

Molecular Epidemiology of Extended-Spectrum β -Lactamase–Producing *Escherichia coli* Pathotypes in Diarrheal Children from Low Socioeconomic Status Communities in Bihar, India: Emergence of the CTX-M Type

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ABSTRACT

BACKGROUND: Childhood diarrheal diseases remain highly endemic in India, but the emergence of extended-spectrum β -lactamase (ESBL)-producing *Escherichia coli* among children with diarrhea in Bihar remains elusive. In this study, we determine and characterize ESBL-producing *E coli* pathotypes among hospitalized diarrheal preschool children living in low socioeconomic level communities in Bihar, India.

MATERIALS AND METHODS: The stool samples were collected everyday throughout the year for 2 consecutive years. In our study, we collected stool samples randomly from every fifth patient. Stool samples were collected from a total of 633 randomly selected diarrheal children (age: 0-60 months) belonging to 17 communities and screened for identification of virulent diarrheagenic *E coli* (DEC) pathotype (viz, enteropathogenic *E coli* [EPEC], enteroaggregative *E coli* [EAEC], enterotoxigenic *E coli* [ETEC], enteroinvasive *E coli* [EIEC], and enterohemorrhagic *E coli* [EHEC]) by a multiplex polymerase chain reaction (PCR) assay. Furthermore, ESBLs were screened by conventional antibiotic resistance pattern testing and later characterized for the presence of β -lactamase (*bla*) genes by PCR and DNA sequencing.

RESULTS: Diarrheagenic *E coli* was detected in 191 cases (30.2%) of the total 633 diarrheic children. Maximum occurrence of DEC was found in ≤ 12 months age group (72.7%) with prevalence of the EAEC pathotype. Most isolates were resistant to ampicillin, ciprofloxacin, piperacillin, levofloxacin, ceftazidime, cefotaxime, ceftriaxone, and gentamicin, whereas over 96% of them were sensitive to amikacin. About 37.6% of total 191 DEC isolates were ESBL producers ($n = 72$), being prevalent among ETEC ($n = 35$; 18.32%), followed by EPEC ($n = 21$; 10.9%), EAEC ($n = 13$; 6.8%), and EIEC ($n = 3$; 1.57%). Interestingly, the commonest β -lactamase was CTX-M type (*bla*_{CTX-M}) in 86.1% ($n = 62$) of the ESBL isolates, followed by *bla*_{SHV} ($n = 49$; 68%), *bla*_{TEM} ($n = 37$; 51.8%), and *bla*_{OXA} ($n = 21$; 29.1%) determinants. Resistance of ESBL isolates was mostly related to ampicillin (100%), ceftriaxone (98.1%), cefotaxime (92.4%), gentamicin (74.1%), and levofloxacin (73.2%), whereas best antimicrobial activities were observed for piperacillin-tazobactam, amikacin, meropenem, and imipenem.

CONCLUSIONS: This study revealed that EAEC (72.1%) is the predominant pathotype in Bihar, significantly high in ≤ 12 months age group children ($P = .04$). Moreover, the widespread prevalence of ESBLs in children, especially the CTX-M type, is of great concern, which requires monitoring of infection control measures through efficient antimicrobial management and detection of ESBL-producing isolates.

KEYWORDS: Acute diarrhea, diarrheagenic *Escherichia coli*, extended-spectrum β -lactamase (ESBL), CTX-M

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Introduction

Diarrheal disease is the second leading cause of death in children under 5 years of age and is responsible for killing around 5 25 000 children every year. Globally, there are nearly 1.7 billion cases of childhood diarrheal disease every year.¹ In India, 212 000 deaths were reported due to diarrhea in children less than 5 years of age.^{2,3} The cause of diarrhea has been studied in both developed⁴⁻⁶ and developing⁷⁻¹⁰ countries and it was shown that enteropathogens implicated in the cause of

diarrhea may vary among regions and populations, even when the same epidemiological and microbiological methods are used.⁷ The causative agents may be an array of bacterial, viral, or parasitic pathogens. Globally, 0.5 million children die annually due to rotavirus-associated diarrhea,¹¹ whereas national approximate from India revealed that rotavirus is the reason for 0.1 million deaths and 0.4 to 0.8 million hospitalizations in children <5 years of age.¹² About 125 million cases of endemic shigellosis caused by enteroinvasive *E coli* (EIEC) or *Shigella* spp. occur every year in Asian countries among children <5 years of age.^{13,14} Bacterial gastroenteritis is very

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common in developing countries of the Southeast Asian regions such as Bangladesh, India, Indonesia, Myanmar, and Nepal. The usual manifestations are vomiting and diarrhea accompanied by abdominal discomfort, nausea, fever, or dehydration. Thus, a better understanding of the role of specific enteric pathogens to the overall burden of diarrheal disease and the antimicrobial sensitivity/resistance pattern of these pathotypes are essential for developing interventions that can effectively reduce the mortality and morbidity associated with diarrhea.

Escherichia coli, from the Enterobacteriaceae family, is the predominant nonpathogenic facultative anaerobic member of the human intestinal microflora.^{15–18} Despite *E coli* being a commensal bacterium among the intestinal microflora of a variety of animals including man, some strains have developed the ability to cause diarrhea (diarrheogenic *E coli* [DEC] groups) and extraintestinal *E coli* (ExPEC) infections including urinary tract infections, meningitis, and septicemia in both humans and animals. Among the bacterial causal agents, DEC plays an important role in causing diarrhea in children below the age of 5 years, particularly preschool children in developing countries.¹⁹ According to the World Health Organization (WHO) report, approximately 11 million children under the age of 5 years die due to *E coli*-mediated gastroenteritis.²⁰ Among DEC, enteropathogenic *E coli* (EPEC) strain accounts for 5% to 10% of pediatric diarrhea in resource-poor countries.²¹

Diarrheogenic *E coli* has acquired greater relevance in recent years; each type of *E coli* caused diarrhea being associated with a different pathotype of *E coli*.^{4,22–25} Interestingly, each pathotype possesses characteristic virulence-associated genes that contribute to its pathogenic mechanisms. Among the different pathotypes of DEC, enterotoxigenic *E coli* (ETEC), enteroaggregative *E coli* (EAEC), and EPEC are the most important enteric pathogens and are responsible for 30% to 40% of acute diarrhea in children below 5 years of age in developing⁶ and developed countries.^{26,27}

Discrimination of virulent DEC strains from the normal fecal flora is difficult using conventional phenotypic methods. In recent years, with the introduction of polymerase chain reaction (PCR) technology, it has become possible to detect presence of different chromosomal and/or plasmid-encoded virulence genes, which are absent in the commensal *E coli* strains,^{28,29} allowing for the rapid detection of DEC strains in clinical samples. Despite progress in the field of enteropathogen detection, use of PCR-based systems is typically limited to reference laboratories, mostly in the framework of outbreak investigations. Based on their pathogenic mechanisms, 5 DEC pathotypes are mostly reported, viz, EPEC, ETEC, EAEC, enterohemorrhagic *E coli* (EHEC) (also known as Shiga toxin-producing *E coli*) and EIEC. The sixth diarrheogenic pathotype is known as diarrhea-associated hemolytic *E coli* or diffusely adherent *E coli* (DAEC), but their role in human diarrhea has not yet been recognized.³⁰

Multidrug-resistant (MDR) DEC strains pose a serious clinical challenge. The MDR phenotype can emerge due to the presence of transmissible metallo- β -lactamase genes in the pathotypes as these β -lactamases can hydrolyze all β -lactams except aztreonam.³¹ However, the predominant mechanism of resistance in DEC to β -lactam antibiotics is due to production of plasmid-encoded extended-spectrum β -lactamases (ESBLs). The ESBL-producing organisms have become widespread globally.^{32,33} The ESBL producers are resistant for most β -lactams, with the exception of cephamycin and carbapenems, but remain susceptible to β -lactam inhibitors.³⁴ In addition, ESBLs comprise diverse group, among which the CTX-M group (so-called cefotaximases) is now the most prevalent ESBLs in most parts of the world.^{35,36} It is very important to determine the prevalence of ESBLs within a geographic area as emergence of ESBL-producing *E coli* leads to higher reliance for treatment with carbapenems (currently ertapenem, imipenem, meropenem, and doripenem, etc).³⁷ Therefore, as no previous reports on molecular epidemiology of ESBL-producing *E coli* pathotypes in diarrheal children exist from Patna, we aimed to investigate the virulence properties and prevalence of ESBL production by DEC pathotypes in children with diarrhea from low socioeconomic communities in Patna, Bihar, India.

Methods

Study subjects

In this study, a total of 633 stool specimens were collected throughout the year from acute diarrheal pediatric outpatients of the preschool age (0–60 months), hospitalized either at the Child Care Hospital or Nalanda Medical College and Hospital, Patna, for 2 consecutive years. Every fifth child, with diarrhea, was enrolled in the study with informed consent from the guardian.

Information about symptoms (eg, number of loose/watery stools, bloody stools, vomiting, fever $\geq 39^\circ\text{C}$) was collected by parental discussion and from medical records. Only the patients of Indian origin and resident of selected area of the Bihar province (Patna) were included in the study. If the parents of any child refused to participate, those patients were excluded from the study. Other exclusion criteria included travelers' diarrhea, chronic diarrhea, antibiotic consumption, immunodeficiency, and chemotherapy treatment in infants.

Stool samples were collected from children of the preschool age with acute community-acquired diarrhea requiring hospitalization in the pediatric ward. Passage of 3 or more watery/loose stools in 24 hours, with or without clinical symptoms of an enteric ailment (nausea, vomiting, abdominal pain or cramps, dehydration, fecal urgency, or dysentery), was considered as diarrhea. Other clinical features and risk factors recorded from patients included vomiting, nausea, abdominal pain, body temperature $\geq 38.0^\circ\text{C}$, duration of diarrhea, feeding type, diarrhea type, drug use, etc. Severity of the disease was based on the WHO classification of dehydration.³⁸ Children with no dehydration were classified as mild diarrhea and those

with mild to moderate dehydration were classified as moderate diarrhea. Children who required intravenous therapy for correction of dehydration and/or electrolyte disturbance were classified as severe. However, the children whose diarrhea could be due to classic pathogens such as *Salmonella* or *Shigella* or rotavirus or gross infestation with parasites were excluded from the study. Next, the patients were stratified by age into 3 groups, viz, group A: ≤ 12 months, group B: > 12 to 36 months, and group C: > 36 to 60 months.

Specimen collection and transport

The specimens were collected in clean, sterile wide-mouthed containers with tight-fitting leak-proof lids and stored at Microbiology Laboratory at the Hospital. Later, the samples were transported to the Microbiology Department of All India Institute of Medical Sciences (AIIMS)-Patna and/or Molecular Biology Department of RMRIMS (Rajendra Memorial Research Institute of Medical Sciences) for further processing within 2 hours of receipt.

Reagents

All media and chemicals were obtained from BD-Difco (Sparks, MD, USA) or Oxoid Inc (Thermo Fisher, Ottawa, ON, Canada), unless otherwise indicated. Oligonucleotide primers used in the study were synthesized from Integrated DNA Technologies (IDT, Coralville, IA). All PCR reagents including *Taq* DNA polymerase were purchased from DSS-Takara (Otsu, Japan).

Conventional screening and identification of DEC

Stool samples were first inoculated into selective enrichment medium (lauryl sulfate tryptose broth) at a ratio of 1:10. After overnight incubation, it was subcultured onto MacConkey agar and sorbitol MacConkey agar plate containing cefixime (0.05 mg/L) and tellurite (2.5 mg/L) and incubated for 24 hours at 37°C to isolate *E. coli*. Typical lactose-fermenting pink colored colonies were selected and subcultured in Luria Bertani agar (LBA) plates. The biochemical identification of *E. coli* was performed according to WHO guidelines.^{39,40} First, standard Gram staining was performed to confirm *E. coli* and later some biochemical tests specific for *E. coli* strains were conducted through the analytical profile index 20E system (BioMérieux, Marcy-l'Etoile, France). Moreover, the stool specimens were inspected for the presence of blood and/or mucus and for parasites using conventional microscopy and staining methods.

Molecular identification of DEC strains

Diarrheagenic *E. coli* virulence genes were identified by molecular methods as described previously with minor modifications.²² Briefly, a sweep of about 5 *E. coli*-like colonies plated on

LBA plates was taken, mixed with 200 μ L of double-distilled water in 1.5-mL microcentrifuge tubes and boiled for 10 minutes in a water bath followed by snap chilling in ice for 5 minutes. The heat-treated bacterial suspensions were centrifuged at 10000 rpm for 5 minutes to pellet down the cell debris, and the supernatants were used as DNA templates in the PCR. The DNA templates were subjected to multiplex PCR with specific primers (Table 2) as previously reported⁴¹⁻⁴⁵ for the detection of different pathotypes such as EPEC, ETEC, EAEC, EHEC, and EIEC.

Polymerase chain reaction experiments were performed with a 20- μ L reaction mixture containing 3 μ L of template DNA and 2.0 μ L of 10 \times PCR buffer containing 25 mM MgCl₂, 2 μ L of a 2.5 mM mixture of deoxynucleoside triphosphates, 0.25 μ L of 5 U/ μ L of *Taq* DNA polymerase, and 0.4 μ L of 20 μ M concentration of each primer except primer *eae*, which was used at 0.44 μ L of a concentration of 20 μ M stock. The thermocycling conditions were as follows: 96°C for 4 minutes, 95°C for 20 seconds, 57°C for 20 seconds, and 72°C for 1 minute for 35 cycles, with a final 7-minute extension at 72°C. The reactions were run in a GeneAmp PCR system 9700 (AB Applied Biosystems, Foster City, CA, USA). The PCR products (15 μ L) were evaluated with a 2% (wt/vol) agarose gel. A molecular marker (100 bp [base pairs] DNA ladder; Invitrogen, Carlsbad, CA, USA) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide. We used multiplex PCR in this study. In every PCR mixture, we added the primers for different genes (of different DEC pathotypes) and used template DNA from reference strain as well as experimental *E. coli* cultures. If the result was negative, the sample was considered negative for DEC. However, if the multiplex PCR was positive, the sizes of the bands on the gel were compared with those of the marker bands in the specific control lanes to identify the types of DEC strain present in the stool sample. The DEC strains were identified by plasmid-encoded gene *aatA* (*cvd*) and chromosomally encoded *aaiC* locus (*aaiC*) for EAEC, heat-labile enterotoxin gene (*elt*) and heat-stable enterotoxin gene (*est*) for ETEC, attaching and effacing gene (*eae*) and bundle-forming pilus gene (*bfpA*) for EPEC, attaching and effacing gene (*eae*), Shiga toxin gene (*stx*), and enterohemolysin gene (*ehxA*) for EHEC and invasion plasmid antigen (*ipaH*) was identified for EIEC (Table 1). All of these loci are known virulence determinants of their respective DEC pathogens.^{6,30} Strains positive for *eae*, but not *bfp*, were designated atypical EPEC. *Escherichia coli* reference strains containing the specific virulence marker for each DEC pathotype was obtained as gift from the National Institute of Cholera and Enteric Diseases, Kolkata, a WHO-recommended reference center for diarrheal diseases in India, and was used as positive controls in the PCR assays.

Antibiotic sensitivity test

The *E. coli* isolates were subjected to antibiotic susceptibility testing by modified Kirby-Bauer disk diffusion method on

Table 1. Age-wise distribution of *Escherichia coli*.

AGE (MONTHS)	NO. OF DIARRHEAL PATIENTS	PERCENTAGE OF <i>E. coli</i>	NON <i>E. coli</i>	PERCENTAGE OF DIARRHEAGENIC <i>E. coli</i>
≤12	481	53.7 (340/633)	141	21.5 (136/633)
>12-36	111	12.9 (82/633)	29	5.4 (34/633)
>36-60	41	6.0 (38/633)	03	3.3 (21/633)
Total	633	72.7 (460/633)	173	30.2 (191/633)

Table 2. Primer sequences and expected size of polymerase chain reaction–amplified gene targets of the pathogenic strains of *Escherichia coli*.

DEC PATHOTYPE	TARGET GENE	PRIMER	PRIMER SEQUENCE (5'-3')	AMPLICON SIZE, BP	REFERENCE
ETEC	<i>elt</i>	LT-F	CACACGGAGCTCCTCAGTC	508	Panchalingam et al ⁴¹
		LT-R	CCCCCAGCCTAGCTTAGTTT		
	<i>est</i>	ST-F	GCTAAACCAGTAG/AGGTCTTCAAAA	147	
		ST-R	CCCGGTACAG/AGCAGGATTACAACA		
EAEC	<i>AatA</i>	CVD432-F	CTGGCGAAAGACTGTATTCAT	630	Panchalingam et al ⁴¹
		CVD432-R	CAATGTATAGAAATCCGCTGTT		
	<i>aaiC</i>	AAIC-F	ATTGTCCTCAGGCATTTAC	215	
		AAIC-R	ACGACACCCCTGATAAACAA		
EPEC	<i>bfpA</i>	BFPA-F	GGAAGTCAAATTCATGGGGG	367	Panchalingam et al ⁴¹
		BFPA-R	GGAATCAGACGCAGACTGGT		
	<i>eae</i>	EAE-F	CCCGAATTCGGCACAAGCATAAGC	881	
		EAE-R	CCCGGATCCGTCTCGCCAGTATTCG		
EIEC	<i>ipaH</i>	EI-1	GCTGGAAAACTCAGTGCCT	424	Tornieporth et al ⁴²
		EI-2	CAGTCCGTAAATTCATTCT		
	<i>astA</i>	Eg1	CCATCAACACAGTATATCCGA	111	
		Eg2	GGTCGCGAGTGACGGCTTTGT		
EHEC	<i>stx</i>	LIN5'	GAACGAAATAATTTATATGT	900	Bastian et al ⁴⁴
		LIN3'	TTTGATTGTTACAGTCAT		
	<i>ehxA</i>	RH35	CACACGGAGCTTATAATATTCTGTCA	321	
		RH37	AATGTTATCCCATTGACATCATTGACT		
ESBL genes	<i>la_{CTX-M}</i>	Forward	TCGTCTCTTCCAG	966	Gniadkowski et al ⁵⁰
		Reverse	CAGCGCTTTTGCCGTCTAAG		
	<i>bla_{SHV}</i>	Forward	ACTGAATGAGGCGCTTCC	210	
		Reverse	ATCCCGCAGATAAATCACC		
	<i>bla_{OXA}</i>	Forward	GGCACCAGATTCAACTTTCAAG	564	
		Reverse	GACCCCAAGTTTCTGTAAGTG		
	<i>bla_{TEM}</i>	Forward	ATAAAATTCTTGAAGACGAAA	1080	
		Reverse	GACAGTTACCAATGCTTAATC		

Abbreviations: DEC, diarrheagenic *E. coli*; EAEC, enteroaggregative *E. coli*; EHEC, enterohemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; EPEC, enteropathogenic *E. coli*; ESBL, extended-spectrum β-lactamase; ETEC, enterotoxigenic *E. coli*.

Mueller-Hinton agar plates as per the Clinical and Laboratory Standards Institute (CLSI) guidelines.⁴⁶ The commercially available minimum inhibitory concentration (MIC) strips containing the following antimicrobials were tested for the DEC isolates: ampicillin, piperacillin, levofloxacin, ciprofloxacin, ampicillin-sulbactam, piperacillin-tazobactam, amikacin, ceftazidime, gentamicin, ceftazidime, cefotaxime, ceftriaxone, cefepime, aztreonam, imipenem, and meropenem (AB Biodisk, Solna, Sweden). The strips were aseptically placed on the surfaces of the sensitivity agar plates and these were incubated for 18 to 24 hours at 37°C. The MIC values (mg/L) were determined (MIC₅₀ and MIC₉₀ were calculated as the MIC point at which 50% and 90% of the isolates were inhibited) and interpreted according to CLSI guidelines as modified in 2013.

Characterization of ESBLs and PCR detection of *bla* determinants

Presence of ESBLs was determined by standard disk diffusion method on Mueller-Hinton agar which is useful for quantitative determination of susceptibility of bacteria to antibacterial agents, recommended by the CLSI guidelines.^{47,48} Here, ESBL activity was determined by the double-disk diffusion method using ceftazidime (30 µg), ceftazidime plus clavulanate (30/10 µg) disks and cefotaxime (30 µg), and cefotaxime plus clavulanic acid (30/10 µg) disks (Oxoid Limited, Basingstoke, UK) on Mueller-Hinton agar (Oxoid Limited, Basingstoke, UK).

Furthermore, all ESBL-producing isolates were investigated for the presence of the β-lactamase (*bla*) genes, viz, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{OXA}, and *bla*_{TEM} by PCR.⁴⁹ Plasmid DNA was extracted from the ESBL isolates using the Qiagen Plasmid Mini Kit (Qiagen) according to the manufacturers' instructions and was used as templates for the following PCR reactions. The PCR experiments were performed with a 25 µL reaction mixture containing 2.5 µL of 10× PCR buffer containing 25 mM MgCl₂, 2 µL of a 2.5 mM mixture of deoxynucleoside triphosphates, 0.25 µL of 5 U/µL of *Taq* DNA polymerase, 0.5 µL of 20 µM concentration of each primer, and 3 µL of template DNA. The specific primers used in 25 µL PCR reaction were described previously⁵⁰⁻⁵³ (Table 2). The PCR amplification conditions for *bla*_{CTX-M} and *bla*_{SHV} gene were 3 minutes at 95°C, 30 cycles of 30 seconds at 95°C, 45 seconds at 55°C, 30 seconds at 72°C, and finally, 5 minutes at 72°C and for *bla*_{OXA} and *bla*_{TEM} gene were 3 minutes at 95°C, 30 cycles of 1 minute at 95°C, 45 seconds at 42°C, 1 minute at 72°C, and final extension for 3 minutes at 72°C in a thermocycler (ABI Biosystems). *Escherichia coli* strain ATCC 25922 was used as negative control and *Klebsiella pneumoniae* strain ATCC 700603 was used as positive control. Products of the expected size were sequenced by Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) using the same primers. The resultant gene sequences were analyzed online using BLAST (Basic Local Alignment Search Tool) ([\[ncbi.nlm.nih.gov/Blast.cgi\]\(http://ncbi.nlm.nih.gov/Blast.cgi\)\) and identified using a β-lactamase database \(<http://www.lahey.org/Studies/>\).](http://blast.</p></div><div data-bbox=)

Statistical analysis

Associations between categorical variables were obtained using χ^2 and Fisher exact tests, with the analysis of variance and Kruskal-Wallis tests used for comparison of medians using GraphPad Prism Software (Version 6) (GraphPad Software, Inc., La Jolla, CA, USA). A *P* value of ≤ 0.05 was considered significant.

Results and Discussion

In India, diarrhea still remains the most common cause of death among children under 5 years of age. Every year, an estimated 2.5 billion deaths occur due to diarrhea; among them, 30% to 40% is contributed only to DEC.^{26,54} Diarrheagenic *E coli* are one of the most common causes for diarrhea worldwide, especially in children and in patients from developing countries.⁵⁵ Regional difference in the prevalence of different pathotypes of DEC is well documented.²⁶ Diarrheagenic *E coli* (46.3%) was the predominant pathogen that was isolated from diarrheal patients in a study by Ballal et al.⁵⁶ In this study, in total, 633 preschool children with diarrhea, admitted at the Child Care Hospital and Nalanda Medical College and Hospital, Patna, were randomly enrolled for 2 consecutive years (Figure 1). Of these, presence of *E coli* was identified in 460 samples (72.7%) (Table 1). Abundance of *E coli* was confirmed by tiny pink rod-shaped gram-negative bacteria and was also confirmed by the weak positive catalase test characterized by appearance of bubbles after 20 to 30 seconds after addition of 3% H₂O₂ on the cultures spread on a glass slide. A recent study on incidence of bacterial enteropathogens among hospitalized diarrheal patients from Orissa, India, also indicated that *E coli* constituted 75.5% consisting of 13.3% pathogenic *E coli* strains.⁵⁷ In our study, a total of 460 *E coli* isolates were enriched, with at least 1 *E coli* isolate being identified in 342 of the samples.

Biochemically confirmed *E coli* colonies from each sample were further set for characterization by pathotype-specific, virulence gene-targeted PCR assays. Of these, 191 (30.2%) isolates of *E coli* were identified as pathogenic types (Table 1) based on the presence of the virulence genes (Table 2). This study suggests the higher prevalence of EAEC, followed by ETEC, EPEC, EHEC, and EIEC, among children with diarrhea in communities across Bihar, consistent with some previous reports.⁵⁸⁻⁶⁰ Among 191 DEC-positive samples, EAEC was found in 132 (69.1%) cases followed by 20 cases of ETEC (10.5%), 16 cases of EPEC (8.4%), 5 cases (2.6%) of EHEC, 3 cases (1.6%) of EIEC, and 15 cases of mixed DEC (7.8%) (Table 3). Our study correlates with another study from South India where EAEC was the most common pathotype found in children with diarrhea (14.7%) followed by EPEC and ETEC.⁶¹ These results are also consistent with a previous study⁶² which described that EAEC infection is responsible for 50% of the cases of persistent diarrhea in India. However, different findings are reported in a

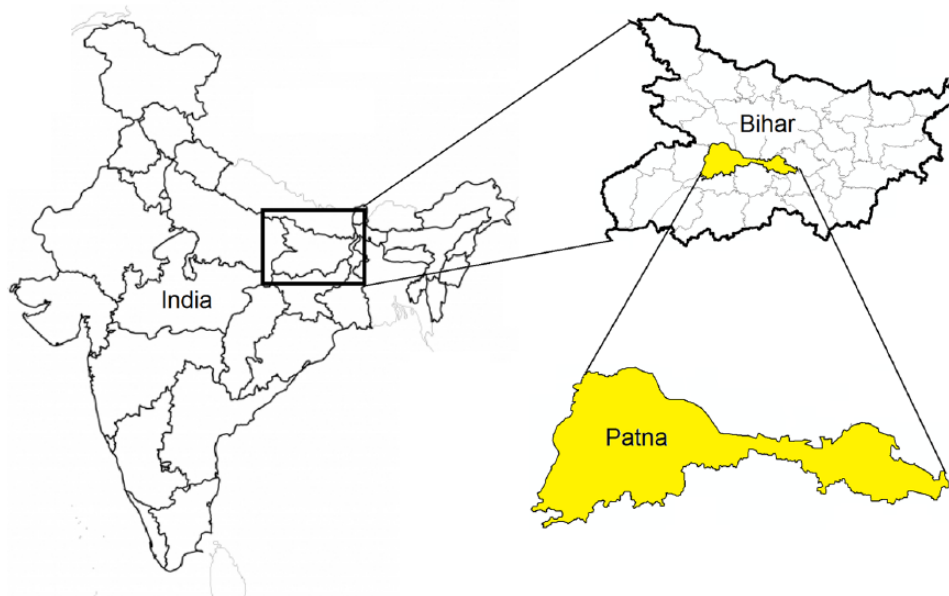


Figure 1. Geographical location of the area of Study. In this study only the patients from the Patna district of Bihar state in India were enrolled.

Table 3. Age-wise distribution of ETEC, EAEC, EPEC, EIEC, and EHEC among total DEC isolates.

AGE, MO	DEC					MIXED
	ETEC	EAEC	EPEC	EIEC	EHEC	
≤12	8.9 (17/191)	48.7 (93/191)	6.3 (12/191)	1.0 (2/191)	1.0 (2/191)	5.5 (10/191)
>12-36	1.05 (2/191)	12.6 (24/191)	0.5 (1/191)	0.5 (1/191)	1.0 (2/191)	2.1 (4/191)
>36-60	0.5 (1/191)	7.8 (15/191)	1.6 (3/191)	0 (0/191)	0.5 (1/191)	0.5 (1/191)
Total	10.5 (20/191)	69.1 (132/191)	8.4 (16/191)	1.6 (3/191)	2.6 (5/191)	7.8 (15/191)

Abbreviations: DEC, diarrheagenic *E coli*; EAEC, enteroaggregative *E coli*; EHEC, enterohemorrhagic *E coli*; EIEC, enteroinvasive *E coli*; EPEC, enteropathogenic *E coli*; ETEC, enterotoxigenic *E coli*.

study conducted in southern India (Mangalore)⁶³ with a prevalence of 11% for EPEC and another study from northern India (Kashmir) with 7.6% prevalence.⁶⁴

In our study, the enrolled patients are of uniform sex distribution. The overall sex distribution among the study subjects was 348 (54.9%) men and 285 (45.02%) women. Next, the preschool children patient data were stratified into 3 age groups, viz, group A: ≤12 months, group B: >12 to 36 months, and group C: >36 to 60 months. Analysis of age-wise distribution revealed that 481 (74.6%) children were of ≤12 months of age, 111 (17.3%) were >12 to 36 months of age, and the rest 41 (8.2%) were >36 to 60 months of age (Table 1). The distribution of different types of DEC pathotypes among different age groups was found to be significant ($P < .05$), whereas no correlation was observed ($P > .05$) between occurrence of DEC and sex of the patient. The distribution of 5 DEC categories (EPEC, ETEC, EAEC, EHEC, and EIEC) and presence of mixed DEC among DEC-positive samples were analyzed among these age groups (Table 3). Interestingly, EAEC and ETEC pathotypes were mostly isolated from the ≤12 months age group followed by >12 to 36 months. The EPEC,

EIEC, and EHEC pathotypes were less comparatively frequent and also mostly found in ≤12 months age group (Table 3).

Enterotoxigenic *E coli* was found in 17 cases (8.9%) of the ≤12 months age group, 2 cases (1.05%) of the >12 to 36 months age group, and only 1 case (0.5%) in the >36 to 60 months age group (Table 3). This finding contradicts a previous work of the highest reporting of ETEC from children >12 months of age than from children <12 months of age.⁶⁸ Simultaneously, EPEC infection is primarily a disease of infants <2 years of age.⁶⁹ We

Enterotoxigenic *E coli* strains are considered to be very important in pediatric patients, especially in the 0 to 5 years age group.⁶⁷ In our study, ETEC was found in the 17 cases (8.9%) of the ≤12 months age group, 2 cases (1.05%) of the >12 to 36 months age group, and only 1 case (0.5%) in the >36 to 60 months age group (Table 3). This finding contradicts a previous work of the highest reporting of ETEC from children >12 months of age than from children <12 months of age.⁶⁸ Simultaneously, EPEC infection is primarily a disease of infants <2 years of age.⁶⁹ We

Table 4. Clinical features and risk factors in diarrheic children infected with diarrheagenic *Escherichia coli* pathotypes.

FEATURES/RISK FACTORS	NO. (%) OF DIARRHEAGENIC <i>E COLI</i> PATHOTYPES				
	ETEC (20)	EAEC (132)	EPEC (16)	EIEC (3)	EHEC (5)
Symptoms					
Vomiting	5 (25)	75 (56.8)	14 (87.5)	2 (66.7)	1 (20)
Abdominal pain	15 (75)	54 (40.9)	7 (43.7)	2 (66.7)	1 (20)
Body temperature					
≥38°C	12 (60)	9 (6.8)	4 (25)	2 (66.7)	3 (60)
Dehydration status					
Mild	1 (5)	17 (12.9)	4 (25)	2 (66.7)	0 (0)
Moderate	16 (80)	112 (84.8)	10 (62.5)	0 (0)	1 (20)
Severe	3 (15)	0 (0)	0 (0)	0 (0)	4 (80)
Others (NA)	0 (0)	3 (2.3)	2 (12.5)	1 (33.3)	0 (0)
Stool color					
Red	2 (10)	17(12.9)	6 (37.5)	2 (67.3)	4 (80)
Green	4 (20)	18 (13.6)	3 (18.7)	0 (0)	0 (0)
Yellow	11 (55)	58 (43.9)	5 (31.2)	1 (33.3)	0 (0)
Black	0 (0)	3 (2.3)	0 (0)	0 (0)	0 (0)
Reddish brown	0 (0)	11 (8.3)	0 (0)	0 (0)	1 (20)
Others (NA)	3 (15)	25 (18.9)	2 (12.5)	0 (0)	0 (0)
Stool consistency					
Semisolid	4 (20)	37 (28)	6 (37.5)	1 (33.3)	1 (20)
Solid	2 (10)	2 (1.5)	0 (0)	0 (0)	0 (0)
Watery	13 (65)	82 (62.1)	2 (12.5)	2 (66.7)	4 (80)
Rice watery	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Others (NA)	1 (5)	11 (8.3)	8 (50)	0 (0)	0 (0)

Abbreviations: EAEC, enteroaggregative *E coli*; EHEC, enterohemorrhagic *E coli*; EIEC, enteroinvasive *E coli*; EPEC, enteropathogenic *E coli*; ETEC, enterotoxigenic *E coli*; NA, not applicable.

found EPEC in 12 cases (6.3%) of the ≤12 months age group, 1 case (0.5%) in the >12 to 36 months age group, and 3 cases (1.6%) in >36 to 60 months age group (Table 3).

Low prevalence of EHEC infection has been reported in developing countries.⁶⁹ Consistently, we found presence of EHEC in only 2 cases (1%) of the ≤12 months age group, 2 cases (1%) in the >12 to 36 months age group, and 1 case (0.5%) in the >36 to 60 months age group (Table 3). Stool samples containing EHEC were both of bloody and nonbloody types. Therefore, the absence of blood in the stool does not rule out presence of EHEC pathotypes. Acute diarrhea has been often related to EIEC pathotypes.⁷⁰ In our findings, EIEC was identified in the 2 cases (1%) in the ≤12 months age group, 1 case (0.5%) in the >12 to 36 months age group, and no cases (0%) in >36 to 60 months age group (Table 3).

Intermediate *E coli* strains presenting specific virulence genes of more than one specific *E coli* pathotype are also reported⁷¹ and were termed mixed DEC. In the study, mixed DEC infections were identified in 10 cases (5.5%) of the ≤12 months age group, 4 cases (2.1%) in the >12 to 36 months age group, and only 1 case (0.5%) in the >36 to 60 months age group (Table 3). Among the mixed combinations, EAEC and EPEC combination was dominantly found.

Analysis of the clinical symptoms of diarrheagenic patients caused by different pathogroups of DEC was done. Watery stool was the most dominant criteria for stool consistency among the study subjects. The findings indicate that infection with EAEC caused moderate (84.8%) to mild (12.9%) dehydration among patients preferably with yellowish (43.9%) stool and watery (62.1%) consistency (Table 4). Infection with

ETEC was mostly characterized by moderate (80%) dehydration status with yellow colored (55%) watery stool (65%), whereas EPEC infection resulted in moderate

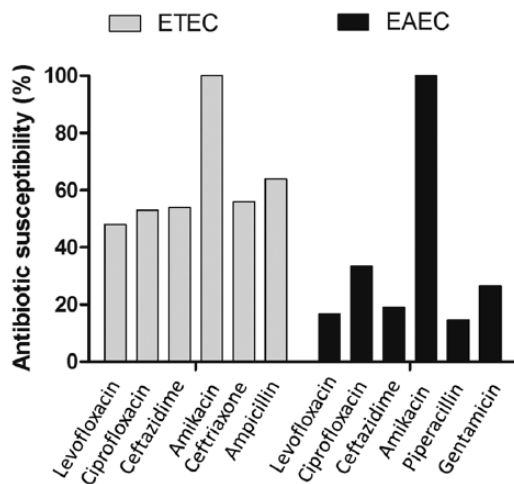


Figure 2. Antibiotic susceptibility pattern of ETEC and EAEC pathotypes. Antibiotic susceptibility test for ETEC and EAEC pathotype was performed according to CLSI guidelines. ETEC pathotypes were found to be sensitive to levofloxacin, ciprofloxacin, ceftazidime, amikacin, ceftriaxone and ampicillin. In case of EAEC pathotypes, comparatively less sensitivity was observed for levofloxacin, ciprofloxacin, ceftazidime, piperacillin and gentamicin, whereas maximum sensitivity was found for amikacin.

dehydration (62.5%) with reddish (37.5%) or yellow colored (31.2%) semisolid (37.5%) or watery (12.5%) stool (Table 4). Other clinical criteria tested were as follows: body temperature of $\geq 38.0^{\circ}\text{C}$ in 3 (60%) EHEC cases, nausea and/or vomiting in 75 (56.8%) EAEC cases and in 5 (25%) ETEC cases, and abdominal pain in 15 (75%) ETEC cases (Table 4).

All antibiotic susceptibility results were interpreted using the break points of the CLSI guidelines. Among the study isolates, typical ETEC pathotypes were found to be sensitive to levofloxacin (48%), ciprofloxacin (53%), ceftazidime (54%), ceftriaxone (56%), ampicillin (64%), and amikacin (100%) (Figure 2). In EAEC strains, sensitivity was found mostly for amikacin (100%), whereas comparatively less sensitivity was observed for piperacillin (14.6%), levofloxacin (16.7%), ceftazidime (19.1%), gentamicin (26.5%), and ciprofloxacin (33.4%) (Figure 2). Most of the EPEC, EIEC, and EHEC strains were resistant to most of the antibiotics, except amikacin and ciprofloxacin (not shown).

Importantly, new classes of enzymes conferring resistance to β -lactam antibiotics have emerged due to antibiotic selection pressure over the past decades, most alarming being the ESBL-producing enteric pathogens.⁷² Typically, ESBLs can hydrolyze third-generation cephalosporins and aztreonam, but not carbapenems, and therefore are inhibited by clavulanic acid and tazobactam. Therefore, in this study, high-level resistance to third-generation cephalosporins were selected for detection of

Table 5. Results of antimicrobial susceptibility tests and resultant MICs for diarrheagenic *Escherichia coli* strains (n= 191) isolated in this study.

ANTIMICROBIALS	TOTAL ISOLATES (N= 191)				ESBL-POSITIVE ISOLATES (N= 72)			
	MIC50, MG/L	MIC90, MG/L	SENSITIVE, %	RESISTANT, %	MIC50, MG/L	MIC90, MG/L	SENSITIVE, %	RESISTANT, %
Ampicillin	256	>256	15.2	83.2	>256	>256	0	100
Piperacillin	64	>256	28.7	51.3	256	>256	0	95
Levofloxacin	4	32	52.4	49.7	16	64	19.7	73.2
Ciprofloxacin	4	64	48.1	54.9	32	>128	31.2	69.4
Ampicillin-sulbactam	16	64	47.2	39.5	32	>128	18.6	72.9
Piperacillin-tazobactam	8	16	87.6	11.8	8	32	84	10.2
Amikacin	2	8	96.8	3.2	2	64	87.3	12.5
Cefoxitin	4	32	77.5	13.8	8	128	69.5	29.6
Gentamicin	16	128	54.2	48.3	64	256	19.8	74.1
Ceftazidime	2.5	32	78.6	21.2	4	64	52.6	45.7
Cefotaxime	2	128	48.2	52.7	64	>128	4.3	92.4
Ceftriaxone	1.25	128	60.3	35.1	64	128	1.2	98.1
Cefepime	1.25	32	82.6	10.5	8	64	62.3	25.8
Aztreonam	16	128	71.8	25.3	32	>128	65.7	32.3
Imipenem	0.125	0.25	100	0	0.125	0.25	100	0
Meropenem	0.064	0.125	100	0	0.064	0.125	100	0

Abbreviations: ESBL, extended-spectrum β -lactamase; MIC, minimum inhibitory concentration.

ESBL-producing DEC pathotypes. Notably, the results demonstrated that 37.6% of total DEC isolates of this study were ESBL producers (n = 72), being prevalent among ETEC (n = 35; 18.32%), followed by EPEC (n = 21; 10.9%), EAEC (n = 13; 6.8%), and EIEC (n = 3; 1.57%). The ESBL-producing *E coli* often display resistance to non- β -lactam antibiotics and chemotherapeutics. The ESBL-producing isolates in our study were also found to be resistant to some antimicrobial agents. Importantly, the isolates were resistant to most oxyimino- β -lactams, viz, ceftriaxone, followed by cefotaxime, ceftazidime, and aztreonam (Table 5). Our findings denoted that resistance of ESBL isolates was also related to ampicillin (100%), piperacillin (95%), gentamicin (74.1%), levofloxacin (73.2%), ampicillin-sulbactam (72.9%), and ciprofloxacin (69.4%) (Table 5). High percentage of ESBL-positive isolates was found to be susceptible to imipenem, meropenem, amikacin, piperacillin-tazobactam (Table 5), and oxyimino- β -lactams in combination with clavulanic acid. This results from the fact that genes coding for ESBLs and those conferring resistance to other antimicrobial drugs often reside within the same conjugative plasmids.

The ESBL-producing pathogens frequently exhibit plasmid-encoded multidrug resistance.⁴⁸ Owing to mutational changes, more than 200 types of ESBLs are currently described in various species of the Enterobacteriaceae family and other nonenteric organisms.^{48,73} All ESBL-producing isolates were investigated for the presence of β -lactamase (*bla*) genes, viz, *bla*_{CTX-M}, *bla*_{SHV}, *bla*_{OXA}, and *bla*_{TEM}, by PCR. In this study, the findings denoted higher MIC values of cefotaxime and ceftriaxone and lower MIC values of ceftazidime. These findings prompted toward the higher presence of CTX-M-type ESBL activity. Notably, the isolates harbored different overlapping combinations of the ESBL types. Interestingly, the commonest β -lactamase was CTX-M type (*bla*_{CTX-M}) in 86.1% (n = 62) of the ESBL isolates, followed by *bla*_{SHV} (n = 49; 68%), *bla*_{TEM} (n = 37; 51.8%), and *bla*_{OXA} (n = 21; 29.1%) determinants. The CTX-M ESBL isolates generally are able to hydrolyze cefotaxime and ceftriaxone. However, their activity against ceftazidime and aztreonam is usually lower.

Conclusions

In summary, this study revealed that EAEC (72.1%) is the predominant pathotype in Bihar, significantly high in ≤ 12 months age group children ($P = .04$). This is the first report of molecular epidemiology of DEC pathotypes and occurrence of ESBL-producing strains among diarrheal preschool children from low socioeconomic communities of Patna, Bihar (India). A report from Katihar/Kosi region of Bihar by Muni et al⁷⁴ showed that among different types of DEC, the maximum number of strains was EPEC (49.1%), followed by DAEC (34.5%), EAEC (10.9%), and ETEC (5.5%). In another study from India, Singh et al⁷⁵ reported DEC in 88.33% of isolates, EPEC (72.5%) being the predominant pathotype followed by EAEC (63.33%), ETEC (45.83%), and EHEC (9.16%). During the past decade, CTX-M β -lactamases have spread rapidly among DEC strains,

and the dominant types of CTX-M have been distributed worldwide. Moreover, the widespread prevalence of ESBLs in children, especially the CTX-M type is of great concern, which requires monitoring of infection control measures through efficient antimicrobial management and detection of ESBL-producing isolates. Active surveillance is recommended to scale up efforts of control strategies to counter multidrug resistance in diarrheal cases of this resource-poor region of India.

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Author Contributions

SD and PD designed the study. SD, AM, AS, and AK performed the experiments. SD, AS, and PD analyzed the data. SD and AM analyzed the sequences and wrote the manuscript which was corrected and approved by all the other co-authors. UKS, AKJ, PD, and SD contributed to reagents/materials/analysis tools. All authors read and approved the final manuscript.

Availability of Data and Materials

All the relevant data of this study have been fully presented in the main manuscript and tables.

Consent to Publication

We state that all the authors have contributed significantly to the work and have approved the content of this manuscript and its submission.

Ethical Approval and Consent to Participate

The study protocol and consent procedure were approved by the Institutional Ethics Committee (IEC) of All India Institute of Medical Sciences (AIIMS), Patna, and Rajendra Memorial Research Institute of Medical Sciences (ICMR), Patna. Informed written consent was obtained from legal guardians of the enrolled children. All clinical investigations were conducted according to the principles expressed in the Declaration of Helsinki.

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