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Diffusely Adherent *E. coli* Burden in Low Socio-Economic Pediatric Population

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ABSTRACT

Background: DAEC seems to be a neglected pathogen among diarrheagenic *E. coli* since its association with pediatric diarrhea is rarely reported. But, the emergence and spread of MDR diarrhea causing bacterial pathogens have become a global burden. This study investigated the prevalence of MDR DAEC among the Mizo pediatric diarrheal patients aged less than 5 years.

Methods: A total of 334 *E. coli* isolates isolated from the fecal samples were subjected to multiplex PCR assays to categorize the pathotypes of diarrheagenic *E. coli* and subjected to antimicrobial sensitivity assay, phenotypic and genotypic ESBL assays (*blaCTX*-M-9, *blaCTX*-M-15, *blaOXA* and CMY-2).

Results: Of 334 *E. coli* isolates, only 0.9% of the isolates were detected as DAEC, when compared with 0.9% and 1.2% of the isolates categorized as ETEC and EAEC from diarrheic samples and 0.6% of the EAEC also detected from the non-diarrheic samples. Among the isolates, 25%, 50% and 8.3% of the isolates were carrying the *blaCTX*-M-9, *blaCTX*-M-15 and CMY-2 respectively and 8.3% isolates from non-diarrheic samples also carried CMY-2.

Conclusion: Though a low frequency of DAEC was observed in comparison to other pathotypes of DEC, majority of them being resistant to commonly used antibiotics and having a high MDR rate, which is a matter of concern to the public health. This pathotype could possibly a natural source for MDR spread among the other diarrheal pathogens of pediatric population.

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Introduction

Acute diarrhea is a common medical problem in India and other developing countries. Almost 2.3 million deaths are reported per year due to diarrhea in the pediatric population in India (1). Enteropathogenic *Escherichia coli* (EPEC), Shiga-toxigenic *E. coli* (STEC) and Enterotoxigenic *E. coli* (ETEC) are the most reported diarrheal pathogens from India; however, scarce information is available about the prevalence of diarrheagenic *E. coli* (DEC) associated with pediatric diarrhea (2, 3, 4). This study region Mizoram is situated in the extreme end of the Himalayan ranges in the North eastern part of India. Aizawl is the capital city, but still very remote and is located at 3715 feet from the sea level, which gets rainfall of 3,000mm with temperature in summer (20 °C to 30 °C) and in winter (11 °C to 21 °C) (5).

A rarely reported but an emerging diffusely adherent *E. coli* (DAEC) pathotype is characterized by the diffuse adherence pattern on cultured epithelial cells like HeLa or Hep-2 (6). A colonization factor that is an afimbrial adhesive sheath (Afa) for intestinal tract infections are used by DAEC strains (7). Similarly, F1845 fimbria consists of at least five genes (daaA–E), which encode the products similar to Dr Adhesin systems, where *DaaE* is termed as the adhesin subunit (8).

The drug resistance in the developing countries has been attributed to the extensive use of antibiotics and poor prescription practices. Only few studies have reported the occurrence of drug resistant DEC in pediatric population of India, but not from the socio economically poor populations, where the awareness and understating of MDR remains limited (9, 10, 11). The present study investigated the prevalence of Diffusely Adherent *E. coli* (DAEC) in Mizoram children who were less than 5 years of age and suffering from diarrhea. In addition, this study examined the seasonal occurrence, multi-drug resistance (MDR) pattern and occurrence of Extended Spectrum β Lactamase (ESBL) variants (*blaCTX-M*-9, *blaCTX-M*-15, *blaOXA*) and *AmpC* enzyme gene *CMY*-2 among the isolates. This report tried to create the awareness among the medical practitioners about the drug resistant Diarrheagenic *Escherichia coli* (DEC) pathogens which could have been the hidden cause for many treatment failures earlier in this region. This report will also help the researchers of the similar study population in developing countries to diagnose and render the timely treatment to avoid the further consequences.

Materials and Methods

Clinical specimens and bacterial strains

A total of 334 *E. coli* isolates were obtained from non-repetitive stool/rectal swab samples from the Mizo children aged less than 5 years from three tertiary care hospitals in Mizoram during November 2013 to October 2015. Out of 334 isolates, 246 were collected from children with acute diarrhea and the remaining 88 isolates were obtained from apparently healthy children who were admitted to the hospital for some non-diarrheal illness. The clinical samples from the patients treated with antibiotics or from those infected with *Salmonella*, *Shigella* and co-infected with parasites, were not included in this study. All the samples were cultured on Mac Conkey's agar (HiMedia, India) and incubated at 37 °C for 24 h. Lactose fermenting colonies were randomly selected from each sample and subjected to conventional microbiological and biochemical tests to identify *E. coli* (12).

Serotyping

The serotyping of the *E. coli* isolates based on 'O' antigen was carried out, as per the method of Edwards and Ewing, at National *Salmonella* and *Escherichia* Centre, Central Research Institute (CRI), Himachal Pradesh, India (13).

Preparation of bacterial DNA for PCR assay

Bacterial DNA was prepared by boiling lysis method. The isolates were inoculated into Luria Bertani (LB) broth (HiMedia, Mumbai, India) and incubated overnight at 37 °C. The broth was centrifuged at 3,000 rpm for 5 min to obtain a pellet that was dissolved in autoclaved distilled water and boiled for 10 min. The resulting bacterial lysate was centrifuged again and the supernatant containing DNA was used as a template for PCR assay (14).

Molecular detection of virulence genes

PCR assay was carried out with the help of following primers: *DaaE* F (5'-GAACGTTGGTTAATGTGGGGTAA-3') and *DaaE* R (5'- TATTCACCGGTCGGTTATC AGT-3') for the detection of DAEC as previously described with minor changes in reaction mix concentrations to yield better PCR products (8). The primers and PCR conditions used for the detection of EAEC and ETEC were as per the method described earlier by Hegde et al. (2012) (2).

HEp-2 Adherence Assay

The method described by Cravioto et al. (1979) (15) was adopted and applied with minor modifications, where the HEp-2 cells grown over night in Dulbecco's Eagle's medium containing penicillin, streptomycin and 2% fetal bovine serum on six-well chamber slides. *E. coli* isolates were grown overnight in Luria broth without shaking at 37 °C (15). The HEp-2 cells were washed with Phosphate buffered saline and then the medium was replaced with Dulbecco's Modified Eagle's Medium (DMEM) containing 1% of mannose and about 10 µL of bacterial suspension was added per well and were incubated at 37 °C in 5% CO2 for 3 h. The monolayers were washed five times with PBS and then fixed with 70% methanol and stained by Giemsa. Strains that adhered to the monolayers were recorded as adhering in localized, diffuse, or aggregative patterns (15).

Antimicrobial Susceptibility test

All the DEC isolates were subjected to antimicrobial susceptibility test against the selected antimicrobials (list of antibiotics as shown in table 4) by disc diffusion method in Mueller-Hinton agar. *E. coli* strain (ATCC 25922) used as a quality control strain and the results were expressed as sensitive, intermediate, and resistant as per CLSI guidelines (16).

Phenotypic and genotypic confirmation of ESBL production

ESBL production in isolates were confirmed phenotypically by using CLSI recommended double disc diffusion tests, where the ceftazidime (30 µg) and ceftazidime plus clavulanate (30/10 µg) disks, cefotaxime (30 µg) and cefotaxime plus clavulanic acid (30/10 µg) disks (HiMedia, India) on Mueller- Hinton agar (HiMedia, India) are placed over the inoculum. After incubation period, the results were read and the isolates are categorized into ESBL producer and non- ESBL producers (16). Furthermore, irrespective of ESBL phenotype test results all the isolates were subjected to PCR assay for the presence of the β-lactamase (*bla*) genes, *viz*, *blaCTX-M*-9, *blaCTX-M*-15, *blaOXA*, and *CMY*-2 by using the previously published primers and PCR reaction conditions (17, 18, 19, 20).

Cloning and sequence analysis

PCR products of all the DAEC isolates carrying *DaaE* gene were purified (QIAGEN kit) and cloned using TA cloning vector (MBI Fermentas) and sequenced in automated sequencer Applied Biosystems 3500 (USA) in the Dept. of Biotechnology, Mizoram University, India. Sequence data were analysed using BioEdit and deposited in NCBI Gene Bank for acquiring the accession Numbers.

Statistical analysis

The data were analysed using SPSS version 17.0 software and the significant differences ($p < 0.05$) between the prevalence of DAEC and other pathotypes in age groups, seasonal variations, Multiple Drug Resistance (MDR) occurrence, ESBL occurrence were compared using chi-square test or Fischer's exact test when appropriate. The p-values less than 0.05 were considered statistically significant.

Results

Clinical features of children affected with DEC

Acute diarrhea lasting for more than one day was recorded of 73.7% (246/334) in children with different clinical symptoms. The occurrence of DEC in females 2.4% (8/334) is little higher than males 1.2% (4/334). Vomiting and abdominal pain were seen in 2.1% (7/334) and 2.7% (4/334) of DEC affected children. All the children infected with ETEC (25%, 3/12) and EAEC (16.6%, 2/12) showed fever at presentation than the DAEC affected children (8.3%, 1/12). In DAEC infection, the stool consistency was mostly watery (16.6%) or with mucous (8.3%) when compared with other types. The DEC affected cases have presented mild (8.3%), moderate (58.3%) to severe (33.3%) dehydration and the DAEC type presented with the severe dehydration in children ($p>0.05$, 95% CI= 0.08536 to 2.314). Although the symptoms were obvious in DAEC; no significant distinct symptoms among the pathotypes were found ($p>0.05$, 95% CI=0.08351 to 2.944). Other major clinical features along with the pathotypes are summarized in table 1.

Isolation and identification of E. coli pathotypes

DEC was significantly detected among diarrheal samples in 2.9 % (10/334) in comparison with non-diarrheic samples 0.6% (2/334) ($p >0.05$, 95% CI = 0.8753 to 1.477). The DEC isolates were categorized as aggregative adherent, locally adhesive or local-like adherent by Hep-2 adherence assay. Among the DEC pathotypes, DAEC and ETEC were less prevalent in the study population 0.9% (3/334) each and detected only from diarrheic children. EAEC was detected from both non diarrheic 0.6% (2/334) and diarrheic samples 1.2% (4/334) respectively ($p >0.05$, 95% CI = 0.8929 to 1.417). Majority of the DEC isolates belonged to the serogroups of O5, O141, O142, O128, O22, O86, Rough and UT (Non-typable) (Table 2). The incidence of diarrhea associated with DAEC was highest during summer (8.3%) and monsoon (16.6%) than in winter (8.3%), among the infants and younger children of Mizoram. DAEC and ETEC were mainly found in the seasons of the summer and monsoon seasons, whereas the EAEC was found in all the seasons.

Occurrence rate of DAEC with other DECs

The occurrence of DEC was significantly high amongst the children aged 13-36 months than in the age group of ≤12 months (figure 1). However, occurrence of DEC in different age groups were greatly varied, DAEC was associated with children aged >12 months (25%) than the infants aged less than 12 months. Whereas EAEC and ETEC occurred in almost all the age groups ranged from 4 -48 months. The incidence of acute diarrhea associated with DAEC increased significantly with the increasing age of the patient and was highest in children aged between 13 to 36 months (16.6%) unlike in EAEC (8.3%) and ETEC (8.3%) where the rate of occurrence does not varied much among the age groups (Table 3).

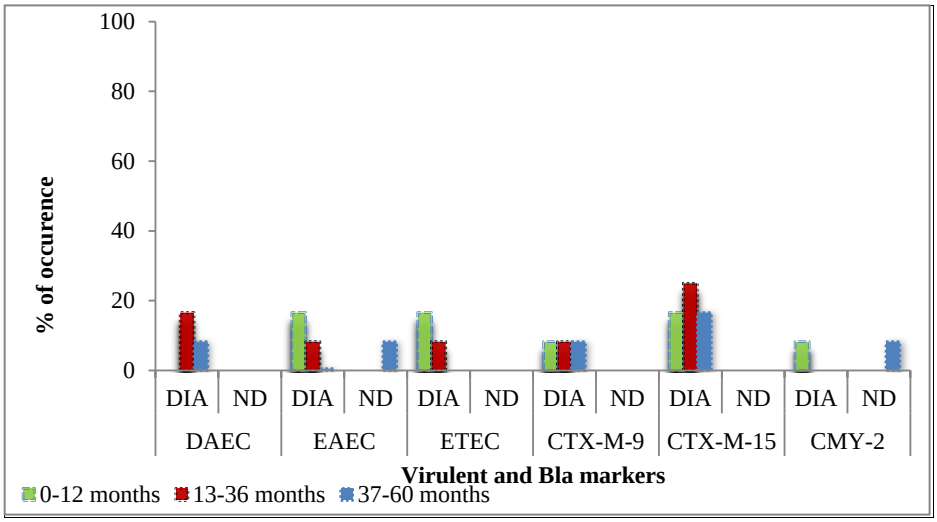

Figure 1. Percentage of occurrence of DEC pathotypes and the *bla* genes in different age group of Mizo children. DIA- Diarrheic, ND- Non- Diarrheic, DAEC- Diffusely adherent *Escherichia coli*, EAEC- Enteroaggregative *Escherichia coli*, ETEC- Enterotoxigenic *Escherichia coli*, Age groups: 0-12 months, 13-36 months and 37-60 months.

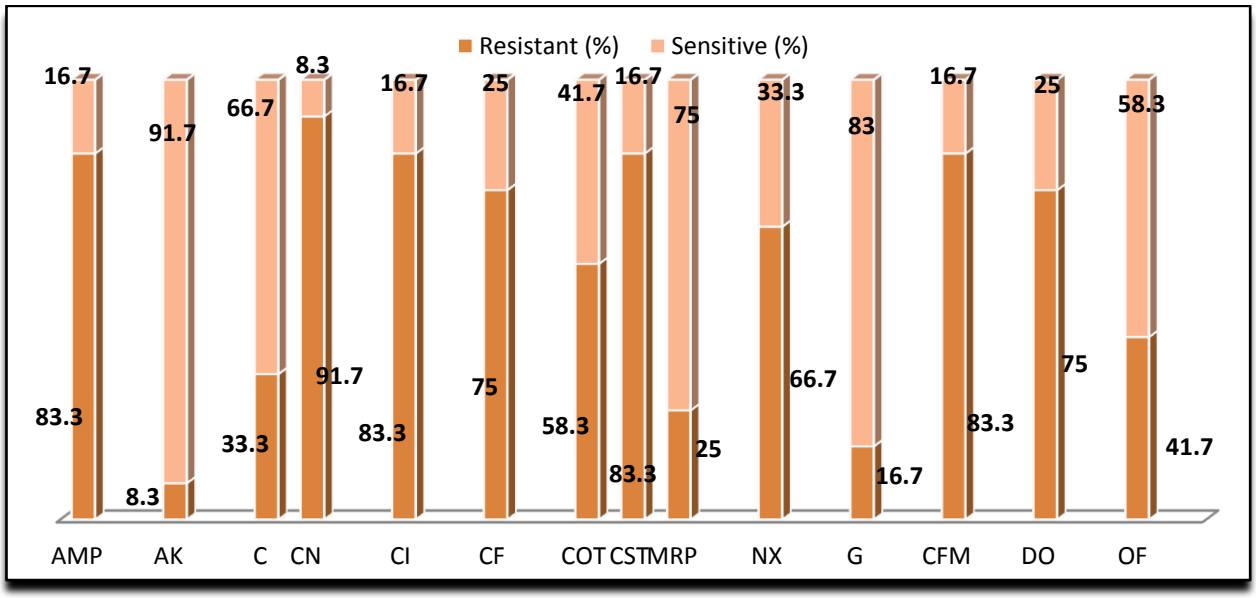

Figure 2. Percentage of Multi drug resistance occurred among the DEC isolates recovered from Mizo pediatric population. MDR - multi drug resistant, µg - microgram, AMP- ampicillin (AMP10 = 10 µg), AK- amikacin (AK10 = 10 µg), C- chloramphenicol (C 30 = 30 µg), CN-cephalexin (CN30 = 30 µg), CI-ceftriaxone (CI 10 = 10 µg), CF- ciprofloxacin (CF10 = 10 µg), COT- co-trimoxazole (COT25 = trimethoprim 1.25 µg and sulphamethoxazole23.75 µg), CST- cefoperazone–tazobactam (CST 75–10 µg), MRP- meropenem (MRP10 = 10 µg), NX-norfloxacin (NX10 = 10 µg), G-gentamicin (G10 = 10 µg), CFM-cefixime (CFM 5 = 5 µg), DO-doxycycline hydrochloride (DO10 = 10 µg) and OF-ofloxacin (OF 5 = 5 µg).

Table 1. Prominent clinical features found in different gender and age group of Mizo children infected with diarrheagenic *Escherichia coli* pathotypes. *

DEC Pathotypes	Gender		Age groups (in months)			Clinical symptoms								
									Dehydration			Stool Consistency		
	Female	Male	0 to 12	13 to 36	37 to 60	Fever	Abdominal pain	Vomiting	Mild	Moderate	Severe	Watery	Mucoid	Bloody
DAEC (n=3)	02	01	_	02	01	01	03	01	_	01	02	02	01	_
EAEC (n=3)	04	02	02	01	03	02	03	04	01	04	01	04	02	_
ETEC (n=6)	02	01	02	01	_	03	03	02	_	02	01	02	01	_
Total (n=12)	08 (66.6)	04 (33.3)	04 (33.3)	04 (33.3)	04 (33.3)	06 (50)	09 (75)	07 (58.3)	01 (8.3)	07 (58.13	04 (33.3)	08 (66.6)	04 (33.3)	

***DEC-** diarrheagenic *Escherichia coli*, **DAEC-** Diffusely adherent *Escherichia coli*, **EAEC-** Enteroaggregative *Escherichia coli*, **ETEC-** Entero toxigenic *Escherichia coli.* Percentage in parenthesis.

Table 2. Seasonal occurrence of serotypes of diarrheagenic *Escherichia coli* recovered from the Mizo pediatric population.*

Summer	138 (41.3)	**DAEC (01):** O142 (1). **EAEC (02):** O 141 (1), O 22 (1). **ETEC (03):** Rough (1), UT (1), O 22 (1).	1 (8.3)	2 (16.6)	3 (25)
Rainy/ Autumn	96 (28.7)	**DAEC (2):** O86 (1), O128 (1). **EAEC (03):** Rough (3). **ETEC (0):** -Nil-	02 (16.6)	3 (25)	–
Winter	100 (29.9)	**DAEC (0):** -Nil- **EAEC (1):** O 5 (1). **ETEC (0):** -Nil-	–	1 (8.3)	–
Total	334 (100)	12 (100)	03 (25)	06 (50)	03 (25)

* DEC- diarrheagenic *Escherichia coli*, DAEC- Diffusely adherent *Escherichia coli*, EAEC- Enteroaggregative *Escherichia coli*, ETEC- Enterotoxigenic *Escherichia coli*. Percentage in parenthesis.

Table 3. Virulent and ESBL gene profiles of Diarrheagenic *E. coli* isolated from the pediatric patients in Mizoram, Northeast India. DAEC- Diffusely adherent *Escherichia coli*, EAEC- Enteroaggregative *Escherichia coli*, ETEC- Enterotoxigenic *Escherichia coli*.

Age groups (Months)	**ESBL (Phenotypic) (n=12)**		**Virulent Genes**						**ESBL genes**					
			***daaE* (DAEC) (n=3)**		***CVD432* (EAEC) (n=6)**		***Elt & Stla* (ETEC) (n=3)**		***$_{bla}$CTX- M-9***		***$_{bla}$TX- M-15***		***CMY-2***	
	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic	Diarrheic	Non Diarrheic
0-12	04 (33.3)	–	–	–	02 (16.6)	–	02 (16.6)	–	01 (8.3)	–	02 (16.6)	–	01 (8.3)	–
13-36	04 (33.3)	–	02 (16.6)	–	01 (8.3)	–	01 (8.3)	–	01 (8.3)	–	03 (25)	–	–	–
37-60	03 (25)	01 (8.3)	01 (8.3)	–	02 (0.6)	01 (8.3)	–	–	01 (8.3)	–	02 (16.6)	–	–	01 (8.3)
Total	11 (91.6)	01 (8.3)	03 (25)	–	05 (41.6)	01 (8.3)	03 (25)	–	03 (25)	–	07 (58.3)	–	01 (8.3)	01 (8.3)

Table 4. Multiple Drug resistance (MDR) Pattern of DAEC isolates recovered from children of Mizoram with acute diarrhea.

Antibiotics (Disc content- µg)	**Resistant (%)**	**Sensitive (%)**	**Multiple Drug resistance (MDR) Pattern**	**No. of MDR isolates (%)**
Ampicillin (AMP 10 µg)	10 (83)	02 (16.7)	C-MRP-G-OF	06 (50)
Amikacin (AK 10 µg)	01 (08.3)	11 (91.7)	G-AK-C-MRP	02 (16.6)
Chloramphenicol (C 30 µg)	04 (33.3)	08 (66.7)	MRP-AK-G	02 (16.6)
Cephalexin (CN 30 µg)	11 (91.7)	01 (08.3)	AMP-DO-NX- CO	10 (83)
Ceftriaxone (CI 10 µg)	10 (83.3)	02 (16.7)	DO-CFM-NX-CST	07 (58.3)
Ciprofloxacin (CF 10 µg)	09 (75)	03 (25)	AMP-CN-CI-CST-CFM	07 (58.3)
Co-Trimoxazole (trimethoprim 1.25µg and sulfamethoxazole 23.75 µg)	07 (58.3)	05 (41.7)	AK-C-G	01 (8.3)
Cefoperazone-Tazobactam (CST-75- 10 µg)	10 (83.3)	02 (16.7)	AMP-CF-MRP-G-OF	06 (50)
Meropenem(MRP 10 µg)	03 (25)	09 (75)	AMP-CFM-OF-CI-NX	08 (66.6)
Norfloxacin(NX 10 µg)	08 (66.7)	04 (33.3)	AMP-CO-NX-DO-G	08 (66.6)
Gentamicin(G 10 µg)	02 (16.7)	10 (83.3)	COT-CST-CF-CN-AMP	10 (83)
Cefixime (CFM 5 µg)	10 (83.3)	02 (16.7)	CFM-NX-DO-CST-CN	11 (91.6)
Doxycycline (DO 10 µg)	09 (75)	03 (25)	AMP-CST-CN-CFM-CI	11 (91.6)
Ofloxacin (OF 5 µg)	05 (41.7)	07 (58.3)	AMP-CI-CF-CST-NX-DO	11 (91.6)
Total (%) (n=12)	08 (66.7)	04 (33.3)		11 (91.6)

Antimicrobial resistance of diarrheagenic E. coli isolates

Altogether, 91.7% of DEC were recorded as multidrug-resistant (MDR), of which >75% isolates were resistant to ampicillin (83%), ceftriaxone (83%), cefoperazone-tazobactam (83%), cefixime (83%), and doxycycline (75%). There are few antibiotics against which the least resistant rate was found against Amikacin (8.3%), gentamicin (16.6%), chloramphenicol (16.6%) and meropenem (25%) (Figure-2) ($p < 0.0001$, 95% CI= 10.105 to 42.872). Similarly, the DEC isolates have also exhibited different drug resistance patterns, of which the most common pattern shown was AMP-DO-NX- CO (83%) and the least was G-AK-C-MRP and MRP-AK-G (16.6%) (Table 4).

Phenotypic and Genotypic detection of ESBL

Significant ESBL producers were detected from the diarrheic samples (91.6%) than the non diarrheic samples (8.3%) by phenotypic detection tests. High ESBL producing DEC was found associated with diarrheic children under the age group of 0-36 (66.6%) than >36 months (33.3%), whereas a less DEC isolates were found ESBL producing among the non- diarrheic (8.3%) ($p > 0.05$, 95% CI = 0.2348 to 226.09). The ESBL producing isolates were found prevalent in almost all the age groups; *blaCTX*-M-9 is been found only in the diarrheic samples (25%) and equally distributed in all the age groups (8.3%). Similarly, the *blaCTX-M*-15 also distributed among the diarrheal cases only, where the age group of 13-36 found to be more vulnerable than other groups 0-12 (16.6%), 13-36 (25%) and 37-60 (16.6%) ($p > 0.05$, 95% CI = 0.06824 to 22.896). In the case of *CMY* -2, it was detected in one isolate each from diarrheic and non- diarrheic samples (8.3%). The ESBL variant of *blaOXA* gene was not found in any of the isolates screened by genotypic PCR assay (figure 1). As a whole the ESBL variant of *blaCTX*-M-15 (58.3%) is detected in significant rate as compared to *blaCTX-M*-9 (25%) and *CMY*-2 (16.6%) (Table 3).

Discussion

Infectious diarrhea among the children and infants aged less than 5 years are still remaining the most common cause of death in India with estimated 1.3 million deaths annually (1). Although DEC pathotypes were well recognized, they are not routinely tested when compared to other diarrheal pathogen and thus the exact burden of diarrhea associated with DEC is less known in hospitalized paediatric patients in India and is unknown in the north east India region. Diarrheagenic *E. coli* was the predominant pathogen that was reported from diarrheal patients in previous studies of this region (14, 21). Similarly in this study, *E. coli* isolates predominates the diarrheal pathogens recovered from diarrheic samples.

Although the symptoms were obvious in DAEC, no significant distinguishing symptoms among the pathotypes were detected and this cannot be used as a superior factor for the symptomatic diagnosis. In DAEC infection, the stool consistency was mostly watery or with mucous when compared with other types with mild, moderate to severe dehydration and the DAEC type shown the severe dehydration in children. Similar clinical symptoms were observed in patients from other studies in India (22).

The prevalence of DAEC varies between different regions and sometimes even between countries. A high prevalence of DAEC was reported in northeast Brazil whereas low prevalence was seen among Chilean children (8, 23). Our finding is similar to those reported by Vidal et al., 2005 and Rajedran et al., 2015, wherein the prevalence of DAEC among the diarrheal patients was only 1% and 0.5%, respectively (3, 8).

Interestingly, EAEC and ETEC pathotypes were mostly isolated from the ≤12 months age group followed by 13 to 36 months age group, whereas, the DAEC pathotype occurred less frequent and mostly found in 13 to 36 months age group (Table 3). Earlier reports shown the occurrence of diarrhea was higher (31.57%) in children who were breastfed for less than 6 months compared to exclusively breastfed children (20.33%) and bottle-fed children (26.08%) (24). Similarly, our data showed a higher rate of DAEC occurrence in the weaning age groups than the breastfed groups which indicates the good hygiene practice among the breast feeding mothers and it also supports the earlier findings of Gupta et al., 2015. Seasonal distribution of the DAEC in Aizawl, Mizoram indicated that most of the cases occurred in monsoon and summer probably due to the increase in temperature. The detection of serogroups belonging to O86, O128, and O142 from DAEC strains are in agreement with the findings of earlier reports (25).

Among the MDR Patterns, AMP-CST-CN-CFM-CI (ampicillin-cefoperazonetazobactam-cephalexin-cefixime-ceftriaxone) pattern was shown by all the DAEC isolates. This is the first study to report the MDR Patterns of DAEC and other DEC in pediatric diarrhea from the entire north east India and our results concord with those reported from other countries (26, 27). Both the developed and developing countries are facing challenges in the treatment of antibiotic-resistant pathogens and especially of those which have beta-lactam resistance, leading to the high mortality rate in hospitals (28). In our study, the E. coli strains containing *blaCTX-M*-15 and *blaCTX-M*-9 were found to be resistant to all the cephalosporin drugs. Similar resistance pattern was also reported earlier in *E. coli* clinical isolates (18, 28). This is the first report to document the occurrence of *blaCTX-M*-15 ESBL resistance among human diarrheagenic *E. coli* isolates from North East India, indicating that the significant antibiotic resistance is the reason behind the failure of treatments earlier.

Utmost the *E. coli* isolates examined here showed the ability to adhere to epithelial cells in vitro; there is a probability that these strains can also colonize the intestinal epithelium of the children. The colonized children may become a potential source for spread of multi drug resistant ESBL producing *E. coli* stains in the hospital environment.

Conclusion

In conclusion, our findings support the significant association of DAEC with diarrheal diseases in children of non breast feeding age group. This study revealed that DAEC is less predominant pathotype but strongly associated with pediatric diarrhea in low socio economic population of Mizoram, India. Although there is controversy about the pathogenesis of DAEC in children and adults, this study proves the pathogenic effect of DAEC in children by finding only from diarrheic cases and also by the pathological effects detected in Hep-2 cell lines. As it is impossible to discriminate the DEC strains by clinical symptoms it is suggested to identify the specific pathotype by specific virulent markers prior treatment. The less prevalence of DAEC in this region may be due to the climatic factors which are different from the neighbouring states and countries from where these pathotypes suspected to be transferred, also it was missed out in earlier studies which may because this is a pathotype affects only the weaning age groups with no breast feeding. Hence, an active surveillance is recommended to scale up efforts of control strategies to encounter multi drug resistance issues of diarrheal cases of this resource-poor region.

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Ethics approval and consent to participate

All authors hereby declare that all the experiments have been examined and approved by the institutional ethics committee and have therefore performed in accordance with the ethical standards.

Conflict of interest

All the authors have no conflict of interest.

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