts for DEC pathotypes [*e.g.* 2,7,12,13]. Here we provide a brief overview of DEC pathotype definitions and provide updates on current and emerging technologies used to detect and define DEC.

DEC PATHOTYPES

In this section we provide a brief overview of the gene targets most commonly used to detect the six DEC pathotypes and discuss hybrid pathotypes. Additional pathotype characteristics are summarized in Table 1.

ETEC

ETEC causes loose, watery stools in children in low- and middle-income countries (LMICs) and travelers to endemic regions. Diagnosis relies on the presence of colonization factors or enterotoxin genes, usually the heat-labile (*lt*) and heat-stable (*sta*) enterotoxins [14]. The presence of either or both the *lt* and *sta* toxin genes defines ETEC strains [14,15], and the contribution of both hemolysins to ETEC pathogenicity has been demonstrated in epidemiological and volunteer studies [*e.g.* 16-18].

EPEC

Like ETEC and most other DEC pathotypes, EPEC is often associated with watery diarrhea in children in LMICs. EPEC virulence genes are encoded on the chromosomal locus of enterocyte effacement (LEE) pathogenicity island [19], and detection centers on the LEE-encoded intimin gene *eae* [20]. Some EPEC strains also carry the EPEC adherence factor plasmid (pEAF), which encodes the bundle-forming pilus gene *bfp* [13]. Strains that are *bfp+* are termed typical EPEC (tEPEC), and strains that are *bfp−* are defined as atypical EPEC (aEPEC) [21].

STEC/EHEC

STEC cause mild or bloody diarrhea, often accompanied by fever and vomiting. Infections are usually self-limiting, but the STEC subtype enterohemorrhagic *E. coli* (EHEC) is frequently linked to life-threatening foodborne disease outbreaks. STEC strains are defined by the presence of the phage-encoded *Shiga* toxin, and EHEC strains have additional

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virulence factors whose expression results in hemorrhagic colitis (bloody diarrhea) and, in some cases, life-threatening hemolytic uremic syndrome (HUS). All EHEC are STEC but not all STEC are EHEC. STEC diagnosis relies on the molecular detection of *Shiga* toxin variants (*stx* genes) and accessory virulence genes, including markers encoded by the LEE pathogenicity island also present in EPEC. Diagnostic methods for EHEC strains often target plasmid-encoded hemolysin genes [22,23]. Serotyping is used for the identification of *E. coli* O157:H7 and other EHEC strains that often cause food-related outbreaks [24,25]."

EIEC

Diarrhea caused by EIEC occurs worldwide but is especially common in children in LMICs [22-24]. The clinical presentation and virulence mechanisms of EIEC are indistinguishable from those initiated by closely-related *Shigella* spp. and both EIEC and *Shigella* carry the pINV F-type plasmid which encodes the genes necessary for enteroinvasive pathogenesis [22,25]. Molecular detection of the pINV-encoded gene *ipaH*, a type-III effector protein, is used to differentiate *Shigella* and EIEC from other pathotypes [26,27]. EIEC isolates are distinguished from *Shigella* using biochemical characteristics [27] or molecular assays, many of which detect the *E. coli*-specific *lacY* gene [22,28,29].

EAEC

EAEC is an emerging diarrheagenic and extraintestinal pathogen that affects all age groups and is prevalent in industrialized and LMIC settings. The pathogenicity and clinical relevance of EAEC are questionable because asymptomatic carriage is common and volunteer studies have inconsistently linked ingestion to diarrhea [30,31]. EAEC cells adhere to each other and host intestinal epithelial cells during infection, forming a characteristic stacked-brick or honeycomb formation when cultured on HEp-2 cells [9,32]. The microscopic HEp-2 cell assay is considered the gold standard for EAEC diagnosis [13]. Several molecular targets have also been used for detection, but there is significant diversity within the EAEC pathotype and a coherent molecular definition of EAEC does not yet exist [33]. Frequently used marker genes for EAEC include *aatA*, a plasmid-encoded gene important for biofilm formation, *aggR*, a plasmid-encoded transcriptional activator, and *aaiC*, a gene located on a genomic island with a type-VI secretion system [34-36]. To date the best definition of EAEC may be the presence of the aggregative pattern in the HEp-2 assay and the lack of markers associated with other pathotypes [13,37]. Whole genome studies of EAEC isolates are needed in order to further define gene targets and the molecular epidemiology of EAEC.

DAEC

There is epidemiological evidence linking DAEC to diarrhea in children in LMICs, but, like EAEC, its status as a diarrheal disease agent is uncertain due to inconclusive challenge studies and high rates of asymptomatic carriage [38-40]. Methodologies for DAEC detection are not well-defined because strains are heterogenous and DAEC-specific molecular characteristics have not been established. DAEC were first defined by their distinctive pattern of diffuse adherence to cells in culture [32,41]. However, this pattern of cell adhesion

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is not suitable for diagnosis because some aEPEC strains also have this phenotype [42]. DAEC strains have also been defined based on the presence of adhesin genes encoded by the *afa*, *dra*, or *daa* operons, which are structurally and functionally similar to one another [8,43]. Molecular assays to detect *daaC, daaE, afaB, afaC*, and other genes in the *afa*, *dra*, and *daa* operons have been developed, but are cross-reactive with well-characterized EAEC genes [8]. As with EAEC, whole-genome sequencing studies coupled with epidemiological data are needed to better characterize DAEC.

Hybrid strains

Several studies have identified hybrid strains that carry genes associated with multiple DEC pathotypes. These include *Shiga* toxin-producing EAEC strains, which have caused disease outbreaks in Europe [44,45], as well as *Shiga* toxin-producing ETEC in livestock [46,47], EPEC strains that carry the ETEC *lt* hemolysin gene [48], and aEPEC strains that encode genes typically found in extraintestinal pathogenic *E. coli* (ExPEC) [49]. Hybrid strains are relatively rare, which is perhaps surprising given the mobile nature of DEC virulence genes [50], but are important examples of the limitations of DEC pathotype designations.

RECENT ADVANCES IN DIAGNOSTIC METHODS

We focus here on research efforts to detect and define DEC pathotypes, citing recent studies that have used established and novel applications of PCR- and sequence-based assays.

Molecular methods

PCR-based molecular methods are widely used to detect and study DEC because they are sensitive, specific, and relatively rapid and easy to use. Both conventional and real-time quantitative PCR (qPCR) are widely used for molecular detection of DEC virulence factors. In contrast to conventional PCR, in which PCR products are visualized using gel electrophoresis, qPCR amplification is measured via fluorescent reporter molecules and targets are quantified relative to a standard curve. Recent qPCR-based DEC research efforts include the development of multiplex qPCR assays for DEC and other enteropathogens [51,52] and new assays to detect all known subtypes of *Shiga* toxin genes [53].

Newer tools are also being used for DEC pathotype detection. These include Luminex and BioFire panels designed to detect and diagnose an array of gastrointestinal pathogens. Both the Luminex and BioFire platforms take advantage of known DEC pathotype targets to deliver fast diagnostic results in clinical settings [54]. In addition to other common diarrheagenic pathogens, the Luminex panel detects ETEC, STEC, EHEC O157:H7, and *Shigella*/EIEC. The BioFire panel also detects ETEC, STEC, EHEC O157:H7, and *Shigella*/EIEC, and has additional gene targets for EAEC and EPEC [55]. Other emerging methods such as digital droplet PCR (ddPCR) and Taqman Array Card technologies have contributed to improved amplification, detection, multiplexing, and automation capabilities for DEC pathotypes. In ddPCR, qPCR reactions are partitioned into thousands of oil droplets, enabling absolute target quantification and reducing sample inhibition. A comparison of ddPCR to conventional and qPCR methods for detecting the EIEC/*Shigella* gene target *ipaH* found that ddPCR shortened detection time and was 10X and 100X more sensitive than

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conventional PCR and qPCR, respectively [56]. TAC relies on a microfluidic card to run qPCR assays for multiple gene targets simultaneously, effectively eliminating variability between methods, requiring less labor and time, and, most importantly, contributing to a better and more complete understanding of the prevalence and clinical relevance of diarrheal disease agents. TAC has most recently been used to identify DEC and other enteropathogens in travelers [57], U.S. military personnel [58], and children in LMICs [59,60], and studies have confirmed that TAC methods have good sensitivity and specificity for DEC gene targets [61-63]. The utility of TAC to study childhood diarrhea in LMIC settings has been demonstrated by its use in the GEMS (Global Enteric Multicenter Study) and MAL-ED (Malnutrition and Enteric Disease) studies [62-66], as well as its recent implementation in CHAMPS (Child Health and Mortality Prevention Surveillance) study sites in Asia and Africa [67].

Unfortunately, molecular diagnostics do not work well for all DEC pathotypes, in particular due to issues defining molecular targets for EAEC and DAEC. In addition, high rates of asymptomatic carriage in epidemiological studies indicate inconsistent relationships between marker gene presence and diarrhea symptoms [4,65]. qPCR and ddPCR are extremely sensitive, allowing for the detection of very small amounts of pathogen, which may or may not represent biologically or clinically relevant infections [11,65]. Also, while molecular methods have been increasingly implemented in LMICs, access to appropriate training, supplies, and equipment may be limited. Nonetheless, both established and emerging PCR-based methods are critical for detecting and diagnosing DEC pathotypes in both clinical and research settings.

Genomics

In contrast to molecular approaches that detect specific gene targets, genomic methods sequence all of the genetic material encoded in the bacterial genome. Genomics can be used to understand the structure, function, and relatedness of DEC pathotype strains, and genome-based analyses can reliably predict DEC pathotypes, serotype, multilocus sequence type, and other characteristics [68,69]. Comparative genome analyses have been used to find new pathotype-specific and diarrhea-associated genes, investigate antibiotic resistance, and examine transmission pathways [70-75]. Results of whole-genome sequencing can be entered into disease surveillance database resources such as the CDC's PulseNet [76] and FDA's GenomeTrakr [77] to track disease outbreaks. Recent research efforts have identified unique plasmids, diverse colonization factors, pathotype and strain-specific gene duplications [17,78-80]. Comparative genomics studies are also used to investigate the virulence potential and origins of hybrid DEC pathotypes [49,81,82].

Microbial genomics continues to advance rapidly, but important limitations remain. Most genomic sequencing approaches require isolation of the microorganism of interest on culture media before sequencing. This culture-based step requires additional effort and time-to-result. However, exceptions to the culture-based step of strain isolation have been demonstrated by studies in which high-quality bacterial genomes have been recovered directly from shotgun metagenomes [83,84] or using single cell sequencing [85]. Also, despite the ongoing rapid development and implementation of user-friendly sequencing

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technologies and analysis pipelines, genome sequencing usually necessitates complex library preparation methods and data processing requires bioinformatics skills.

Perhaps the largest limitation of genomics is that taxonomic and functional annotation of sequence-based datasets relies on large public databases that have high levels of misannotation and uneven coverage of taxonomic groups [86]. While *E. coli* is the most widely studied organism in the world, DEC pathotypes are not evenly represented in sequencing databases. We demonstrated this by performing a blastn analysis of DEC pathotype genes against all complete and draft *E. coli* genomes in the NCBI Genome Database (Table 2). This exploration of DEC pathotype marker gene presence in published *E. coli* genomes indicates that some pathotypes (especially STEC/EHEC) may have higher representation in NCBI than others. STEC/EHEC prevalence in the NCBI Genome Database is perhaps unsurprising given their importance as foodborne pathogens in industrialized nations. However, EPEC and ETEC are the DEC pathotypes with the highest global burden of disease, and are closely linked to childhood mortality in LMICs [3,87], and additional sequencing efforts are needed for these pathotypes. Importantly, the global prevalence of DAEC and EAEC has been difficult to determine because the gene targets used to detect these pathotypes are not specific. Additional genome sequencing efforts are warranted in order to better define molecular targets for DAEC and EAEC.

DEC and the gut microbiome

Sequence-based gut microbiome studies of DEC infection can characterize pathogen-induced shifts in the composition of the complex microbial communities of the gut. The two most widely used approaches for analyzing the gut microbiome are marker-based amplicon sequencing and whole-metagenome shotgun sequencing. Amplicon sequencing studies usually target the 16S ribosomal RNA (rRNA gene) and are used to characterize the taxonomic composition and diversity of microbial communities in the gut [88]. 16S rRNA sequencing has been used to investigate shifts in the gut microbiome during ETEC infection [89], but 16S gene studies generally do not have sufficient resolution to identify bacteria to the species level.

Shotgun metagenomes are obtained by sequencing the genome content of all microbes present in a sample and provide both taxonomic and functional gene information. In contrast to 16S rRNA marker-based sequencing, strain-level taxonomic resolution can be inferred using metagenomic data [90]. Recent studies demonstrate how metagenomic methods can be used to understand the implications of DEC infections. For example, a study that relied on shotgun metagenomes and PCR-pathotyped DEC isolates from an epidemiological study of DEC-associated diarrhea in Ecuador assembled high-quality *E. coli* genomes from metagenome data and used these to directly measure the population diversity of *E. coli* in the gut and the functional and virulence characteristics of DEC [84]. The potential and power of shotgun metagenome-based DEC pathotype detection has also been demonstrated in food and water samples [91,92].

As with genomics, annotation of gut microbiome data relies on database comparisons, and, though *E. coli* are well represented in reference databases, it is important to consider the implications of database biases and misannotations. Gut microbiome methods are not

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currently practical in many LMIC settings due to significant equipment and training requirements, and are usually more time- and computationally-intensive than PCR-based detection methods. In addition, both marker-based and shotgun metagenome sequencing are considered only semi-quantitative. Thus, pathogen detection and quantification usually rely on quantitative PCR-based methods. However, emerging approaches for sequencing and analysis are contributing to faster and more quantitative gut microbiome methods [93-95]. Gut microbiome methods are helping to untangle the complex relationships between commensal and pathogenic bacteria in the host, and these efforts have important implications for future development of diagnostics, therapeutics, and vaccines.

CONCLUSIONS

Grouping DEC into pathotypes based on shared characteristics is useful in order to understand differences in the virulence mechanisms, prevalence, modes of transmission, and other characteristics associated with various subtypes. However, pathotype designations have limited capacity to accommodate DEC strains that do not neatly fit pathotype definitions, and genomics and metagenomics have and will continue to highlight the imperfections in classic DEC pathotype definitions while concurrently providing new information to help improve diagnostic methods and definitions. *E. coli* virulence determinants are horizontally acquired, and it is likely that gene losses and gains will continue to result in emerging pathogenic strains of DEC that do not align with current pathotype definitions. It is important to acknowledge and understand exceptions to DEC pathotype definitions and to continue to use molecular and sequence-based tools to understand the breadth of DEC diversity at both the strain and population levels. Continuing to do this in combination with well-designed epidemiological studies will help us to better understand DEC-associated disease outcomes and design treatment and intervention strategies to improve health outcomes.

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KEY POINTS

- Current diarrheagenic *E. coli* (DEC) pathotype definitions are based on molecular detection of pathotype-specific virulence genes, but these have not been well defined for all pathotypes.
- Existing and emerging PCR and sequence-based technologies continue to move forward efforts to diagnose and study DEC pathotypes.
- Additional sequencing efforts are needed for DEC pathotypes of global importance.
- Pathotype categorization is imperfect but useful for understanding differences in DEC pathogenicity, transmission, clinical presentation, and epidemiology.

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Table 1.

Characteristics of DEC pathotypes

Pathotype	Clinical description	Pathogenesis	Epidemiology	Diagnostic genes	Other diagnostic methods	Transmission	Reference(s)
ETEC	Loose/watery stools, may be accompanied by fever and vomiting	Relies on adhesins and plasmid-encoded enterotoxins	Commonly diagnosed in children in LMIC settings and traveler's diarrhea in industrialized nations; asymptomatic carriage is common	The *lt* and/or *sta* hemolysins	n/a	Human-to-human fecal-oral transmission	[15,97,98]
EPEC	Mild to severe loose/ watery stools	Virulence genes on the LEE pathogenicity island cause attaching and effacing lesions on enterocytes	Commonly diagnosed in children in LMIC settings; aEPEC has been linked to persistent diarrhea lasting >14 days; asymptomatic carriage is common	The LEE pathogenicity island-encoded *eae* adhesin	tEPEC strains also have the plasmid-encoded *bfp* bundle-forming pilus gene	Human-to-human fecal-oral transmission; animal-to-human transmission for aEPEC	[20,99,100]
STEC/ EHEC	Mild to severe loose/ watery stools; EHEC causes bloody stools and can advance to life-threatening HUS	*Shiga* toxin inhibits protein synthesis and causes host cell death in the large intestine	Associated with foodborne disease outbreaks, often linked to leafy greens, milk, or beef products	The *Shiga* toxin *stx* gene defines STEC and additional targets may be used, including genes encoded by the LEE-pathogenicity island also present in EPEC	Serotyping, specific and differential media and molecular assays for EHEC strains, especially EHEC O157:H7	Human-to-human and animal-to-human fecal-oral transmission; cattle are considered a major reservoir	[101-104]
EIEC/ *Shigella*	Mild to severe loose/ watery stools, rarely accompanied by fever, tenesmus, blood, mucus, and leukocytes in the stool	Enteroinvasive virulence mechanism involves crossing the intestinal epithelial barrier, killing macrophages, invading enterocytes, intracellular and cell-to-cell propagation	Commonly diagnosed in LMIC settings and traveler's diarrhea in industrialized nations	The gene for the type-III effector protein *ipaH*	EIEC and Shigella isolates can be differentiated using biochemical characteristics or molecular assays	Human-to-human fecal-oral transmission	[22,25,27,105]
EAEC	Mild loose/watery stools, may be accompanied by fever, nausea, tenesmus, borborygmi, or mucus in the stool	Colonization is facilitated by fimbriae, followed by biofilm formation and toxin release	Associated with childhood diarrhea in LMICs; inconsistent results in adult challenge studies and asymptomatic carriage is common	The plasmid-encoded transcriptional activator *aggR*, pathogenicity island-associated gene *aaiC*, and the plasmid-encoded dispersin transporter *aatA*	Characteristic stacked-brick or honeycomb pattern in microscopic HEp-2 cell assay	Human-to-human fecal-oral transmission; EAEC has been isolated from animals but transmission has not been established	[9,33,35,106,107]
DAEC	Mild loose/watery stools	Adhesins trigger signaling pathways that promote cell lesions, loss of intestinal microvilli and inflammation	Associated with childhood diarrhea in LMICs; adult challenge studies failed to cause diarrhea and asymptomatic carriage is common.	Genes in the *afa*, *dra*, or *daa* adhesin operons; cross-reactivity with EAEC genes frequently reported	HEp-2 cell assay	Human-to-human fecal-oral transmission	[8]

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Table 2.

Blastn results for DEC pathotype-specific genes against all complete *E. coli* genomes (*n*=1,060) and all *E. coli* draft assemblies (*n*=18,195) in the NCBI Genome Database (accessed March 9, 2020).

Pathotype	Marker gene[a]	Gene description	Number of complete *E.coli* genomes with blastn hits[b]	Number of *E. coli* draft genomes with blastn hits[b]	Number of total diarrheal deaths in children <5 (%)[c]
ETEC	*eltA*	Heat-labile enterotoxin, subunits A and B	24	474	23,649.8 (4.7)
	eltB		24	465	
	sta	Heat-stable enterotoxin	27	279	
EPEC	*eaeA*	LEE-encoded intimin protein	9	159	11,284.3 (2.3)
	bfpA[d]	Plasmid-encoded bundle-forming pilus	1	91	
EAEC	*aggR*	Plasmid-encoded transcriptional activator	17	216	Not measured
	aaiC	Pathogenicity island-encoded secreted protein	2	127	
DAEC	*afaE-I*	Afimbrial adhesin subunit, human-specific variants	7	70	Not measured
	afaE-III		0	17	
EIEC/*Shigella*	*ipaH*	pINV plasmid-encoded type-III effector protein	2	128	54,905.5 (11)[e]
STEC/EHEC	*stx1A*	Shiga-like toxin variants, subunits A and B	126	2104	Not measured
	stx1B		126	2095	
	stx2A		202	2970	
	stx2B		202	2438	

[a]Reference sequences downloaded from the Virulence Factors Database [96]

[b]>90% query coverage and >90% sequence identity

[c]From Global Burden of Disease Study 2015 [3], only reports values for ETEC, EPEC and *Shigella*

[d]present in typical EPEC (tEPEC), absent in atypical EPEC (aEPEC)

[e]for *Shigella* only