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Regular paper

Co-existence of Extended Spectrum β-Lactamase and carbapenemase-producing genes from Diarrheagenic Enteric pathogens isolated in a tertiary care hospital

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Multiple drug resistance (MDR) among bacterial pathogens is a growing concern that clinicians are facing worldwide. Diarrhea among infants is frequent and is caused by various bacterial and viral infectious agents. Two hundred and twelve stool specimens were collected from pediatric patients from a rural quaternary hospital in Barshi, Sholapur, India, between March and December 2017. Total 180 specimens were positive for various bacterial pathogens, while the remaining 32 diarrhea cases may have been caused by a viral or uncultured bacterial pathogen. Identification of the bacterium and its antibiotic susceptibility were primarily carried out with VITEK-2. Distribution of diarrhea-causing bacteria among the 180 samples was as follows: 61.11% (110) *Escherichia coli*, 30.55% (55) *Klebsiella pneumoniae*, 4.44% (8) *Proteus mirabilis*, 2.22% (4) *Shigella* spp. 1.11% (2) *Morganella morganii* and 0.55% (1) each for *Enterobacter cloacae* and *Citrobacter koseri*. There was a co-existence of multiple genetic traits conferring extreme drug resistance (XDR) status to 19 isolates, 17 of which were determined to be *E. coli* and one each of *E. cloacae* and *C. koseri*. Antibiotype determination using VITEK-2 and polymerase chain reaction (PCR) amplification of the genetic traits indicated the co-existence of bla_{TEM} and $bla_{CTX\text{-}M15}$ isolates in all 19 isolates, with the exception of *E. cloacae*. Results showed that 10 out of 19 strains expressed the AmpC cephalosporinase $bla_{CMY\text{-}2}$ gene, whereas metallo-carbapenemase was expressed in four isolates. Distribution of $bla_{NDM\text{-}11}$ and acquired penicillinase $bla_{SHV\text{-}1}$ resistance among 180 clinical isolates is discussed in the light of ESBL traits. This is the first report from the rural part of Maharashtra India showing that as many as 10.55% of the pathogenic strains were XDR, a step ahead of MDR.

Key words: diarrhoea paediatric, *Escherichia coli*, extended spectrum *β*-lactamase (ESBL)

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Abbreviations: AST, Antibiotic susceptibility testing; CLSI, Clinical and Laboratory Standards Institute; DEC, Diarrheagenic *Escherichia 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*; HCAI, Healthcare-associated infections; MBL, Metallo-β-Lactamase; MDR, Multiple drug resistance; MIC, Minimum inhibitory concentration; NAP, National Antimicrobial Stewardship Programs; NDM, New Delhi metallo β-lactamase; PCR, Polymerase chain reaction; XDR, Extreme drug resistance

INTRODUCTION

Diarrhea remains a leading killer of young children all over the world, despite the availability of simple and effective solutions to prevent and control it. Bacterial resistance to antibiotics has become a major public health issue worldwide. The reality of this threat has been acknowledged in the WHO 2014 report on antibiotic resistance (www.who.int/drug resistance/en). More than one billion diarrheal episodes occur every year among children younger than five years in socioeconomically developing countries, causing 2 to 2.5 million deaths (O-Ryan *et al.*, 2005). Diarrheagenic *Escherichia coli* (DEC) is the major cause of gastroenteritis in children in the developing world and is associated with high resistance levels to antibiotics (Gebre Silasie *et al.,* 2018). Most *Escherichia coli* strains live harmlessly in the intestines and rarely cause disease in healthy individuals. Nonetheless, a number of pathogenic strains can cause diarrhea or extra-intestinal diseases, both in healthy and immunocompromised individuals.

Diarrheal illnesses are a severe public health problem and a major cause of morbidity and mortality in infants and young children, especially in developing countries. When the body has an infection, the immune system encourages increased metabolism and waste removal, causing diarrhea. The common symptoms of diarrhea include having three or more loose stools in one day, bloody stools, gas and bloating, fever, stomach cramps and loss of appetite.

Extended spectrum *β*-lactamase (ESBL) is a major mechanism of antibiotic resistance belonging to the *Enterobacteriaceae* — a family of bacteria that normally lives in the gastrointestinal tract without causing infection. For this reason, many ESBL-related infections irritate the gastrointestinal lining. Diarrheagenic *E. coli* strains are classified on the basis of their virulence properties and serological characteristics (Alikhani *et al.*, 2012). Currently at least five virotypes have been studied in detail (Todar, 2007): enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC). Enterotoxigenic *E. coli* are responsible for community-acquired diarrheal disease in areas of poor sanitation and are the most common cause of travellers' diarrhea. The EAggEC causes chronic diarrheal disease in developing countries, while EPEC causes infantile enteritis, especially in tropical countries. EHEC strains are known to cause a disease that is similar to dysentery (bloody diarrhea). These *E.coli* types are responsible

for diarrheal diseases that can be lethal, particularly in children in developing countries. The *E. coli* has several pathotypes which cause diseases. These diseases manifest into different symptoms in the gastrointestinal tract and extra-intestinal sites.

Emergence of bacterial resistance against commonly used antibiotics has become a serious global concern. Antimicrobial resistance is a significant public health problem, particularly in developing countries (Kaper *et al.*, 2004). This consequently leads to challenges in the treatment of infectious diseases among the public. The number of clinical multi-drug resistant (MDR) isolates of pathogenic *E. coli* strains is increasing and represent a major healthcare problem; increasing morbidity and mortality worldwide. Antibiotic resistance can be intrinsic or acquired. Intrinsic resistance refers to bacteria that are resistant to an antibiotic in their natural state, without acquiring resistance determinants. They have inherent structural or functional characteristics that allow the tolerance of particular antimicrobial agents. This type of resistance may lead to failure of treatment with certain antibiotics (Vernet *et al.*, 2014).

Antibiotic resistance is a problem of deep scientific concern both in the hospital and community settings. The developed countries have established National Antimicrobial Stewardship Programs (NAP) for the surveillance of antimicrobial resistance, which is helpful in devising policies to keep the hospital-acquired (nosocomial) and healthcare-associated infections (HCAI). In developing countries such as India, in the absence of such a system, the true burden of antimicrobial resistance remains poorly understood. Indiscriminate use, misuse, and abuse of antibiotics are postulated to have been the major reasons for development and spread of antibiotic resistance. Sporadic reports describing the antibacterial susceptibility towards commonly prescribed drugs show a high level of resistance in Indian hospitals overall. The other major reason for high microbial resistance in Indian setup is the unprescribed use and/ or self-medication leading to drug abuse (Krishna and Kharat 2022 –Personal Communication – MS submitted to JEPH-Hindawi).

The objectives of present investigation were to find the antibiotic resistance in bacteria causing pediatric diarrhea and to find out mechanism(s) for antibiotic resistance using molecular methods. In this investigation we used second/ third generation cephalosporins and carbapenem antibiotics to assess antibiotic resistance

METHODOLOGY

Patients

Between March and December 2017, a total of 212 stool samples were collected from 212 infant and child patients admitted to the pediatric wards of the Dr. Jagdale Mama Hospital, Barshi, and other hospitals in the Barshi town, District of Solapur, Maharashtra State, India. Since patients had entered either into convalescent phase or completely recovered within 5 days, no second sampling was needed. These samples have been included in the present study. A microscopic examination of the stool samples was conducted for consistency, color, presence of mucus and

Table 1. Antibiotic resistance of *Escherichia coli* isolates by Kirby-Bauer method.

Antibiotic	Disk (in µg)	No of Isolates			Total	Percent Resistant (%)
		Sensitive	Intermediate	Resistant		
Ampicillin	10	0	0	110	110	100.00
Ticarcillin	75	0	0	15	15	100.00
Piperacillin	100	0	0	16	16	100.00
Cefoperazone/Sulbactam	75/10	0	0	1	1	100.00
Cefazolin	30	3	0	61	64	95.31
Ceftriaxone	30	7	0	139	146	95.21
Cefuroxime Axetil	30	3	1	71	75	94.67
Ceftazidime	30	2	0	35	37	94.59
Cefuroxime	30	3	2	77	82	93.90
Aztreonam	30	9	0	81	90	90.00
Ampicillin/Sulbactam	10/10	7	7	66	80	82.50
Cefepime	30	28	4	144	176	81.82
Amoxicillin/Clavulanate	20/10	15	14	45	74	60.81
Piperacillin/Tazobactam	100/10	67	21	83	171	48.54
Imipenem	10	119	1	46	166	27.71
Meropenem	10	126	0	48	174	27.59
Doripenem	10	16	1	6	23	26.09
Ertapenem	10	111	0	26	137	18.98

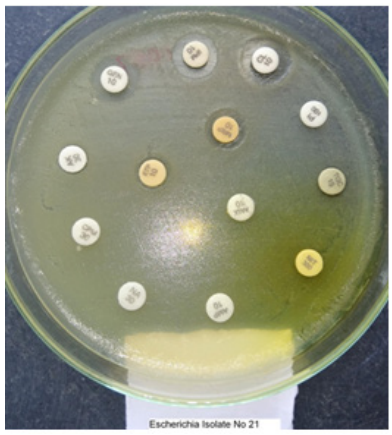

Figure 1. Multi-drug resistant isolate of *Escherichia coli*, Isolate No. ESBL21.
Antibiotics used were amikacin, amoxicillin, ampicillin, ciprofloxacin, colistin, etrapenem, gentamicin, imipenem, meropenem, nalidixic acid, netilmicin, piperacillin, and tigecycline.

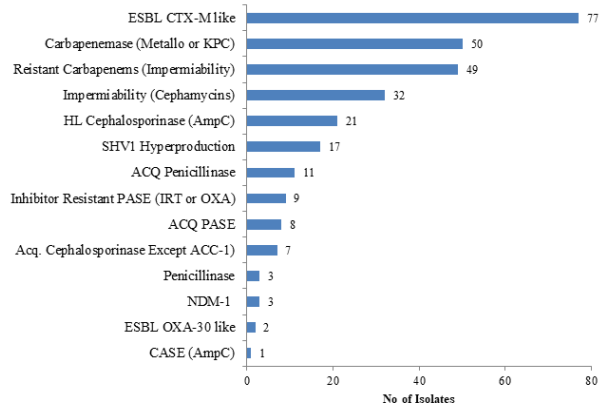

Figure 2. β-lactam antibiotic-resistant phenotypes observed in 171 isolates as identified in VITEK-2 System.

blood. All the samples were examined microscopically for the presence of pus cells, red blood cells and ova/cysts. All the samples were from patients having acute diarrhea.

Isolation and identification of the organism causing diarrhea

The stool samples received in sterile containers were processed for isolation of etiological bacterial pathogen using the recommended standard methods. The samples were inoculated on Blood agar plates and MacConkey's agar plates and incubated overnight at 37°C. The purified isolates were preserved in 25% glycerol, at –80°C, for further analysis. Identities of the isolates were established by using biochemical tests described in Bergey's Manual of Systematic Bacteriology (Brenner *et al.*, 2012).

Serotyping of selective MDR isolates

Nineteen out of 180 specimens contained multiple drug resistant bacteria. Based on morphological, colony, and biochemical characterization, 17 out of 19 were *E. coli* isolates. We conducted serotyping of all 17 *E. coli* using Prolex *E. coli* O167:H7 and non O157:H7 kit (Prolex USA). Bacterial isolates were grown on MacConkey's agar supplemented with 1% Sorbitol as the carbon source. Colonies developed after 24 h at 37°C were used for preparation of 0.3 McFarland suspension and used to decipher serotype with Prolex *E. coli* O157:H7 and *E. coli* non 157: H7 kit as per manufacturers protocol.

Susceptibility test determination by the disc diffusion method

Antimicrobial susceptibility testing was done on the Mueller-Hinton agar using the disc diffusion method, according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2012). The isolates were subjected to antimicrobial susceptibility testing and the results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2012). All the antibiotic discs: ampicillin, ampicillin/sulbactam amoxycillin/clavulanate, ticarcillin, piperacillin, piperacillin/tazobactam, cefoperazone/sulbactam, cefazolin, ceftriaxone, cefuroxime, cefuroxime axetil, ceftazidime, cefepime, imipenem, doripenem, meropenem, ertapenem, colisin, tigecylin, amikacin, chloramphenicol, ciprofloxacin, levofloxacin, nitrofurantoin, and trimethoprime/sulmethoxazone were obtained from HI Media Ltd, Mumbai, India. The disc potency of the β-lactam antibiotics is presented in Table 1.

Antibiotic susceptibility and Minimum Inhibitory Concentration (MIC) determination of diarrheagenic MDR isolates with micro-broth dilution method

Nineteen diarrheagenic selected isolates were subjected to antibiotic susceptibility testing (AST) and

Table 2. Primers used for amplification of the β-lactamase trait(s)

β-lactamase gene	Primer Sequence	Amplicon (in bp)	Annealing temp (°C)	Reference
$bla_{CTX-M15}$	F-TTGTTAGGAAGTGTGCCGCT	302	55	Karim *et al.*, 2001
	R-ATCGTCCCATTGACGTGCTT			
bla_{CMY-2}	F-ATAACCACCCAGTCACGCAG	417	56	Bauernfeind *et al.*, 1996
	R-TCCAGGTATGCGCCAGTTTT			
bla_{NDM-11}	F-GGCCAGCAAATGGAAACTGG	443	56	Rahman *et al.*, 2015
	R-AATACCTTGAGCGGGCCAAA			
bla_{NDM-1}	F-GGTTTGGCGATCTGGTTTTC	621	55	Poirel *et al.*, 2011
	R-CGGAATGGCTCATCACGATC			
bla_{TEM-1}	F-GAGTATTCAACATTTCCGTGT	849	55	Dallenne *et al.*, 2010
	R-AATCAGTGAGGCACCTATC			

determination of MIC according to the CLSI (M07-A10)-recommended micro broth-dilution method. Antibiotic susceptibility tests for 19 diarrheagenic isolates was carried out as described in CLSI, (2012). The MIC were determined for the commonly prescribed antibacterial agents, as well as, against certain novel combinations of drugs. The drugs used for MIC determination were β-lactam antibiotics: ceftazidime standalone and in combination with clavulanic acid or avibactam 4 mg/L, cefepime standalone and in combination with tazobactam 8 mg/L, piperacillin in combination with tazobactam 4 mg/L, imipenem standalone and in combination with relebactam 4 mg/L, meropenem standalone and in combination with EDTA 200 mg/L, and non β-lactam antibiotics: amikacin, levofloxacin, tigecycline, colistin and sulfamethoxazole-trimethoprime. . The MICs were determined in triplicates.

Genotype Determination

The enzymatic β-lactam resistance mechanism was confirmed through the *β*-lactamase gene specific PCR. The primers used were as reported in the literature and shown in Table 2. (Bauernfeind *et. al.,* 1996; Karim *et. al.,* 2001; Dallen *et al.*, 2010; Poirel *et al.*, 2011; Rahman *et. al.,* 2015). The method used in this study was that of Unno and others (Unno *et al.,* 2010).

RESULTS

A total of 212 stool samples were collected from infants and children admitted to the pediatric wards. Of these, 180 bacterial isolates were cultured and identified. Results in Table 1 show that bacterial cultures included *Escherichia coli* (110; 61.11%), *Klebsiella pneumonia* (55; 30.55%), *Shigella spp* (4; 2.22%), *Proteus mirabilis* (8; 4.44%), *Morganella morganii* (2; 1.11%), *Enterobacter cloacae* (1; 0.55%) and *Citrobacter koseri* (1; 0.55%). The antimicrobial susceptibility testing (AST) of these 180 cultures was done on a Mueller-Hinton agar, using the disc diffusion method according to the recommendation of the Clinical and Laboratory Standards Institute (CLSI, 2012); the results are summarized in Table 3. Resistance to β-lactam antibiotics was observed in 171 isolates, as identified with the VITEK-2 System (Fig. 2). A total of 19 isolates, comprising of 17 *E. coli,* 1 *E. cloacae* and 1 *C. koseri,* exhibited resistance to cephalosporins, trimethoprim/sulfamethoxazole, and levofloxacin, and were classified as MDR isolates and used as representative isolates fin further studies. All of these 17 *E. coli* isolates were identified by the IMViC test, growth on EMB agar, and serotyping, to characterize their type. The *E. coli* ESBL3, *E. coli* ESBL 13, *E. coli* ESBL 15, *E. coli* ESBL 22 and *E. coli* ESBL 25 isolates expressed serotype of *E. coli* O157:H7. *E. coli* O26, *E, coli* O121 and *E. coli* O45 serotype was expressed in one *E. coli* ESBL29, *E. coli* ESBL 28 and *E. coli* ESBL 21 isolates, respectively. No serotypes were detected for *E. coli* O111, *E. coli* O103 and *E. coli* O145. MIC against the panel of β-lactam and non β-lactam antibiotics was performed on representative isolates using micro-broth dilution method. All the 19 isolates were resistant to third and fourth-generation cephalosporin, namely ceftazidime and cefepime. According to Amblers' classification, there are four Extended Spectrum *β*-Lactamase (ESBL) enzymes that can be differentiated phenotypically (Ambler 1980). The combination of ceftazidime and clavulanic acid, an ESBL inhibitor, suggested the ESBL expression as a mechanism of ceftazidime resist-

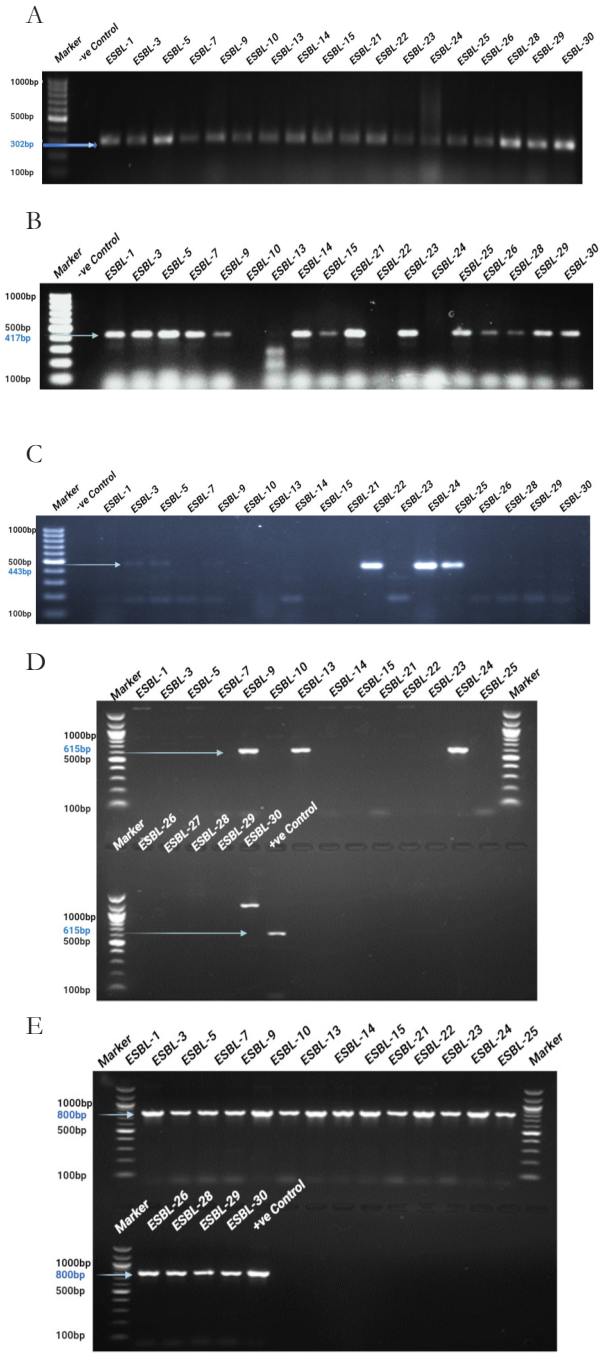

Figure 3. PCR amplification of ESBL traits. Figure 3A: bl$a_{CTX-M15}$, Figure 3B: bla_{CMY-2}, Figure 3C: bla_{NDM-11}, Figure 3D: bla_{NDM-1}, Figure 3 E: bla_{TEM-1}.
Amplicons, prepared as described in Methods and in Table 1, were loaded on the gel in the following order: in Fig. 3A, 3B and 3C; Lane 1 M -100 bp ladder. Lane 2: Negative control, Lane 3 ESBL 1, Lane 4 ESBL3, Lane 5 ESBL 5, Lane 6 ESBL 7, Lane 7 ESBL 9, Lane 8 ESBL 10, Lane 9 ESBL13, Lane 10 ESBL 14, Lane 11 ESBL 15, Lane 12 ESBL 21, Lane 13 ESBL 22, Lane 14 ESBL 23, Lane 15 ESBL 24, Lane 16 ESBL 25, Lane 17 ESBL 26, Lane 18 ESBL 28, Lane 19 ESBL 29, Lane 20 ESBL 30. Loading order for Fig. 3D and 3E; **Top Panel:** Lane 1 100 bp ladder, Lane 2 ESBL 1, Lane 3 ESBL 3, Lane 4 ESBL 5, Lane 5 ESBL 7, Lane 6 ESBL 9, Lane 7 ESBL 10, Lane 8 ESBL 13, Lane 9 ESBL 14, lane 10 ESBL 15, Lane 11 ESBL 21, Lane 12 ESBL 22, Lane 13 ESBL 23, Lane 14 ESBL 24, Lane 15 ESBL 25, Lane 16 100 bp ladder. **Bottom Panel**: Lane 1 100 bp DNA ladder, Lane 2 ESBL 26, Lane 3 ESBL 28, Lane 4 ESBL 29, Lane 5 ESBL 30 and Lane 6 Positive control.

Table 3. Antibiotic Sensitivity Test against β-lactam & non-β-lactam antibiotics

Strain	CAZ	CLV	FEP	PTZ	MEM	MED	IPM	CST	TIG	AMK	LEV	SXT	NIT	C	CIP
E. coli 1	R	R	R	I	S	S	S	S	S	S	R	R	S	R	R
E. coli 3	R	S	R	S	S	S	S	S	S	S	R	R	S	R	R
E. coli 5	R	R	R	R	s	S	S	S	S	S	R	S	S	S	R
E. coli 7	R	R	R	R	S	S	S	S	S	S	R	S	S	S	R
E. coli 9	R	R	R	R	R	S	R	S	S	R	R	R	I	S	R
E. coli 10	R	S	R	S	S	S	S	S	S	R	R	R	S	S	R
E. coli 13	R	R	R	R	R	S	R	S	S	R	R	R	R	R	R
E. coli 14	R	S	R	I	S	S	S	S	S	S	R	R	S	S	R
E. coli 15	R	S	R	I	S	S	S	S	S	S	R	R	S	S	R
E. coli 21	R	R	R	R	S	S	S	S	S	S	R	S	S	S	R
E. coli 22	R	2	R	I	S	S	S	S	S	S	R	R	S	S	R
E. coli 23	R	S	R	S	S	S	S	S	S	S	R	R	S	S	R
E. cloacae 24	R	R	R	R	R	S	R	S	S	I	R	R	I	R	R
E. coli 25	R	4	R	I	S	S	S	S	S	S	R	R	S	S	R
E. coli 26	R	S	I	S	S	S	S	S	S	S	R	R	S	S	R
E. coli 27	R	S	R	S	S	S	S	S	S	S	R	R	S	S	R
E. coli 28	R	S	R	S	S	S	S	S	S	S	R	R	S	S	R
E. coli 29	R	S	R	S	S	S	S	S	S	S	R	R	S	S	R
C. koseri 30	R	S	R	S	S	S	S	S	S	S	S	R	S	R	R

The AST was carried out on 19 MDR diarrhea isolates with Kirby Bauer disc diffusion method using the following antibiotics: β-lactams: CAZ, Ceftazidime; CLV, Clavulanic Acid; FEP, Cefepime, PTZ, Piperacillin, Tazobactam; MEM, Meropenem; MED, Meropenem, EDTA; IPM, Imipenem; and non β -lactams: CST, Colistin; TIG, Tigecyclin; AMK, Amikacin; LEV, Levofloxacin, SXT, Trimethoprim/Sulfamethoxazole; NIT, Nitrofurantoin; C, Chloramphenicol; and CIP, Ciprofloxaxin. Status Sensitive (S), Intermediate (I) and Resistant (R) was assigned as per CLSI (2012) standards. Antibiotic acronyms in red denote β -lactams while purple denote non-β-lactams. The non-E.coli ESBL isolates are highlighted in green.

ance in 11 isolates (ESBL A). Eleven isolates showed reduced susceptibility to piperacillin–tazobactam (Table 4). Furthermore, four isolates showed an ESBL C phenotype, where MIC remained high to β-lactam even after combining it with clavulanic acid, a β-lactamase inhibitor, while carbapenem remained active against these isolates (Table 4). Metallo-*β*-lactamase phenotype (ESBL B) in three isolates was evident, as the MIC of meropenem standalone was over 4-fold higher than MIC of a combination of meropenem/EDTA (Table 4). Among the non-β-lactam antibiotics, colistin and tigecycline were the most active agents (Table 3/Table 4). Amikacin showed a high level of resistance against two MBL isolates and one AmpC-producing isolate, while the other isolates were susceptible at the CLSI breakpoint (Table 4). Nearly all of the isolates except one, *C. freundii,* waere levofloxacin-resistant, while 3 out of all 19 were susceptible to trimethoprime/sulfamethoxazole (Table 3). Another three out of19 isolates were nitrofurantoin-resistant (Table 3).

The presence of *β*-lactamase gene in these 19 isolates was verified with PCR, which showed co-existence of multiple *β*-lactamase enzymes in all the isolates. All of the isolates had the $bla_{\text{CTX-M15}}$ gene variant (Fig. 3A) and bla_{TEM} gene (Fig. 3E). The AmpC cephalosporinase $bla_{\text{C-MY-2}}$ gene was amplified in 14 isolates (Fig. 3B). A weak metallo-*β*-lactamase, $bla_{\text{NDM-11}}$ and a strong metallo-carbapenemase, $bla_{\text{NDM-1}}$ were present in three isolates each (Fig. 3C and Fig. 3D). Antibiotic resistance phenotype inferred from the antibiotic susceptibility test (disc diffusion) is shown in Table 3. MIC against 13 antibiotics (7 β-lactams and 6 non-β-lactams) and genotype data (Fig. 3A to E) showed a good correlation. β-lactam antibiotics blocked the synthesis and growth of the bacterial cell wall by inhibiting penicillin-binding proteins on the cytoplasmic membrane. The results shown in Table 3 demonstrated the MDR feature, while the results presented in Table 4 suggested the XDR feature in these 19 isolates.

DISCUSSION

Treating infections caused by antibiotic-resistant pathogens is a global challenge. Antimicrobial resistance is a major challenge facing Indian and global clinicians. Studies reported herein were conducted on pediatric diarrhea patients from tertiary care hospital from rural part of Maharashtra, India. Veeraraghavan and Walia (Veeraraghavan & Walia, 2019) in their surveillance review reported antimicrobial susceptibility of Indian *E. coli* isolates to: amoxicillin/clavulanic acid (11–38%), cefotaxime (14–76%), ceftazidime (7–50%), piperacillin/tazobactam (21–89%), cefperazone/sulbactam (88–100%), imipenem (43–100%), meropenem (67–89%), amikacin (27–88%), gentamicin (21–86%), ciprofloxacin (15–67%), levofloxacin 13–25%), and colistin (97–100%). The global rise in antibiotic resistance is attributed to the emergence of extended Spectrum *β*-lactamase (ESBL) enzymes. The prevalence of ESBL-producing pathogens has been rapidly increasing in acute care hospitals (Spadafino *et al.*, 2014). The ESBL enzyme produced by the $bla_{\text{TEM-1}}$ and $bla_{\text{CTX-M15}}$ genes has been prevalent in global *E. coli* isolates, and while carbapenems are the treatment of choice for serious infections caused by ESBL-producing bacteria, the carbapenem resistance is becoming prevalent

Table 4. Attributing MDR status to the strains based on MIC with Micro-broth dilution method

Organism	MIC (μg/mL)												
	CAZ	CLV	FEP	PTZ	MEM	MED	IPM	CST	TIG	AMK	LEV	SXT	NIT
E. coli 1	>64	16	>64	64	0.03	0.03	0.25	0.25	0.25	2	64	>128	16
E. coli 3	>64	1	64	8	0.03	0.03	0.25	0.12	0.25	2	64	>128	16
E. coli 5	>64	>64	>64	128	0.06	0.03	0.5	0.25	0.5	2	16	0.12	8
E. coli 7	>64	>64	>64	128	0.03	0.03	0.5	0.25	0.25	2	16	0.12	8
E. coli 9	>64	>64	>64	>128	64	0.12	16	0.25	0.25	>256	32	>128	64
E. coli 10	>64	>64	>64	16	<0.03	4	0.5	0.25	0.25	128	32	>128	16
E. coli 13	>64	>64	>64	>128	>64	0.06	32	0.25	0.25	>256	32	>128	128
E. coli 14	>64	2	>64	64	0.03	0.03	0.25	0.25	0.25	2	32	>128	16
E. coli 15	>64	2	>64	64	0.03	0.03	0.25	0.25	0.25	1	64	>128	16
E. coli 21	>64	>64	>64	>128	0.03	0.03	0.25	0.25	0.5	2	16	0.12	8
E. coli 22	>64	2	>64	64	0.03	0.03	0.25	0.25	0.5	2	32	>128	16
E. coli 23	32	0.12	16	8	0.03	0.03	0.06	0.25	0.5	4	32	>128	8
E. cloacae 24	>64	>64	64	>128	32	0.03	16	0.12	1	16	8	>128	64
E. coli 25	>64	4	>64	32	0.03	0.03	0.5	0.12	0.25	0.5	32	>128	16
E. coli 26	32	0.25	8	8	0.03	0.03	0.12	0.25	0.5	2	32	>128	8
E. coli 27	32	0.25	32	16	0.015	0.03	0.06	0.25	0.25	4	32	>128	8
E. coli 28	32	0.25	32	16	0.03	0.03	0.12	0.25	0.25	16	16	>128	8
E. coli 29	32	0.5	16	8	0.03	0.03	0.12	0.25	0.5	16	16	>128	8
C. koseri 30	64	0.5	32	16	0.03	0.03	0.12	0.25	1	2	1	>128	32

The MIC was estimated with micro-broth double dilution method. The β-lactam antibiotics included: CAZ, Ceftazidime; CLV, Clavulanic Acid; FEP, Cefepime; PTZ, Piperacillin, Tazobactam; MEM, Meropenem, MED, meropenem-EDTA; and IPM, Impenem; while non β-lactams were: CST, Colistin; TIG, Tigecyclin; AMK, Amikacin; LEV, Levofloxacin; SXT, Trimethoprime-Sulfmethoxazone; NIT, Nitrofurantoin. The β-lactam antibiotics are typed in red while non β-lactam antibiotics are typed in purple.

as well, based on the production of New Delhi metallo β-lactamase (NDM) and carbapenem hydrolyzing $bla_{\text{OXA-48}}$ enzymes. Interestingly, bla_{VIM}, bla_{IMP} and bla_{KPC} are less commonly reported among Indian *E. coli* isolates. An ertapenem-resistant *E. coli* isolate has been isolated from the peritoneal fluid of a patient, who had been treated with imipenem/cilastatin for 10 days. Ertapenem resistance may be explained by a defect in the outer membrane protein and production of extended-spectrum *β*-lactamase CTX-M2 (Lartique *et al.*, 2007).

The presence of a dominant extended spectrum *β*-lactamase or the conditional expression of multiple extended spectrum *β*-lactamases imposes therapeutic limitations for clinicians. All of the nineteen isolates analyzed in this study carried more than one ESBL trait (Table 5). However, the functional characterization of ESBL by MIC (Table 3 and Table 4) allowed the classification of isolates on the basis of phenotypic dominance – the MIC and sequence *β*-lactamase trait classification, shown in Table 5. Our study demonstrate that although there are more than one ESBL trait in a strain, the functional diversity still exists, reflecting antibiotic sensitivity and MIC of β-lactam antibiotics.

In the present investigation, we found $bla_{\text{NDM-1}}$ type of β-lactam antibiotic resistance in three out of 19 isolates (16.7%) (Fig. 3D). Originally described in 2009, the $bla_{\text{NDM-1}}$ gene is now widespread in *E. coli* and *K. pneumoniae* isolates from India, Pakistan, Eastern Europe and United Kingdom (Kumarasamy *et al.*, 2010). Our studies also show the presence of metallo-*β*-lactamase variant, encoded by $bla_{\text{NDM-11}}$ gene, in 3 out of 19 isolates (Fig. 3C). Colistin, another treatment possibility, is quite toxic and therefore rarely used. Currently, there is no drug available for the treatment of multi-drug resistant, Gram-negative bacteria in patients with diarrhea. The incidence of infections caused by β-lactam-resistant organisms, due to the production of various enzymes, has increased in recent years. Infection-control practitioners and clinicians need a clinical laboratory to identify and characterize different types of resistant bacteria. This is required to minimize the spread of these bacteria and help select the appropriate antibiotics.

Veeraraghavan and Walia (Veeraraghavan & Walia, 2019) summarized that resistance to non-β-lactam antibiotics is common among Indian *E. coli* isolates. Mutations in the *mcr*-1, *mcr*-2, *mcr*-3 and *mcr*-4 have been reported to cause colistin resistance. Tetracycline resistance was acquired based on *tetA* and *tetB* genes, trimethorpirm based on *dfr*A1, *dfr*A17, sulphonamides; *sul*1, *sul*2; chloramphenicol; *cat*A1, *cat*B3, *cat*B4; streptomycin; *strA/B;* and quinolone resistance based on plasmid genes *aac*(6')-*ib-cr*, *qnr*B1, and *qnr*B1 and *qnr*S1.

Multi-drug-resistant *E. coli* is commonly encountered in hospital settings during daily clinical practice. The management of such infections is extremely important for the future, with particular care to prevent the new antibiotic resistance. In our study, we selected 19 multi-drug resistant strains of which 17 were *E. coli* isolates — Isolate Nos, ESBL 1, ESBL 3, ESBL 5, ESBL 7, ESBL 9, ESBL 10, ESBL 13, ESBL 14, ESBL 15, ESBL 21, ESBL 22, ESBL 23, ESBL 25, ESBL 26, ESBL 27, ESBL 28, and ESBL 29, one *Enterbacter cloaceae* isolate

Table 5. Ambler's phenotypic classification of diarrheagenic *E. coli* strains supported by the PCR-based ESBL traits.

Organism	Ambler Class		Genotype
	Resistome Phenotype	β-lactamse trait based	
E. coli 1	Class C	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 3	Class A	ESBL A, ESBL B, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},bla_{NDM-11}
E. coli 5	Class C	ESBL A, ESBL B, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2}, bla_{NDM-11}
E. coli 7	Class C	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 9	Class B	ESBL A, ESBL B, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY2}, bla_{NDM-1},
E. coli 10	Class A	ESBL A	bla_{TEM-1}, $bla_{CTX-M15}$,
E. coli 13	Class B	ESBL A, ESBL B	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{NDM-1},
E. coli 14	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 15	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 21	Class C	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 22	Class A		bla_{TEM-1}, $bla_{CTX-M15}$, bla_{NDM-11}
E. coli 23	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. cloacae 24	Class B	ESBL A, ESBL B,	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{NDM-1}, bla_{NDM-11}
E. coli 25	Class A	ESBL A, ESBL B, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2}, bla_{NDM-11}
E. coli 26	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 27	Class A	ESBL A	bla_{TEM-1}, $bla_{CTX-M15}$,
E. coli 28	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
E. coli 29	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},
C. koseri 30	Class A	ESBL A, ESBLC	bla_{TEM-1}, $bla_{CTX-M15}$, bla_{CMY-2},

ESBL 24 and one *Citrobacter koserii* isolate ESBL 30. We report that all of these isolates carried the $bla_{CTX-M-15}$ and bla_{TEM-1} type β-lactamase genes, which agrees with earlier reports from the globe and Indian isolates as well. The bla_{NDM-1} gene was detected in ESBL 9, ESBL13, ESBL 24 and ESBL 29 isolates. The presence of the bla_{NDM-1} at about 25% can cause concern as this is higher percentage than summarized in Veeraraghavan and Wali (Veeraraghavan & Wali, 2019). All of these isolates were resistant to trimethoprime/sulfamethoxazole and levofloxacin, most of them were resistant to ciprofloxacin, and at least one to the β-lactam antibiotics. All of the isolates were sensitive to colistin, which agrees with Veeraraghavan and Walia (2019).

CONCLUSIONS

This study reports isolation and molecular characterization of 19 MDR diarrheagenic isolates, of which 17 were *E. coli.,* from the patients in rural part of Maharashtra, India. The broth dilution MIC studies indicated these isolates had very high MIC against certain β-lactamase antibiotics, qualifying as XDR and demanding a combination therapy for a successful outcome. Co-existence of ESBL within these isolates attributed to their XDR status. The growing prevalence of XDR-MDR *E. coli* when there is no suitable anti Gram-negative antibiotic available for the treatment, demands more intensified hospital surveillance and search for a new effective antibiotic.

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