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Full Length Research Paper

Co-existence of multiple β-lactamase traits among clinical isolates of *Escherichia coli* from rural part of Maharashtra, India

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Acquisition of multidrug resistance and the spread of MDR among pathogenic microorganisms is a growing concern to world. Extended-spectrum β-lactamases (ESBLs) strains of the Enterobacteriaceae; ESBL trait expressing *Escherichia coli* poses major threat and ESBL *E. coli* infections are increasingly reported from hospital settings as well as community settings with rising mortality and morbidity rates. The objective of this study was to study distribution of various types of β-lactamases expressed within the clinical isolates of *E. coli*. In this work, clinical specimens: urine, stool, sputum, pus were collected over a period of three months from patients from rural area of Maharashtra State, India. Out of number of isolates, only thirty-six *E. coli* isolates were chosen for determining existence of ESBL, MBL, AmpC and Carbapenamase producing ability. Choice of isolates included resistance to at least of the three third generation cephalosprins used in this study. About 75% of *E. coli* strains were found to express ESBL trait, while remaining 25% expressed non-ESBL trait. About 28% isolates produced AmpC phenotype. Seven of 36 isolates (18%) indicated co-existence of ESBL and AmpC, while strain AKA43 was found to contain MBL and AmpC traits together. The prevalence of ESBL, MBL, AmpC and carbapenemase producing *E. coli* was studied. A few of these isolates were found to harbor more than one type of β-lactamases.

Key words: Extended spectrum ß-lactamases (ESBLs), ampCß-lactamases, metallo-ß-lactamases (MBLs), antibiotic resistance.

INTRODUCTION

The absence of new, effective anti-gram-negative antibiotics makes infection control the most important counter measure against multidrug-resistant gram-negative pathogens. Infection control can prevent

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additional infections and the spread of resistant pathogens and thereby reduce the need to use antibiotics (Anderson, et al., 2007; Arakawa et al., 2000; Bhavnani et al., 2006). Because susceptibility tests may be unreliable, special tests are required to detect the resistance mechanisms involved. The mechanisms include extended-spectrum-β-lactamases (ESBLs), AmpC β-lactamases and carbapenemases of molecular classes A and B (Anderson et al., 2007; Arakawa et al., 2000; Mohamed et al., 2006; Black et al., 2005; Ambler et al., 1991).

Extended-spectrum β-lactamases (ESBLs) strains of Enterobacteriaceae have emerged as significant pathogens (Rawat, 2010). Reports of infection or colonization with ESBL-producing Enterobacteriaceae strains have focused mainly on hospitalized patients or nursing home residents (Birgy et al., 2013; Paterson et al., 2005; Kenneth, 2010). Unlike most other anti-microbial-resistant pathogens that are associated with admission to hospitals, ESBL-producing *Escherichia coli* are predominantly community-onset pathogens (Cosgrove et al., 2002). Third world countries are considered as epicenter of this antimicrobial resistance primarily because the factors like overcrowding, poor nutrition and hygiene status and lack of infection control measures combined with antibiotic misuse/overuse has led to high endemic levels of resistant bacteria. Continuous and improper use of third generation cephalosporins has induced mutations in β-lactamases and has led to the emergence of ESBL producing Enterobacteriacae members (Fair, 2014; Arora, 2005).

Paterson (2005) stated that ESBL producing *E. coli* poses major threat and ESBL *E. coli* infections are increasingly reported from hospital settings as well as community settings with rising mortality and morbidity rates. Major risk factors for colonization or infection with ESBL producing organisms are long term antibiotic exposure, prolonged intensive care unit (ICU) admission, nursing home residency, severe illness, residence in surroundings with significant use of ceftazidime or other third generation cephalosporins (Ahammad, 2014). Besides these classical risk factors, the trait has been carried across the world by international travelers, food and animal trade form high to low prevalence region. Impact of international travelling is great; the best example is rapid spread of New Delhi Metallo β-lactmases (NDM-1) from India to other western countries (Kenneth, 2010). The Carbapenemases are diverse enzymes that vary in the ability to hydrolyze carbapenems and other β-lactams. Detection is a crucial issue because (i) they are often associated with extensive, sometimes total, antibiotic resistance and (ii) more-resistant organisms such as strains of *Pseudomonas* and *Acinetobacter* species that have acquired a carbapenemase can be vectors responsible for carbapenemase transmission to members of the family Enterobacteriaceae in which the resistance mechanism is not recognized (Thomson, 2010).

In this work, clinical specimens: urine, stool, sputum, and pus were collected over a period of three months from patients from slow progressing area of Maharashtra State, India. The objective of this study was to isolate and classify MDR *E. coli* straining exhibiting resistance to third generation cephalosporins, study distribution of various types of β-lactamases expressed within these clinical isolates of *E. coli*. To dissect what proportion of isolate make use of single and more than one types of β-lactamases.

MATERIALS AND METHODS

Bacterial isolates

Outdoor patients from the major hospitals of Barshi, Maharastra, India were grouped based on nature of samples and the symptoms. The clinical samples like urine, pus, stool and sputum were collected from patients for the microbial and biochemical analysis (Table 1). From these samples, bacteria were isolated by standard microbiological procedures and stored at 4°C for further experiments. Bacterial isolates were confirmed to be *E. coli* by VITEK-2 analyzer as well as standard biochemical properties as per Bergey's manual of systematic bacteriology.

Antibiotic susceptibility test

The bacterial isolates were screened for susceptibility to third generation antibiotics along with first and second generation β-lactams. The sensitivity of isolates were detected by Kirby-Bauer disc diffusion method (Bauer, 1966) on Mueller Hinton agar plates containing Ampicillin (25 µg), Oxacillin (1 µg), Penicillin-G (10 U), Ceftazidine (30 µg), Cefepime (30 µg), Tetracycline (30 µg), Cloxacillin (30 µg), and Cefotaxime (30 µg) (Himedia, Mumbai). The experiment was done as per the guidelines provided by Clinical and Laboratory Standards Institute (CLSI). Briefly, overnight grown culture density was adjusted to 0.5 MacFarland constant and plated on Mueller Hinton agar plates with sterile cotton swab. Four discs for antibiotics were placed on seeded agar plates. Zone diameter breakpoints recorded and organisms were classified as sensitive, intermediate and resistant as per the CLSI guidelines provided with *E. coli* NCTC 11954, strain harboring plasmid borne TEM-1 non ESBL type β-lactamase.

Detection of ESBL producers

Isolates exhibiting resistance to CTX, CAZ or CPD alone or together were tested for sensitivity in combination with clavulanic acid (CA). When inhibitory zones of CTX-CA, CAZ-CA or CPD-CA were found to be larger than 5 mm to that of zone for corresponding antibiotic without CA then these isolates were referred to as ESBL producers (Garrec et al., 2011).

Detection of MBL producer

The metallo β-lactamase (MBL) production was detected by combined disc method (Picao et al., 2008) using Imipenem (10 µg)

Table 1. Drug resistant bacterial isolates from hospital patients.

S/N	Species Name	Specimen	Gender
1	*E.coli*AKA41	Urine	F
2	*E.coli*AKA42	Pus	F
3	*E.coli*AKA43	Other	F
4	*E.coli*AKA44	Urine	M
5	*E.coli*AKA45	Urine	M
6	*E.coli*AKA46	Sputum	M
7	*E.coli*AKA47	Pus	F
8	*E.coli*AKA48	Pus	F
9	*E.coli*AKA49	Pus	M
10	*E.coli*AKA50	Urine	F
11	*E.coli*AKA51	Urine	M
12	*E.coli*AKA52	Pus	M
13	*E.coli*AKA53	Pus	M
14	*E.coli*AKA54	Pus	M
15	*E.coli*AKA55	Pus	M
16	*E.coli*AKA56	Pus	F
17	*E.coli*AKA57	Urine	M
18	*E.coli*AKA58	Urine	F
19	*E.coli*AKA59	Urine	F
20	*E.coli*AKA60	Pus	M
21	*E.coli*AKA61	Pus	M
22	*E.coli*AKA62	Urine	M
23	*E.coli*AKA63	Urine	F
24	*E.coli*AKA64	Urine	M
25	*E.coli*AKA65	Urine	F
26	*E.coli*AKA66	Pus	F
27	*E.coli*AKA67	Stool	F
28	*E.coli*AKA68	Urine	F
29	*E.coli*AKA69	Pus	F
30	*E.coli*AKA70	Urine	F
31	*E.coli*AKA71	Urine	F
32	*E.coli*AKA72	Urine	F
33	*E.coli*AKA73	Urine	M
34	*E.coli*AKA74	Stool	F
35	*E.coli*AKA75	Urine	F
36	*E.coli*AKA76	Urine	F

and Imipenem-EDTA. Both the IMP and IMP+EDTA discs were placed 30 mm apart from each other on seeded agar plates, plates were then incubated at 37°C for 24 h. Zones of inhibition were observed after 24 h. An isolate showing increase of inhibitory zone by more than 5 mm for IMP+EDTA than that of IMP alone considered to be MBL positive isolate, producing metallo β-lactamase enzyme.

Detection of AmpC producer

The AmpC production was detected by disc potentiation test using phenylboronic acid (PBA) as an enzyme inhibitor (Coudron, 2005; Tsakris et al., 2009). Phenyl boronic acid was dispensed onto commercially available Cefoxitin disc–CX (30 µg). These discs were dried and used within 30 min. The two discs CX and CX+PBA were placed on seeded agar plates by 30 mm apart from each other. An increase in inhibitory zone of ≥5 mm for the disc with PBA than that of CX alone was considered as an AmpC producer as the inhibitor potentiated the inhibitory effect of the cephalosporins by inactivating the AmpC enzyme.

Modified Hodge test for NDM1

The carbapenemase production among the isolates was detected

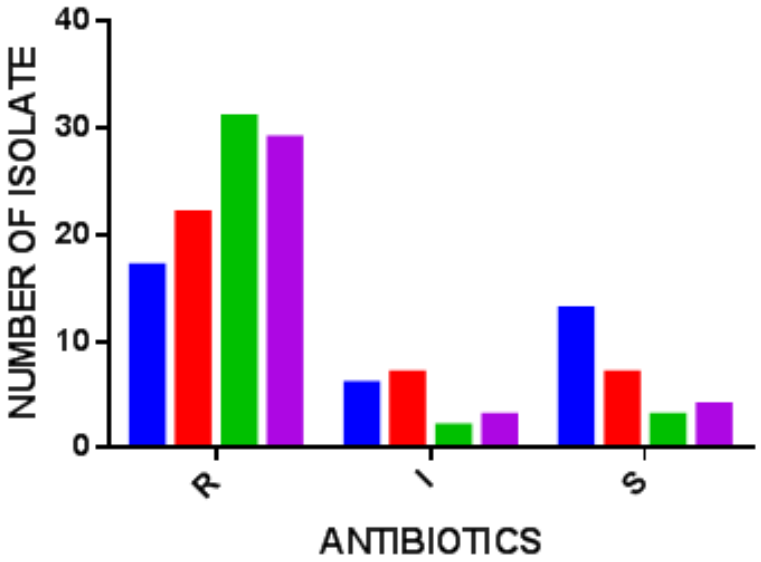

Figure 1. Antibiotyping for *E. coli isolates*. Antibiotic sensitivity estimated with disc diffusion assay. R denotes resistant phenotype, I denotes intermediate resistance while S denotes sensitive phenotype. The antibiotype expressed as histogram for various antibiotics; blue represents Tetracycline, red represent Cefepime, green represents Cefotaxime while purple indicates Ceftazidime.

by modified Hodge test as recommended by Clinical and Laboratory Standards Institute CLSI (Amjad et al., 2011). A lawn of 1:10 dilution of *E. coli* ATCC 25922 was spread on Muller Hinton agar plate seeded with *E. coli* strain ATCC 25922. Meropenem or Ertapenem (Dewees et al., 1970) (10 µg) susceptibility disc was placed in the centre of the ATCC 25922 seeded agar plate. In a straight line, test organism was spread from the edge of the disc to the edge of the plate. Plates were incubated at 37°C for 24 h. After incubation, the plates were examined for a clover-leaf type indentation at the intersection of the test organism and the *E. coli* ATCC 25922, within the zone of inhibition of the carbapenem susceptibility disc. The enhanced growth of *E. coli* ATCC 25922 toward the Ertapenem or Meropenem disc due to enzyme production by test organism was interpreted as a positive result for carbapenemase production.

Plasmid curing

Plasmid curing experiment was performed as described in Kharat and Mahadevan (1999). In brief, isolates exhibiting resistance to either Ceftazidime, Cefepime or Cefotaxime were subjected to cure the plasmid with low concentrations of ethidium bromide (EtBr). Overnight grown test organism 1:100 proportions to LB broth containing 10 µg/ml EtBr. Test organisms were incubated at 37°C for overnight. Grown cultures were serially diluted up to 10^{-6} with sterile physiological saline and 100 µl dilution was spread inoculated on LB agar plates. Plates were then incubated at 37°C for 24 h for the appearance of colonies. Well isolated colonies were picked and patched on LB agar medium plates supplemented with CTX (30 µg/ml) with a numbered grid line attached on the bottom of each plate. Plates were incubated at 37°C for 24 h. Plasmid was said to be cured from strain if a few of the isolated colonies could grow on LB agar plate but not on CTX agar plates, while parent strain could grow happily on LB agar and TCX agar plates.

Plasmid analysis

Plasmid DNA was isolated with standard alkali hydrolysis protocol. Plasmid were separated by agarose gel electrophoresis along with molecular weight marker and visualized under UV transilluminator. Purified plasmid DNA was used as donor to transform *E. coli* DH5α (NEB) with $CaCl_2$ method. Transformation mixture was plated on LB agar supplemented with 30 µg/ml CTX. The CTX^R transfromants were then tested for resistance to unselected antibiotics for which parent strain exhibited resistant phenotype (Upadhyay et al., 2015).

RESULTS

Susceptibility of *E. coli* isolates

Antibiotic susceptibility pattern for different β-lactams antibiotic was performed on *E. coli* isolates obtained from different clinical samples collected during May 2013- to July 2013. The group of antibiotics included were Penicillin, Ampicillin, Oxacillin, and Coxacillin, partially broad spectrum and third generation β-lactam members, namely, Ceftazidime, Cefotaxime and fourth generation Cefepime along with a non β-lactam protein synthesis inhibitor Tetracycline. The presence of ESBL, MBL, AmpC and carbapenemase activity by phenotypic confirmatory test was analyzed.

Majority of the isolates were obtained from female patients with urinary tract infection (urine) followed by sepsis (pus) sample (Table 1). Results shown in Figure 1

depict antibiotic susceptibility profile for all 36 *E. coli* isolates obtained from urine, pus, and stool. It is evident from Figure 1 that resistance to third generation cephalosporins was differential and found to be high ranging from 60 to 81% among the clinical isolates. Resistance to broad spectrum antibiotic Tetracyclin was comparatively low accounting for 47%.

ESBL producers

Out of these ESBL producers, 7 isolates were found to exhibit resistance to TET, accounting for tetracycling resistant strains to 16 out of 36 (Figure 1). The strain AKA46 and AKA49 were found to exhibit resistance to TET, CPM, CAZ, and CTX, whereas AKA50 and AKA54 were found to exhibit resistance to TET and CAZ; and TET and CTX, respectively. All nine isolates not exhibiting ESBL phenotype were also found to exhibit TET resistance phenotype (Figure 1). Resistance to selected antibiotics was differential, meaning β-lactamase producers were either resistant to one of the three cephalosporins and yet exhibited sensitivity to remaining two cephalosporins (Figure 1). Results represented in Figure 2 show that out of 36 *E. coli* isolates 75% could express ESBL trait while 25% were non-ESBL producers.

MBL and AmpC producers

Only AKA43 strain indicated existence of both MBL and AmpC (Figure 2B). In contrast, 10 isolates (28%) exhibited AmpC production ability (Figure 2A). Coexistence of ESBL and AmpC trait was observed in 7 isolates (Figure 2A). Coexistance of ESBL and AmpC trait in four of the seven strains is as shown in Figure 2C-1, strain AKA46, Figure 2C-2 for strain AKA51, Figure 2C-3 for strain AKA54 and Figure 2C-4 for strain AKA61. Interestingly, differential resistance to antibiotics was experienced among AmpC producers. Five of the AmpC producers were resistant to TET, seven were resistant to CPM, eight each were resistant to CTX and CAZ.

Carbapenem

Carbapenems such as imipenem and meropenem are powerful antibiotics that are not inactivated by ESBLs and AmpC lactamases and therefore are regarded as the last line of treatment for infections caused by ESBL producers. Recently, the rapid emergence and dissemination of carbapenem resistance in Enterobacteriaceae was reported due to production of carbapenemase enzyme. Presence of carbapenemase producers among the isolates was checked by Modified Hodge Test, none of the 36 isolates could exhibit carbapenamase production ability.

Plasmid curing

The EtBr is a mutagenic agent primarily causing frame shift by intercalating into the template DNA. The concentration required to impart mutation on genomic DNA is usually higher than it is required for low molecular weight extrchromosomal DNA. At lower concentrations, EtBr is known to interfere plasmid replication leading to plasmid loss in the next generations. We sought to test whether or not CTX resistance exhibited in clinical isolate is encoded on plasmid. The bacterial growth curve was performed at various concentrations to estimate lowest EtBr to be used in plasmid curing experiment. Bacterial growth was perturbed at 100 µg/ml EtBr, but unaffected at lower concentration, thus, we decided to use EtBr at 1/10 concentration (10 µg/ml) in plasmid curing experiment. Bacteria were grown and plasmid curing experiment was carried out as described in materials and methods. Plasmid curing was initially tested with replica plating for antibiotic markers; ceftazidime, cefepime and cefotaxime. All of the 36 clinical strains were able to gain sensitivity to cephalosporin antibiotics upon plasmid curing. These observations suggested that resistance exhibited in clinical isolates of *E. coli* is likely due to extrachromosomal genome.

Plasmid preparation

Existence of plasmid in clinical isolates was further substantiated as the demonstration of plasmid DNA. About eleven *E. coli* isolates were selected as sample for plasmid studies. Isolation of plasmid DNA with alkali lysis method was conducted on these 11 isolates. An aliquot of plasmid DNA was subjected for agarose gel electrophoresis. Results shown in Figure 3 indicate that all of the eleven plasmid preparations were successful substantiating observations of plasmid curing, which serves as second evidence. These observations are in agreement that cephalosporin resistance in *E. coli* isolates is likely due to expression of enzyme from the plasmid.

Transformation of antibiotic resistance

The DH5α cephalosporin naive competent cells were used as recipient and the plasmid DNA isolated from the earlier experiment served as the donor. Transformation mixture was plated onto LB agar supplemented with cefotaxime 30 µg/ml and incubated at 37°C for 24 h of the appearance of transformant colony. Experiments performed with controls indicated that plasmid DNA

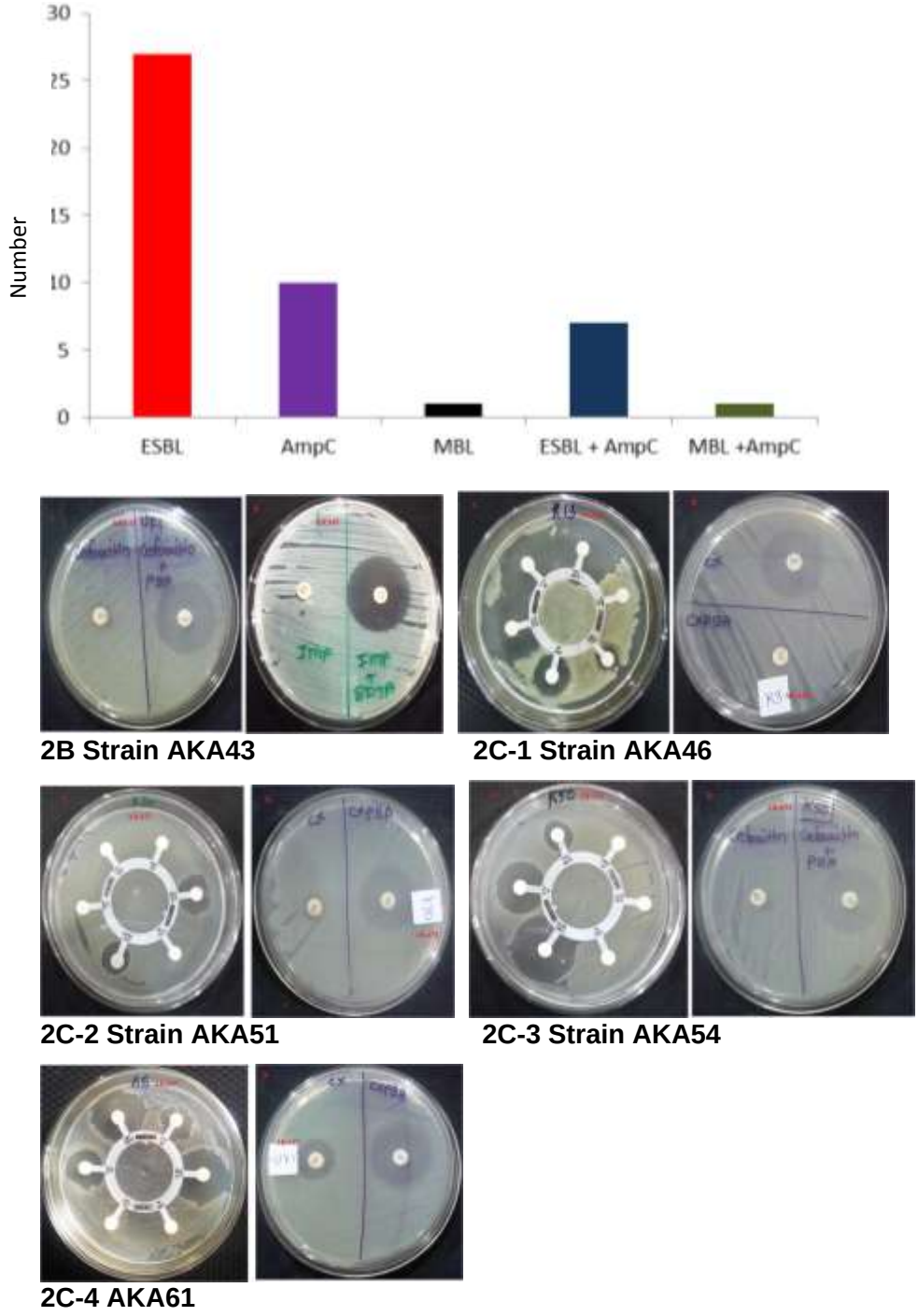

Figure 2. (A) Phenotypic classification of β-lactamase trait. Prescribed tests for detection of expression of ESBL, MBL, AmpC and Carbapenamase tests were conducted. In depicted histograms, red denotes ESBL, purple denotes AmpC, black denotes MBL, blue denotes dual ESBL and AmpC while green denotes MBL and AmpC expressing β-lactamase strains. (B) Phenotypic co-expression of MBL and AmpC trait in strain AKA43. Expression of the MBL was indicated by noticing enhanced zone of inhibition with Imp + EDTA whereas when enhanced with cefoxitin + phenylboronic acid it was treated as AmpC. (C) Phenotypic co-expression of ESBL and AmpC trait in 1. Strain AKA46, 2. Strain AKA51, 3. Strain AKA54 and 4. Strain AKA61. Expression of AmpC was indicated by noticing increased zone of inhibition for the disc with cefoxitin and phenyl boronic acid whereas when increased with cefotaxim + clavulanic acid it was treated as ESBL trait.

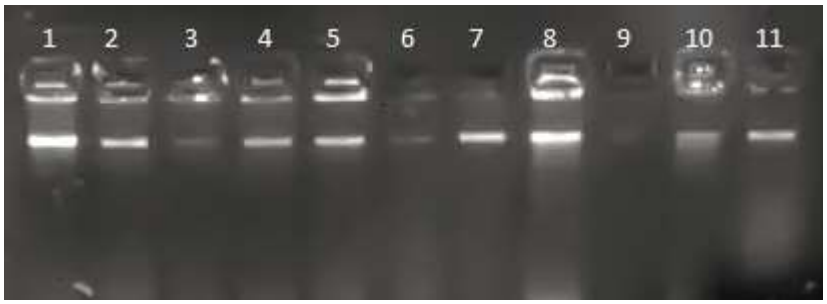

Figure 3. Plasmid analysis from a few □-lactamase producing *E. coli*. Plasmids isolated from the bacterial isolates were subjected for agarose gel electrophoresis. In the picture lane 1-AKA75, lane 2-AKA41, lane 3-AKA63,lane 4-AKA73, lane 5-AKA65, lane 6-AKA46, lane 7-AKA43, lane 8-AKA74, lane 9-AKA67, lane 10-AKA54, lane 11-AKA51, lane 1-AKA75, lane 2-AKA41,lane 3-AKA63,lane 4-AKA73, lane 5-AKA65, lane 6-AKA46, lane 7-AKA43, lane 8-AKA74, lane 9-AKA67, lane 10-AKA54, lane 11-AKA51.

isolated from eleven *E. coli* cephalosporin resistant strains was able to transfer CTX resistance. This experiment was again in agreement with results described in plasmid curing and yet provided another evidence to substantiate the proposition of cephalosporin resistance exhibited among 36 clinical *E. coli* isolates which is a plasmid borne trait.

DISCUSSION

ESBL detection is not routinely carried out in many microbiology laboratories of hospitals in developing countries. In our study, the prevalence of various β lactamases in the *E. coli* isolates was alarmingly high. The ESBL production was 75% found to be maximum as compared to the other β lactamases. Similar findings are in agreement with earlier reports (Bandekar et al., 2011) and 39.8% in burn ward patients (Ibrahim, 2013).

In this study, high resistance rates among ESBL-producing *E. coli* to first line antimicrobial therapy such as trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid ciprofloxacin and ofloxacin were observed (data not shown). Similar findings have been reported in developing countries (Bandekar et al., 2011; Al-Muharrmi et al., 2008; Hussain, 2011; Idowu, 2011; Kader, 2004) as well as in developed countries (Moyo, 2010; Winokur, 2001). Significantly high rates of resistance to such commonly used oral antimicrobials have been previously described making these agents clinically ineffective for empirical treatment of infection caused by ESBL-producing strains (Kader, 2004; Moyo, 2010; Winokur, 2001; Ventola, 2015). Earlier studies reported that ESBL-producing isolates exhibited significantly higher resistant rates to non-β-lactamase antimicrobials agents including fluoroquinolones, aminoglycosides, tetracyclines and trimethoprim-sulfamethoxazole, compared to non-ESBL producing isolates. The possible explanation for this observation may be the fact that ESBLs are encoded on plasmids and can be mobile and therefore, easily transmissible as resistance gene elements for other antimicrobials from one organism to another (Bradford, 2001; Kader, 2004; Al-Agamy et al., 2006).

In this study, the AmpC production was seen in 27% isolates, which is in agreement with that of earlier AmpC producer reports. It was 17.3% in Kolkata (2005) and 22.9% in the study by Bandekar et al. (2011) from burn ward patients. In parallel studies, Bhattacharjee et al. (2008) reported that of total *Pseudomonas aeruginosa* isolates of 22% carrying AmpC trait. The only β-lactams which were active against the AmpC and the ESBL coproducers were the carbapenems. However, recently, the resistance to the carbapenems has been increasing, which is mostly due to the production of the metallo β-lactamases. In the index study, the MBL producers were 10.98%. In our studies, only strain AKA43 could express both MBL and AmpC traits.

Multidrug resistance producing Enterobacteriaceae strains in rural water reservoir in Guantao China (Zhang et al., 2015). About 42% ESBL-E (Enterobacteriaceae) carriers were detected from 18 villages of 3 counties located in Shandong, China (Sun et al., 2014; Chandel et al., 2011), while studying neonatal sepsis from rural and urban setting in India they identified the presence of ESBL producing strains in community of infants with no prior history of hospitalization or antibiotic use. Similar to our methodology and observations, co-existence of ESBL and MBL was confirmed with phenotypic and VITEK 2 advanced expert characterization by (Kotwal et al., 2016). In their studies of total *Pseudomonas aeruginosa* strains, only 2% strains could co-express ESBL and MBL. In the present study, the AKA43 strain could exhibit expression of two traits, but MBL and AmpC.

In our study, the occurrence of ESBL-producing *E. coli*

among urine specimens is of great concern, since *E. coli* is one of the main causative agents of urinary tract infections and consequently due to the use of antimicrobial agents, ESBL producing *E. coli* is more likely to spread. This was also true in the present study, because prevalence of ESBL producing *E. coli* isolates varied among the participating hospitals from 45.1 to 75%. Although, earlier studies from different countries have reported that ESBL producing Enterobacteriaceae, *P. aeruginosa*, *Klebsiella pneumoniae* and *E. coli* may be found in rural area, this is a first report from rural part of Maharashtra, Western India. This article collectively describes that *E. coli* isolates not only exhibit resistance to third generation cephalosporins, but also co-expression of at least two β-lactamases. These observations express dire need of cautious antibiotic prescription to be avoided when not indicated on the basis of clinical microbiology investigation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

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