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RESEARCH ARTICLE

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Molecular characterization of diarrheagenic *Escherichia coli* pathotypes: Association of virulent genes, serogroups, and antibiotic resistance among moderate-to-severe diarrhea patients

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Background: Diarrheagenic *Escherichia coli* (DEC) signifies as an important etiological agent of moderate-to-severe diarrhea. This study was primarily focused on molecular identification of DEC pathotypes; their association with serogroups and estimates of resistance profiles against different antibiotics regime.

Methods: Five hundred seventy-two stool specimens from diarrhea patients were investigated for DEC pathotypes. Molecular pathotypes were identified by amplification of virulence genes associated with distinct pathotypes followed by sequencing. Diarrhea is a self-limiting disease, however, severity and persistence of infection suggest antibiotic use. Therefore, AST and MIC were determined against common antibiotic regimen. Correlations between molecular pathotypes and serogroups were analyzed by somatic "O" antigen serotyping.

Results: The present findings reveal incidence of DEC as an etiological agent up to a level of 21% among all diarrheal age groups. DEC infection rate was higher in children. Enteropathogenic *E. coli* EPEC, a molecular pathotype of DEC, was found as a predominant pathotype with highest frequency of 13.7%. Two other molecular pathotypes enterotoxigenic *E. coli* (ETEC) and enteroaggregative *E. coli* (EAEC) accounted for 5.7% and 1.3%, respectively for all diarrhea incidences. Serological analysis deciphered somatic antigens O26, O2, and O3 as major serogroups identified among EPEC, ETEC, and EAEC pathotypes, respectively. All DEC pathotypes exhibited high levels of antibiotic resistance except for cotrimoxazole and norfloxacin.

Conclusion: Comprehensive molecular characterization of DEC pathotypes, their incidence estimates, and antibiogram patterns will help in ascertaining better diagnostic and therapeutic measures in management of diarrheal diseases.

KEYWORDS

antimicrobial resistance, childhood diarrhea, diarrhea, diarrheagenic *E. coli*, serogroups

1 | INTRODUCTION

Diarrheal diseases are major cause of morbidity and mortality in low- to middle-income countries and estimated to be second leading cause of mortality among children < 5 years of age, resulting in 0.5 million deaths globally.[1] Sub-Saharan and South East Asian regions account for highest burden of the disease (>72%).[2] Unfortunately, India bears highest toll of the disease which demands acceleration in interventions

for diarrhea prevention and cure.[2] Despite the well-known fact that diarrheal diseases are transmitted by fecal oral route[3] inexorable outbreaks continue to be a scourge globally.[4] Infectious milieu of diarrhea shed light on its multifactorial nature and vast array of disease etiology.[5] Virulence arsenal of etiological agents varies with geographical features and become significantly evident in choice of treatment protocol. Therefore, for a successful treatment regime, identification of etiological agents is of utmost significance.

Escherichia coli is enormously versatile bacterium which elaborates its commensal and pathogenic potential in human host. Diarrheagenic *E. coli* (DEC) is reported as one of the leading causes of gastrointestinal disorders worldwide and signified as an important issue to address in public health.[6-9] In low- to middle-income countries, >40% of diarrheal episodes among children are caused by diarrheagenic *E. coli* pathotypes.[10] These pathotypes also play a considerable role in diarrhea morbidity in the Indian population.[11-13] Remarkably, distinct DEC pathotypes display specific virulence arsenal which transforms the predominant repertoire available for diagnostic and therapeutic approaches. DEC is further catalogued into various pathotypes based upon occurrence of these unique virulence determinants contributing to specific pathophysiology,[14] viz. Enteropathogenic *E. coli* (*eae*, *bfpA*), enterotoxigenic *E. coli* (*eltB*, and *estA*), enteroaggregative *E. coli* (*pCVD*), enterohemorrhagic *E. coli* (*vt1* and *vt2*), and enteroinvasive *E. coli* (*ial*).[14-16]

Enteropathogenic *E. coli* (EPEC) is frequently associated with diarrhea incidences from both community and healthcare settings. EPEC has been categorized into atypical and typical EPEC by the presence of *eae* gene alone and simultaneous expression of *bfpA* and *eae* genes, respectively.[14,15]

Enterotoxigenic *E. coli* (ETEC), another *E. coli*, has been reported as a significant pathogenic form responsible for diarrhea in travelers and population inhabiting endemic regions globally.[12,17,18] ETEC in the stool specimen may be confirmed by amplification of two marker genes *estA* and *eltB*, which encode heat stable and heat labile secretory enterotoxins, respectively.

Since the last decade, several reports have been published for identification of adherent enteroaggregative *E. coli* (EAEC) as an emerging enteropathogen responsible for adult and childhood diarrhea worldwide.[19-22] The plasmid-encoded gene probe *pCVD* which elucidate aggregative phenotype is utilized for identification of EAEC in diagnostic and epidemiological studies.[12,18,23]

Another molecular pathotypes of *E. coli*, enterohemorrhagic *E. coli* (EHEC), a subgroup of Shiga toxin-producing *E. coli* and enteroinvasive *E. coli* (EIEC) cause a devastating form of gastrointestinal infections which may lead to severe life-threatening complications like hemolytic uremic syndrome (HUS). EHEC colonizes large intestine and secretes toxins.[14] EIEC invades small bowel enterocytes and is regarded a true intracellular pathogen. However, both EIEC and EHEC display low levels of incidences.[6,24]

Escherichia coli serogrouping is used as a conventional method for pathogen characterization and diagnosis.[14,15] Besides, potential use of O antigen characterization, DEC associations with O antigens varies across different geographical regions.[25,26] As DEC pathotypes possess a large number of different "O" somatic antigen; therefore, their continuous monitoring is helpful in subtyping strains and enhancing phylogenetic studies.

As diarrheal disease is generally self-limiting, antidiarrheal agents are not usually recommended for treatment of diarrhea.[27] However, traveler's diarrhea, persistent diarrhea, and acute invasive diarrhea display high severity of infection and extended recovery periods which reinforce the use of antimicrobials such as ampicillin, norfloxacin, nalidixic acid, cefixime, and cotrimoxazole.[27-30]

Several investigations have been conducted to study the prevalence of DEC pathotypes in different parts of India.[12,13,31,32] However, comprehensive study regarding DEC-mediated diarrhea is sparse, and inclusive epidemiological studies are not available from Northern hilly regions of the country. This study focuses on investigating the prevalence of diarrheagenic *E. coli* pathotypes with exhibited serogroups in Himachal Pradesh, a northern hilly state of India. Molecular methods were utilized to better define DEC incidences, their etiology and clinical outcomes. Correlation of DEC pathotypes with different age groups and clinical symptoms was also analyzed. The study also encompasses resistance patterns of identified *E. coli* pathotypes, which will be useful in treatment regimes for tackling these specific pathogens.

2 | MATERIALS AND METHODS

2.1 | Culture media and reagents

MacConkey agar, eosin methylene blue agar, Muller Hinton agar, nutrient agar, LB broth, and agar for conventional culture techniques were purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India. Agarose, saturated phenol, sodium acetate, antibiotic disks (ampicillin 10 μg, cefixime 5 μg, cotrimoxazole 75 μg, norfloxacin 10 μg, and nalidixic acid 30 μg), and E-strips were also purchased from Hi-media Laboratories Pvt. Ltd., Mumbai, India. PCR master mix and 100-bp DNA ladder were purchased from Promega, Thermo Fisher Scientific, and New England Biolabs (NEB). Chloroform, isoamyl alcohol, and ethanol of analytical grades were purchased from Merck. Primers utilized in this study were purchased from Integrated DNA Technologies, Bangalore, India.

2.2 | Study sites and clinical specimens

From February 2013 to April 2016, a total of 572 stool specimens of diarrheal patients aged between 13 days and 85 years were collected. Samples were collected from patients with primary complaint of three or more loose stools/day who were admitted to Regional hospital, Solan and tertiary care hospital Indira Gandhi medical college (IGMC), Shimla. Information on gender, age, geographic origin, and clinical symptoms was obtained by means of standard questionnaire. Patients presented with loose stool as chief complication but also reported to have other clinical manifestations such as dehydration, vomit, fever, abdominal pain, and mucus, in common. Written informed consents were taken from patients or patient's parent or legal guardians in case of children.

Stool specimens from diarrheal patients were collected in sterile containers and transported immediately to the laboratory after collection. All experiments included in the study were authorized by the Institutional Ethics Committee (IEC/Project no-04-2014).

2.3 | Isolation and detection of DEC

The stool specimens were enriched in Luria Broth and streaked onto MacConkey agar, eosin methylene blue agar and incubated for 24 to 48 hours at 37°C. Typical lactose fermenting pink colored colonies from MacConkey agar were selected and subcultured on Luria-Bertani agar. Following overnight incubation, lactose fermenting colonies were subjected to a series of standard biochemical tests; IMViC (indole, methyl red, Voges-Proskauer, citrate), triple sugar iron agar, urease agar, and motility tests.[18] Bacterial strains with characteristic IMViC pattern ++−− were biochemically characterized as *E. coli* strains.

2.4 | DNA extraction and 16S rRNA gene characterization

Biochemically confirmed strains were initially molecularly characterized using 16S rRNA gene for *E. coli*.[33] DNA extraction of *E. coli* strains was performed by phenol-chloroform method. PCR thermo cycling conditions for 16S rRNA gene were standardized at following condition. Initial denaturation was performed at 94°C for 5 minutes, final denaturation 94°C for 30 seconds, primer annealing at 52°C for 30 seconds, and initial extension at 72°C for 30 seconds for 35 cycles, with a final extension of 7 minutes at 72°C. PCR was set up with 25 μl reaction mixture having 12.5 μL 2 × PCR Master Mix, 0.2 μmol/L of each primer, 300 ng/μL of template DNA, and nuclease-free water. PCR products were evaluated with a 1.5% 1XTAE (Tris-acetic acid-ethylene diamine tetracetate buffer) agarose gel at 50 mV for 30 minutes. A 100-bp molecular marker was run concurrently. PCR products were visualized under ultraviolet light trans-illumination.

2.5 | Molecular characterization of DEC pathotypes

DEC molecular pathotypes were identified by amplification of virulent genes. Primer sequences were selected from two published studies and are given in Table S1.[17,18] Initially, molecular pathotypes were amplified in a multiplex PCR followed by single gene PCR for identification and reproducibility of specific DEC pathotypes. Different molecular pathotypes were identified on the basis of amplification of following amplicons of genes; ETEC encoded heat stable (*estA* 147bp[17,18]) and heat labile toxin (*eltB* of 322bp[17] or 508 bp[18]) genes, EPEC encoded bundle pilus-forming gene (*bfpA* 367bp[17,18]) and intimin gene (*eae* of 830[18] or 376 bp[17] amplicon), EHEC encoded verocytotoxins (*vt1* 130bp[17] and *vt2* 298bp[17]), EIEC encoded invasion gene (*ial* 320bp[17]), and EAEC plasmid encoded aggregative phenotype specific (*pCVD* 630bp[17,18]) were targeted in the PCR. During EPEC pathotype identification, *eae* and *bfpA* genes amplify at 367bp and 376 bp, respectively,[17] which could not be resolved by agarose gel electrophoresis; therefore, *eae* gene (830 bp) and *bfpA* gene (367 bp) were stringently amplified by single gene PCR. PCR thermocycling conditions for pathotypes were same as described above for amplification of 16S rRNA gene. Amplified PCR products were further confirmed by commercial Sanger sequencing at various time intervals during study. Sequenced DEC pathotypes were taken as positive control in PCR.

2.6 | Serological characterization

Identification of bacterial somatic O antigen was performed by standard agglutination test using 176 "O"-specific antisera.[34] For serogroups characterization, biochemically and molecularly confirmed *E. coli* isolates were screened at National *Salmonella* and *E. coli* center at Central Research Institute, Kasauli (H.P.). Briefly, test strain was inoculated into 5 mL nutrient broth and incubated at 37°C for overnight with agitation. Bacterial growth was boiled at 100°C for one hour, and then formalin was added to a final concentration of 0.3% (Test antigen). For testing with pooled sera, 50 μL of 16 pools of O antisera was added to 96-well plate. Then 50 μL of test antigen was added to each well. A negative control well was set up with 50 μL each of antigen and saline, respectively. Plates were incubated at 37°C overnight and observed for agglutination reaction. Test strain showing agglutination in all wells including negative control, strain was regarded "rough." If agglutination was seen with single pool, then next agglutination test was set up with factor sera constituting the pool. But if agglutination was seen with more than one pool, then antigen was titrated against all sera constituting the pools. The test antigen which even did not show agglutination following antigen preparation at 121°C for 2½ hours was regarded as "untypeable."

2.7 | Antimicrobial susceptibility test (AST) and minimum inhibitory concentration determination (MIC)

The antimicrobial susceptibility of the PCR-positive *E. coli* pathotypes was determined by standard Kirby Bauer's disk diffusion method[35] against ampicillin (10 μg), cefixime (5 μg), cotrimoxazole (25 μg), nalidixic acid (30 μg), and norfloxacin (10 μg) according to CLSI and ICMR guidelines.[30,36] Minimum inhibitory concentrations for ampicillin (0.016-256 μg), cefixime (0.016-256 μg), cotrimoxazole (0.016-256 μg), nalidixic acid (0.016-256 μg), and norfloxacin (0.016-256 μg) were determined using the E test. *Escherichia coli* ATCC 25922 was used as reference strains for quality control in AST and MIC tests. Results were interpreted according to CLSI and ICMR guidelines.[30,36]

2.8 | Statistical analysis

The age of the patients was classified into five groups, viz., <2 years, 3-5 years, 6-17 years, 18-65 years, and >65 years. In statistical analysis >65 years age represented the most normative group because elderly from developing countries are more prone to diarrheal infection due to immunocompromised status.[37] In a similar study by Dutta et al,[12] 2012, elderly age group comprising subjects >65 years of age were also taken as reference for comparative statistical analysis.

TABLE 1 Comparative analysis of clinical features associated with DEC-positive and DEC-negative patients by chi-square test

Clinical symptoms observed	DEC positive (n = 120,%)	DEC negative (n = 452,%)	*P* value (at 95% CI)
Vomiting	30 (25)	109 (24.1)	.840
Fever	25 (20.8)	62 (13.7)	.0536
Dehydration	25 (20.8)	107 (23.6)	.5117
Watery diarrhea	20 (16.6)	11 (2.4)	.0001*
Mucus	6 (5)	4 (0.8)	.0022*
Abdominal pain	3 (2.5)	6 (1.3)	.3589

*Statistically significant values.

Similarly, categorized DEC was compared among each age group with >65 years as reference group. Fisher's exact test was performed to establish mutual relatedness among the three types of DEC pathotypes. *P* values of ≤.05 were considered as statistically significant and calculated the odds ratio (OR) at the 95% confidence interval (CI). Chi-square (bivariate analysis) was used to compare clinical symptoms in DEC-positive and DEC-negative populations.

3 | RESULTS

3.1 | Identification and molecular characterization of *E. coli*

During February 2013 to April 2016, a total of 572 stool specimens were collected from diarrheal patients admitted to regional (Govt. hospital Solan) and tertiary care hospital (Indira Gandhi Medical College, Shimla) in Himachal Pradesh, a hilly state of India. Hospitalized patients presented a very wide age group window ranging from 13 days to 85 years. A total of two hundred forty-seven (n = 247) patients < 5 years of age and three hundred twenty-five patients (n = 325) aged >5 years were analyzed in this study. Standard microbiological techniques and biochemical assays showed the presence of diarrheagenic *E. coli* in stool specimens of diarrhea patients.

3.2 | Incidences of DEC molecular pathotypes in study population

Identification of diarrheagenic *E. coli* pathotypes was performed on the basis of biochemical, molecular, and serological assays. All biochemically characterized *E. coli* were also confirmed by 16S rRNA gene amplification (Figure S1A). Further, DEC molecular pathotypes were identified by amplification of virulence gene-specific primers selected according to pathotype classification system devised by Nataro and Kaper[14] (Figures S1B-D). Overall, diarrheagenic *E. coli* accounted for a proportion of approximately 21% (n = 120/572) in hospitalized patients. Among distinct DEC pathotypes, EPEC (n = 79/572, 13.8%) was found to be predominant pathotype followed by ETEC (n = 33/572, 5.8%) and EAEC (n = 8/572, 1.4%). Pathotypes belonging to classes EHEC and EIEC of DEC were not found in analyzed specimens. The amplified products of PCR were further confirmed by sequencing, and partial coding sequences obtained were found to be 100% similar to targeted reference genes. The gene sequences were submitted to NCBI database (NCBI accessions: KX911251, KX911252, KX911253, and KX911255) and were utilized as positive control in subsequent PCR analysis (Data S1–S6).

3.3 | Distribution of virulent genomic elements among DEC molecular pathotypes

Characterization of DEC molecular pathotype was ascertained on amplification of either distinct gene or combination of genes (Table S1). In this study, *eae* gene of atypical EPEC (62.5% *eae* gene, n = 75/120) was most prevalent as compared to typical EPEC (3.3% *eae* & *bfpA*, n = 4/120). In case of ETEC-infected patients, strains harboring *estA* (18.3%, n = 22/120) were more prevalent than strains possessing both *estA* and *eltB* genes (10%, n = 12). All EAEC strains (n = 8) possessed *pCVD* (6.6%, 8 of 120) gene.

3.4 | Clinical symptoms Vs DEC molecular pathotypes

Clinical symptoms of DEC pathotype-mediated infection vary from acute to persistent diarrhea, febrile, or afebrile, with or without symptoms of dehydration. Besides, loose stools as a common illness among study population, symptoms of fever, vomit, dehydration, mucus, and abdominal pain were also observed. To ascertain DEC molecular pathotype-specific clinical symptoms, chi-square analysis was performed, and *P* values and odds ratio (OR) at 95% confidence interval (CI) were calculated (Table 1). For comparison, DEC-positive population (n = 120) was taken as positive control, and population without DEC infection (n = 452) was utilized as negative control. Symptoms of watery stools, visible mucus were found statistically associated with DEC pathotype infection. Other pathophysiological features such as vomiting, severe dehydration, and fever were also observed with higher frequency in EPEC and ETEC pathotypes, but similar cases were also observed in DEC-negative population therefore statistically insignificant. While, EAEC infection was found primarily associated with the frequent bowl movements (>6 episodes of watery stool), fever, vomiting, and dehydration.

3.5 | Age group distribution of DEC molecular pathotypes

For determination of high-risk age groups, study population was stratified into five various age groups, viz. children 0-2 years (n = 202) and 3-5 years (n = 45), adolescent 6-17 years (n = 41), adult 18-65 years (n = 257), and elderly >65 years (n = 27). Our study revealed uniform abundance of EPEC and ETEC infections in all age segments, however, children < 5 years (<2 years & 3-5 years) of age showed higher incidence rates as compared to any other age group (Figure 1). Although EAEC pathotype was detected with low frequency, but enteropathogen was predominantly found in children population (5.2%, 7/572) as compared to adult diarrheal patients (0.3%, 1/572) (Figure 1).

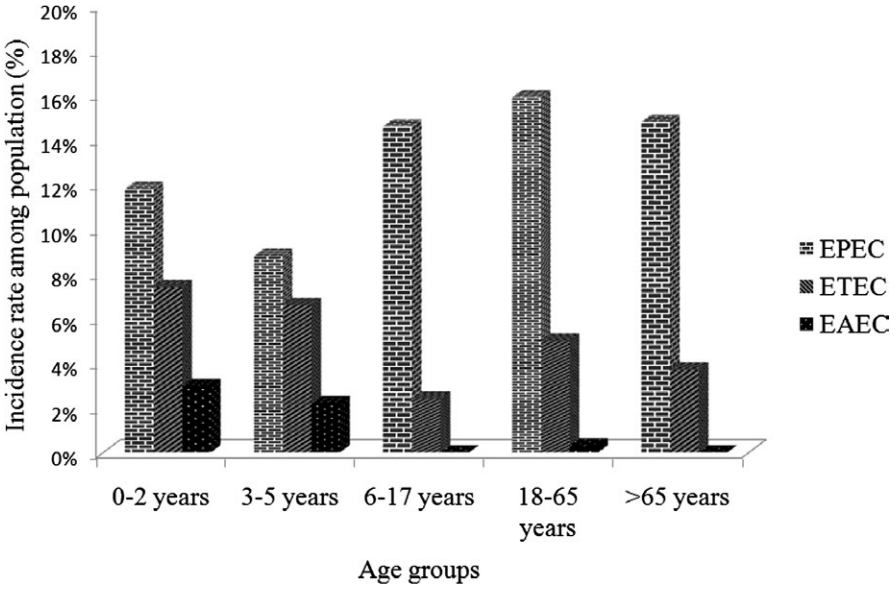

FIGURE 1 Prevalence of diarrheagenic *Escherichia coli* (DEC) pathotypes among different age groups. *X*-axis represents different age groups under study, and *Y*-axis represents proportions of different pathotypes. EPEC= Enteropathogenic *E. coli*, ETEC= Enterotoxigenic *E. coli*, EAEC= Enteroaggregative *E. coli*

To recognize specificity of any DEC pathotype to particular age groups, bivariate Fisher analysis was performed (Table 2). Statistically significant correlations were observed for EPEC, ETEC, and EAEC pathotypes with those of children <2 years of age. However in adult age group (17-65 years), only EPEC and ETEC prevalence were correlated significantly.

3.6 | Serogroup analysis of DEC pathotypes

Molecular pathotypes of DEC were characterized for *E. coli* somatic O antigen and were found associated with at least twenty-three different O serogroups (Figure 2). Serologic analysis revealed 60.8% (73/120) of *E. coli* isolates as diarrhea-associated serotypes. Serogroups O2, O26, O35, and O41 were the most commonly characterized with a prevalence of 41% (30/73).

TABLE 2 Bivariate analysis of age wise distribution of diarrheagenic *Escherichia coli* pathotypes using Fisher's exact test

Age group	DEC pathotype	Odd Ratio (at 95% CI)	*P*- value
0-2 yrs	EPEC (24/79)	8.182 (2.684-24.94)	.0001*
	ETEC (15/33)	26.67 (3.248-219)	.0001*
	EAEC (6/8)	44.20 (1.794-1089)	.0070*
3-5 yrs	EPEC (4/79)	1.00 (0.2411-4.418)	1.0000
	ETEC (3/33)	3.1 (0.3050-31.50)	.6312
	EAEC (1/8)	3.4 (0.1194-96.78)	1.0000
6-17 yrs	EPEC (6/79)	1.541 (0.4175-5.688)	.7480
	ETEC (1/33)	1.000 (0.05988-16.70)	1.0000
18-65 yrs	EPEC (41/79)	20.23 (6.743-60.69)	<.0001*
	ETEC (13/33)	20.80 (2.522-171.5)	.0005*
	EAEC (1/8)	3.400 (0.1194-96.78)	1.0000
	>65 yrs as reference category		

*Statistically significant values.

Figure 3 shows O26 and O2 as most commonly isolated among EPEC and ETEC pathotypes, respectively. O26 is often associated with classical attaching and effacing group (EPEC) and non-O157-EHEC strains.[14] We observed serogroups O2, O25, and O128 in ETEC strains only. Apart from classical serogroups, EAEC predominantly belonged to serogroup O3 (2.5%). However, higher proportions of strains belonging to EPEC, ETEC, and EAEC pathotypes remained untypeable (30%) and did not agglutinate with O antiserum (9%).

Correlation between DEC virulent genes and O serogroups revealed *eae* gene of EPEC was most commonly associated with more number of O serogroups (Figure 4). *estA* and *eltB* genes of ETEC toxins were observed with O2, O20, O25, O102, and O141 serogroups while serogroup O9 was observed with *estA* only. The *pCVD* gene of EAEC was found to be associated majorly with one serogroup O3.

3.7 | Antimicrobial resistance in DEC molecular pathotypes

Several studies have been performed for analyzing antibiotic resistance patterns among diarrheagenic *E. coli* isolates.[27-30] Therefore, molecular pathotypes of DEC were also screened for antibiogram patterns by antimicrobial susceptibility test (AST) and minimum inhibitory concentration (MIC) according to CLSI guidelines.[36] Antibiotics utilized in screening were chosen on the basis of ICMR recommendations[30] (Figure S2A & Figure S2B). AST and MIC revealed that a majority of diarrheagenic *E. coli* strains were sensitive for cotrimoxazole (36%), while <20% were sensitive for cefixime, norfloxacin, ampicillin, and nalidixic acid (Table 3). Proportions of intermediate strains against all five antibiotic were <5%. DEC pathotypes exhibited alarming rates of resistance against widely used antimicrobials; ampicillin, cefixime, nalidixic acid, and norfloxacin (approximately 80%).

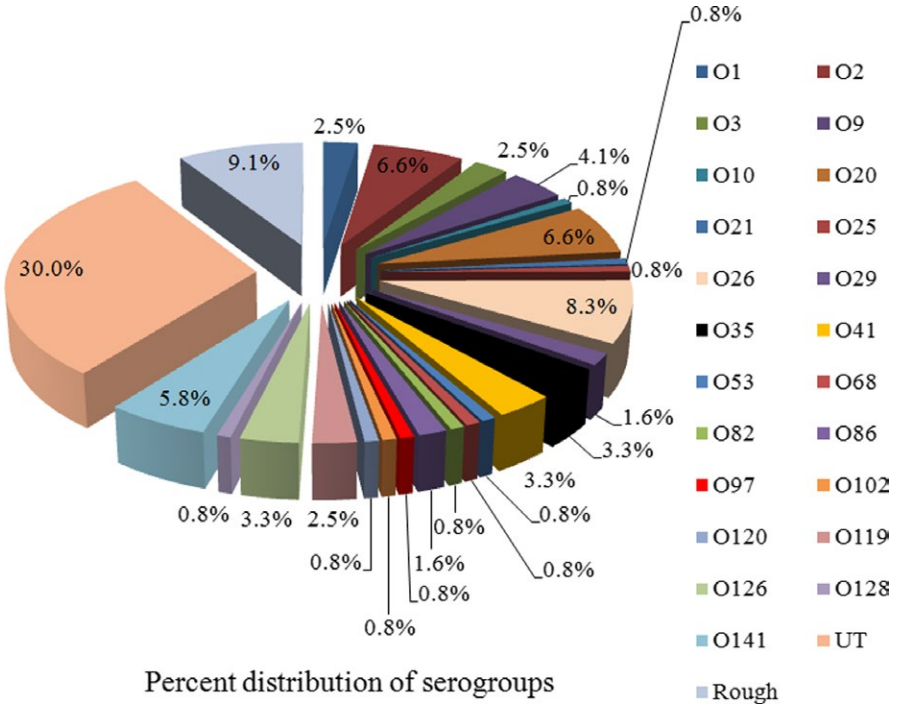

FIGURE 2 Distribution of different O serogroup among diarrheagenic *Escherichia coli* (DEC) isolates. UT (untypeable), Rough (Nonagglutinable with antisera)

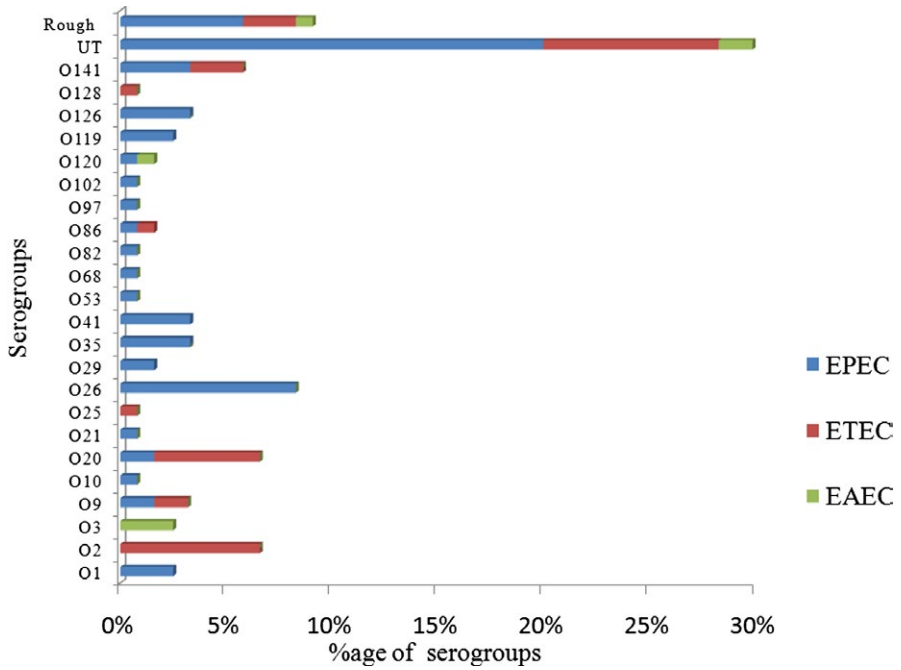

FIGURE 3 Relationship of virulent genotype with serogroups of diarrheagenic *Escherichia coli* strains characterized from diarrhea patients

On correlating resistance pattern with distinct DEC pathotype, EPEC strains were observed to be most resistant against all tested drugs. ETEC strains were found 18-49% sensitive against all tested antibiotics. EAEC pathotypes showed highest sensitivity for cotrimoxazole (62.5%), norfloxacin (37.5%), nalidixic acid, and cefixime (25% in both). Table 3 showed statistical analysis using chi-square test performed to evaluate pathotype-specific antibiotic resistance level. All molecular DEC pathotypes were found to possess significant levels of antibiotic resistance against all antibiotics.

4 | DISCUSSION

Successful interventions in management of infectious diseases need identification of the etiological agent and treatment of clinical symptoms manifested during disease. Diarrhea is a multifactorial illness associated with wide spectrum of pathogens including viruses, bacteria, and parasites.[38,39] Conventional microbiological techniques combined with molecular identification system incredibly increases reproducibility and scalability of etiological agent characterization.[40] However,

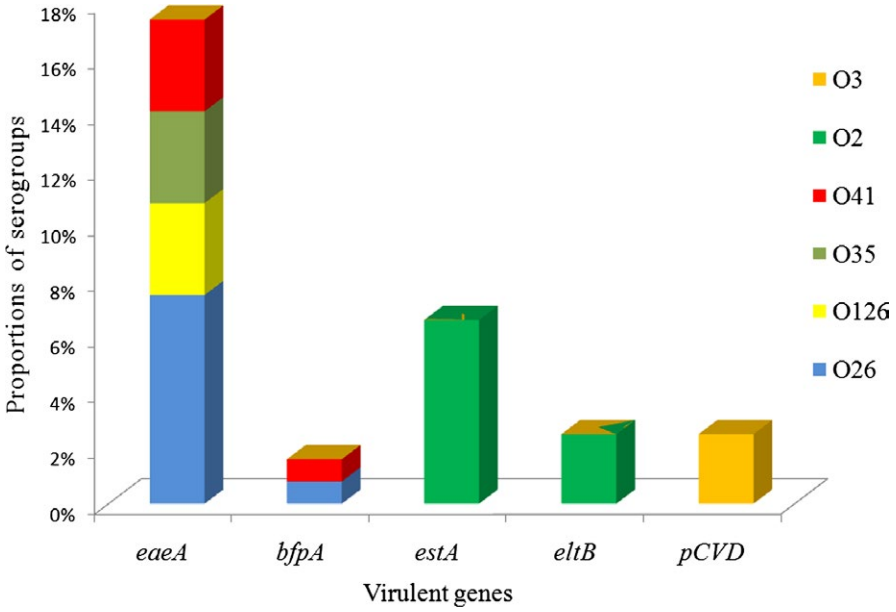

FIGURE 4 Representing association of diarrheagenic *Escherichia coli* (DEC) virulent genes with top six O serogroups

TABLE 3 Antibiotic resistance among the different diarrheagenic *Escherichia coli* groups in patients with diarrhea

	Characteristics (Resistant)	*P* value	OR (95% CI)
AMP	EPEC 82.2% (65)	.0026**	2.792 (1.456-5.353)
	ETEC 78.7% (25)	< .0001**	3.473 (1.866-6.462)
	EAEC 75% (6)	< .0001**	4.895 (2.668-8.979)
CPM	EPEC 77.2% (61)	.0311*	2.052 (1.108-3.801)
	ETEC 81.8% (26)	.0004**	3.329 (1.721-6.440)
	EAEC 75% (6)	< .0001**	4.895 (2.668-8.979)
NAL	EPEC 79.7% (67)	.0078**	2.452 (1.299-4.627)
	ETEC 81.8% (27)	< .0001**	4.205 (2.209-8.005)
	EAEC 62.5% (5)	< .0001**	3.807 (2.078-6.974)
NOR	EPEC 79.7% (67)	.0078**	2.452 (1.299-4.627)
	ETEC 69.6% (22)	.0135*	2.154 (1.205-3.849)
	EAEC 62.5% (5)	< .0001**	3.807 (2.078-6.974)
COT	EPEC 62% (49)	Reference category	
	ETEC 51.5% (16)		
	EAEC 37.5% (3)		

AMP, ampicillin; COT, cotrimoxazole; CPM, cefixime; NOR, norfloxacin, and NAL, nalidixic acid. Statistically significant values (*$P < .05$, **$P < .01$ (chi-square test), for the comparison of the resistance percentage among the different *E. coli*).

in context of diarrhea, the viral agents have been explored to a greater extent as compared to others.[41-43] By virtue of phenotypic and genotypic attributes, *E. coli* elaborates capacities from important gut commensal to pathogen of intestinal as well as extraintestinal infections.[15,44] *E. coli* possesses a repertoire of virulent elements which lead to segregation of this bacterium into diverse kinds of pathotypes and genotypes. Lack of uniform surveillance system for bacterial pathogens underestimates their role in diarrhea incidences. In our previous study, we have deciphered the role of viral agents of diarrhea in Himachal Pradesh, a Northern hilly state of India.[45] The current study was aimed at elucidating the frequency of DEC pathotypes using virulence gene markers in moderate-to-severe diarrhea population of Himachal Pradesh. Prior to this study, there have been no reports from present region addressing DEC incidences. Therefore, it is of utmost importance to address DEC-associated diarrheal incidences to provide a comprehensive view of diarrhea etiology within the region which will facilitate further epidemiological and therapeutic prospects.

The diarrhea study cases involved in comprehensive investigation belonged to a broad window of age, from 13 days to 85 years. Therefore, the study population was stratified into five different age groups (0-2 years, 3-5 years, 6-17 years, 18-65 years, and >65 years), and age group >65 years was taken as reference group for statistical analysis. The incidence rates of diarrheagenic *E. coli* were observed up to 21% as the sole pathogen and approximately 6% as mixed infection

with Rotavirus. Our study shows moderate DEC infection rates, similar to the reports from developing world.[46-49] However, reports from other parts of India and neighboring countries showed 10-35% variation in DEC incidence rates.[11-13,50,51] Sporadic outbreaks with 42% to 65% of incidences are also reported from different regions of India.[52-54] Globally, prevalence of *E. coli* as an etiological agent of diarrhea is well reported between 30% and 40% cases.[55,56] DEC coinfection with other enteric pathogens is greatly known to aggravate symptoms and duration of diarrhea.[50,57]

Our observations indicate higher proportions of DEC pathotypes associated with childhood diarrhea than any other age set. In a recent study conducted in Mexico, Canizalez-Roman and coworkers[49] also reported higher DEC incidences in children population. In addition, higher frequencies of DEC pathotypes in moderate-to-severe cases of childhood diarrhea are reported all over the globe.[5,12,29,56,58] Previous studies established that DEC preponderance among children may be due to their compromised immune level and intimate attachment of pathogens to the tender epithelial mucosa.[14,59] DEC infection-induced alterations in intestinal physiology and microbiota composition remain restricted to the postnatal period also.[59] Therefore, DEC infection might predispose children < 5 years to sequelae of diarrheal episodes.

By molecular identification approach, DEC molecular pathotype EPEC is observed with highest frequency among all diarrheal patients. Recurrent isolation of EPEC from severe diarrhea cases is implicated especially in pediatric populations.[60] Persistent diarrhea is the most common clinical presentation in EPEC infection, and this enteropathogen possesses an innate propensity to persist longer in intestine than other pathotypes.[14] EPEC is typically categorized into two classes, atypical EPEC having *eae* gene and typical EPEC possess combination of *bfpA* and *eae* genes.[61] High frequencies of the *eae* gene in current study underpin the importance of atypical EPEC as predominant diarrheal pathogen in the region. Low frequency of *bfpA* observed in the present study suggests its fewer incidence rates in the population similar to the previous reports.[62,63] Both *eae* and *bfpA* genes are responsible for intimate attachment to the surfaces via intimin and bundle-forming pilus. In addition, EPEC also possesses different combination of fimbriae and type III secretion system protein for producing attaching and effacing phenotypes. On global level, EPEC alone contributes for 5%-10% cases of pediatric diarrhea.[58,61,64] Our observations coincide with various epidemiological studies from different parts of world which reported EPEC as the main DEC pathotype affecting children and adults with similar frequency.[65-68]

Another DEC molecular pathotype ETEC-specific clinical outcomes rely upon the secretion of two enterotoxins, viz heat labile (*estA* gene) and heat stable (*eltB* gene) toxins. These toxins result in secretory diarrhea via Cl^- secretion through the cystic fibrosis transport receptor (CFTR) and cyclic guanosine monophosphate (cGMP).[14] Among the ETEC-positive patients, *estA* gene was more frequently isolated than *eltB* alone, or *estA* and *eltB* in combination are similar to other studies.[12,69] For many years, ETEC has been implicated as the major cause of traveler's diarrhea[70]. In the present study, ETEC showed varying prevalence among all ages, and similar observations were reported from the northern part of the country.[69,71]

EAEC pathotype is known to cause disease via multiple mechanisms; adherence to mucosa, secretion of toxins, and mucosal inflammation.[22] The EAEC enteropathogen was identified by using *pCVD* gene probe. We observed EAEC predominantly in children (n = 7/8) followed by elderly age group (1/8). Other studies have also shown prevalence of *pCVD*-positive *E. coli* in the stool specimens of adults and childhood diarrhea, and this can be as high as 11%.[72] Current findings strengthen evidences that EAEC is an emerging diarrheal agent in the South East Asian children population.[73]

As different DEC molecular pathotypes exhibit surface to invasive pathophysiology resulting in different clinical outcomes. We found that clinical symptoms of watery stools and mucus were significantly associated with DEC pathotype infection. Present observations reinforce the conviction that DEC pathotype is considerably responsible for severe gastrointestinal infections associated with childhood and adult diarrhea.[39]

Characterization of *E. coli* somatic "O" antigen still appears to be useful technique for conventional identification of certain DEC pathotypes.[14,74-76] The serogroup O26 was most commonly observed followed by O2, O41, O35, O126, and O1. Similar to our study, serogroups O26, O2 were found to be associated severe diarrhea cases.[61] Interestingly, few isolates belong to untypeable or rough classes in various categories of DEC pathotypes. From the literature, *E. coli* serogroups are much related to identification of clonal variant of DEC pathotypes rather than precise identification.[14,15,75,76]

The global spread of antimicrobial resistant strains threatens the effective prevention and treatment of enteric infections caused by Gram-negative bacteria. *E. coli* has become increasingly resistant to conventional and commonly used antibiotics in hospital and community settings,[26,77] and certainly poses serious threat to the management of infectious diseases.

We examined the DEC pathotypes resistance against five antibiotics: ampicillin, cefixime, norfloxacin, nalidixic acid, and cotrimoxazole belonging to class quinolones and β-lactams. These antibiotics are in accordance with Centre for Disease Control and Prevention and also advised by ICMR.[27,30] In the present study, EPEC was found as most the resistant pathotype, and highest levels of antibiotic resistance were observed against ampicillin. These observations are concordance with previous studies analyzing DEC resistance.[78,79] We observed lowest resistance rates against cotrimoxazole among all DEC pathotypes. However, Sadeghabadi and coworkers reported approximately 80% resistance against cotrimoxazole in diarrheagenic *E. coli*. Similar reports across the globe also elucidated high levels of resistance against DEC pathotypes.[71,79-82] Although in our study, proportions of DEC as diarrheal pathogen are limited to a moderate level; however, current study revealed high levels of resistance among DEC pathotypes in hospitalized patients. The observed high resistance rates to antibiotics may be a result of extreme disease severity and persistence of infections among hospitalized patients.

The present study is to our knowledge, the first comprehensive research in the region addressing associations of molecular DEC pathotypes with clinical outcomes and antibiogram patterns. Our findings

highlight the importance of continuous DEC pathotype surveillance programs for therapeutic approaches and not the least the benefit of employing comprehensive inspection of antimicrobial resistance in the region. In relation to treatment, a very few studies have evaluated comprehensive importance of drugs for the management of DEC pathotype infection. After introduction of the rotavirus vaccines into national immunization program of India, the next priority must be to identify diarrheal pathogens owing to high morbidity and mortality rates. The study would help in prioritizing diagnostic and therapeutic measures against predominant DEC pathotypes. Exploring the resistant phenotypes would aid in management and spread of multidrug-resistant strains.

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AUTHOR'S CONTRIBUTIONS

Jitendraa Vashistt and Harish Changotra designed the study and fieldwork. Nutan Thakur and Swapnil Jain performed fieldwork with assistance from Neelam Grover in stool specimens collection and transport. Nutan Thakur carried out laboratory research experiments and manuscript writing. Yashwant Kumar performed serogrouping of *E. coli* strains. Rahul Shrivastava was involved in protocol designing and data presentation.

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SUPPORTING INFORMATION

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