PREVALENCE AND ANTIMICROBIAL SUSCEPTIBILITY OF ESBL AND AMPC β-LACTAMASES PRODUCING ESCHERICHIA COLI AND KLEBSIELLA PNEUMONIAE FROM VARIOUS CLINICAL SAMPLES: AN EMERGING THREAT

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ABSTRACT

Resistance to broad spectrum β-lactams mediated by Extended Spectrum β-lactamases (ESBL) and AmpC β-lactamases enzymes is a growing threat worldwide.

AIM

The aim of the study was to detect the prevalence and antimicrobial susceptibility of ESBL and AmpC β-lactamase producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from various clinical samples.

MATERIALS AND METHODS

A total of 288 isolates comprising of 180 *Escherichia coli* and 108 *Klebsiella pneumoniae* isolated from various clinical samples were included. ESBL was detected by Phenotypic Confirmatory Disc Diffusion Test (PCDDT) and Double Disk Synergy Test (DDST). AmpC detection was done by AmpC disk test.

RESULTS

Out of 180 *Escherichia coli* and 108 *Klebsiella pneumoniae* isolates 91 (50.5%) and 63 (58.3%) were confirmed to be ESBL producers by PCDDT and 81 (45%) and 57 (52.7%) by DDST respectively. AmpC was detected in 35 (19.4%) of *Escherichia coli* and 33 (30.5%) of *Klebsiella pneumoniae* isolates. Co-production of ESBL and AmpC was detected in 6 (3.3%) *Escherichia coli* and 11 (10.2%) of *Klebsiella pneumoniae* isolates. Majority of ESBL producers were from blood in both organisms. Multidrug resistance (MDR) was seen in 79.1% of ESBL *Escherichia coli* and 63.5% of ESBL *Klebsiella pneumoniae* isolates. MDR was seen in 28 (96.5%) of AmpC producing *Escherichia coli* and all AmpC producing *Klebsiella pneumoniae* isolates.

CONCLUSION

It is essential to report ESBL and AmpC beta lactamase production along with routine susceptibility, which will aid the clinicians in prescribing antibiotics. Strict adherence to the hospital antibiotic policy and good infection control practices would go a long way in curtailing the menace of drug resistance.

KEYWORDS

AmpC β-lactamase, *Escherichia coli*, Extended-spectrum β-lactamase, *Klebsiella pneumoniae*, Multidrug resistance.

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INTRODUCTION

The rapid global dissemination of Enterobacteriaceae harbouring plasmid borne extended-spectrum β-lactamases (ESBL) and plasmid mediated AmpC β-lactamases represents a significant clinical threat.[1,2] The predominant mechanism for resistance to β lactam antibiotics in gram negative bacteria is by synthesis of β-lactamases. Among the β-lactamases, the production of ESBLs and AmpC β-lactamases are the most common.[3]

ESBLs are plasmid-mediated β-lactamases that are capable of efficiently hydrolyzing penicillin, narrow and broad-spectrum cephalosporins and monobactams (aztreonam), but they do not hydrolyse cephamycin or carbapenems (imipenem, meropenem). β-lactamase, inhibitors such as clavulanic acid, sulbactam and tazobactam generally inhibit ESBL producing strains. They have evolved from genes of TEM-1, TEM-2 or SHV-1 by mutation that alter the amino acid configuration around the active site of these β-lactamases rendering them susceptible to hydrolysis by these enzymes. There are also new families of ESBLs including the CTX-M and OXA-type enzymes as well as novel unrelated β-lactamases. ESBL producing isolates are most commonly found in *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli).*[1]

AmpC β-lactamases are primarily chromosomal and plasmid-mediated and are resistant to β-lactamase inhibitors such as clavulanic acid, but can hydrolyse cephamycin. Plasmid mediated AmpC β-lactamases (PMABLs) have evolved by the movement of chromosomal genes on to plasmids and are found in *E. coli, K. pneumoniae*, *Salmonella* spp., *Proteus*

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mirabilis, *Citrobacter freundii*, *Enterobacter aerogenes* which confer resistance similar to their chromosomal counterparts. Carbapenems are one of the antibiotics of last resort for many bacterial infections, such as *E. coli* and *K. pneumoniae* producing AmpC and ESBL.[4]

These organisms are responsible for a variety of infections like urinary tract infections, septicaemia, hospital acquired pneumonia, intra-abdominal abscess, brain abscess and device related infections and are typically associated with multidrug resistance. Treatment failures after instituting β-lactam antibiotic therapy for infections caused by ESBL producing gram negative bacilli have been reported.[5] It has been demonstrated that ESBL and AmpC production by infecting organisms adversely affects the clinical outcome.

Distinguishing between the AmpC and the ESBL producing organisms has epidemiological significance and it may have a therapeutic importance as well.[6] Moreover, these strains are no longer confined to the hospital environment, but of late are being isolated from the community at increasing frequencies.[7,8] Therefore, it is necessary to know their prevalence so as to enable the clinician to select appropriate antibiotic regimen at the earliest. The routine susceptibility tests performed by clinical laboratories fail to detect these strains making treatment options difficult. With this background, the current study was conducted to determine the prevalence of ESBL and AmpC β-lactamases in *E. coli* and *K. pneumoniae*, which were isolated from various clinical samples from both in-patients and out-patients who attended a tertiary care hospital in North-West India.

MATERIAL AND METHODS

A total of 288 consecutive, non-repetitive isolates comprising of 108 *K. pneumoniae* and 180 *E. coli* were recovered from different clinical samples between January 2014 and May 2014 (Table 1). The isolates were identified by standard biochemical methods.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility of the isolates was done by Kirby Bauer disc diffusion method following the Clinical and Laboratory Standards Institute (CLSI) guidelines.[9] using commercially available discs (HiMedia, Mumbai, India). Cefepime (30 µg), ceftriaxone (30 µg), ceftazidime (30 µg), cefoxitin (30 µg), amikacin (30 µg), gentamicin (10 µg), cefuroxime (30 µg), ciprofloxacin (5 µg), doxycycline (30 µg), meropenem (10 µg), norfloxacin (10 µg), nitrofurantoin (300 µg) and cefoperazone/sulbactam (75/10 µg).

Screening for ESBLs and AmpC β-lactamases

As per CLSI recommendation, isolates showing resistance (zone ≤ 22 mm for ceftazidime and ≤ 25 mm for ceftriaxone) by disc diffusion method were considered potential ESBL producers and further preceded for confirmation.[9]

Isolates showing resistance to cefoxitin (inhibition zone < 18 mm) by disc diffusion method were considered potential AmpC producers and further tested for presence of AmpC β-lactamase enzyme by AmpC disk test.

Detection of ESBLs and AmpC β-lactamases

The Phenotypic Confirmatory Disc Diffusion Test (PCDDT)

All strains that were potential ESBL producers were subjected to confirmation using the PCDDT as recommended by CLSI.[9] A disc of cefotaxime (30 µg) and ceftazidime (30 µg) alone and a disc of cefotaxime/clavulanic acid (30 µg/10 µg) and ceftazidime/clavulanic acid (30 µg/10 µg) were placed independently 30 mm apart center to center on a lawn culture of 0.5 McFarland turbidity of the test isolate on Mueller-Hinton Agar (MHA) plate and incubated for 18-24 hours at 35^0 C. A ≥5 mm increase in zone diameter for either antimicrobial tested in combination with clavulanic acid versus its zone when tested alone confirmed ESBL production (Figure 1).

Double Disc Synergy Test

A 0.5 McFarland suspension of the test isolate was swabbed on MHA plate and 30 µg antibiotic discs of ceftazidime, ceftriaxone and cefotaxime were placed on the plate 15 mm (center to center) from the amoxicillin/clavulanate (20 µg/ 10 µg) (augmentin) disc and incubated at 37^{0}C for 18-24 hrs. Clear extension of the edge of the inhibition zone of any of these cephalosporin discs towards the augmentin disc was interpreted as positive for ESBL production (Figure 2).

AmpC Disk Test

Lawn cultures of ATCC *E. coli* 25922 were prepared on MHA plate and a 30 µg cefoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc moistened with sterile saline (20 µL) and inoculated with several colonies of the test organism was placed besides the cefoxitin disk almost touching it. After overnight incubation at 35°C, the plates were examined for either an indentation or a flattening of the zone of inhibition indicating enzymatic inactivation of cefoxitin (positive result) or the absence of a distortion indicating a negative result.[10] (Figure 3).

Quality Control

Every batch of media prepared was checked for sterility for 24 hours. CLSI reference strains of ESBL positive *K. pneumoniae* ATCC 700603 and ESBL negative *E. coli* ATCC 25922 were included in the study.

STATISTICAL ANALYSIS

Chi square test was applied for analysis of categorical data. All statistical calculations were done by using MedCalc Statistical Software, version 14.12.0 (MedCalc Software bvba, MedCalc Ostend, Belgium). $P < 0.05$ was taken as significant for interpretation.

RESULTS

Out of 288 non-repetitive isolates that were included in the study, 180 were *E. coli* and 108 were *K. pneumoniae*. The number of ESBL and AmpC β-lactamase producers detected by screening test was 250 and 145 respectively.

Out of 250 screen positive isolates, 154 (61.6%) were confirmed as ESBL producers. DDST detected 138 (55.2%) ESBL producers, while all 154 were detected by PCDDT. (Table 2) Ten strains of *E. coli* and six strains of *K. pneumoniae* were not detected as ESBL producers by DDST. ESBL production was seen in 91/180 (50.5%) of *E. coli* and 63/108 (58.3%) of *K. pneumoniae*. Distribution of ESBL producers from various clinical samples is shown in Table 1. Maximum number of ESBL producers was isolated from blood accounting for 80% and 82.1% of *E. coli* and *K. pneumoniae* respectively.

Detection of AmpC β-lactamases

Out of 145 screen positive isolates, 68/145 (46.89%) were confirmed as AmpC β-lactamase producers by AmpC disk test. AmpC β-lactamase production was seen in 35/180 (19.4%) of *E. coli* and 33/108 (30.5%) of *K. pneumoniae* isolates (Table 2).

Co-production of ESBL and AmpC β-lactamases

Among the 154 ESBL positive isolates, 17 also tested positive for AmpC β-lactamase. Co-production of ESBL and AmpC was observed in 17/288 (5.9%) isolates. It was higher in *K. pneumoniae* (10.2%) than in *E. coli* (3.3%).

Antimicrobial Sensitivity Pattern

A wide spectrum of antimicrobial resistance pattern to various antimicrobial agents were detected in ESBL positive *E. coli* and in *K. pneumoniae* (Figure 4, 5). Both *E. coli* and *K. pneumoniae* strains showed a high degree of resistance to 4th generation cephalosporin cefepime accounting for 91.7% and 94% respectively. Least resistance was seen with meropenem in *E. coli* isolates accounting for 1.2%. However, among *K. pneumoniae* isolates the resistance was 14.3%. Among the urinary *E. coli* isolates, a high resistance of 89.2% was seen with norfloxacin.

A high multi-drug resistance (MDR) of 79.1% and 65.2% respectively was observed among ESBL producing strains of *E. coli* and *K. pneumoniae*. MDR was significantly higher in ESBL *E. coli* strains than non-ESBL strains. (P=0.036) multi-drug resistance was seen in 28/29 (96.5%) of AmpC producing *E. coli* and 22/22 (100%) of *K. pneumoniae* isolates.

Name of Sample	***Escherichia coli***				***Klebsiella pneumonia***			
	Total No.	**ESBL Positive (Pure) No. (%)**	**AmpC Positive (Pure) No. (%)**	**AmpC & ESBL Co-producers No. (%)**	**Total No.**	**ESBL Positive (Pure) No. (%)**	**AmpC Positive (Pure) No. (%)**	**AmpC & ESBL Co-producers No. (%)**
Urine	134	65 (48.5%)	22 (16.4%)	4 (2.9%)	14	07 (50%)	02 (14.3%)	03 (21.4%)
Pus	13	04 (30.7%)	04 (30.7%)	00	31	10 (32.2%)	09 (29.0%)	03 (9.6%)
Blood	10	08 (80%)	01 (10%)	00	28	23 (82.1%)	02 (7.1%)	00
Sputum	09	04 (44.4%)	00	01 (11.1%)	05	02 (40%)	02 (40%)	02 (40%)
Tracheal swab	02	00	01 (50%)	00	11	04 (36.3%)	04 (36.3%)	01 (9.1%)
Suction tip	02	01 (50%)	00	00	08	01 (12.5%)	03 (37.5%)	01 (12.5%)
Vaginal Swab	04	01 (25%)	00	00	01	00	00	00
CSF	02	01 (50%)	00	00	03	01 (33.3%)	00	01 (33.3%)
Others	04	01 (25%)	01 (25%)	01 (25%)	07	04 (57.1%)	00	00
Total	**180**	**85(47.2%)**	**29(16.1%)**	**06(3.3%)**	**108**	**52(48.1%)**	**22(20.3%)**	**11(10.1%)**

Table 1: Distribution of ESBL and AmpC β-lactamases in Different Clinical Samples

Name of Microorganism	**Total No. of Isolates**	**No. of Isolates Resistant to 3GCs in Screening Test**	**No. of Isolates Positive by PCDDT***	**No. of Isolates Positive by DDS†**	**No. of Isolates Resistant to Cefoxitin**	**No. of Isolates Positive by AmpC disk Test**
Escherichia coli	180	148	91	81	81	35
Klebsiella pneumoniae	108	102	63	57	64	33
Total	**288**	**250**	**154**	**138**	**145**	**68**

Table 2: Results of Screening and Confirmatory Tests for ESBL and AmpC Production

*PCDDT= Phenotypic Confirmatory Disc Diffusion Test; †DDST= Double Disc Synergy Test

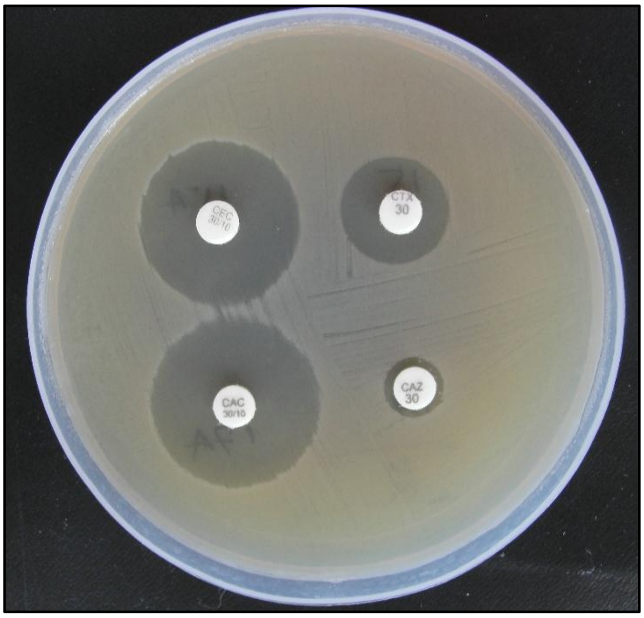

Fig. 1: Phenotypic Confirmatory Disc Diffusion Test. ESBL production confirmed by an increase in Zone of ≥5 mm for Ceftazidime (CAZ) and Ceftazidime/Clavulanic Acid (CAC) and Cefotaxime (CTX) and Cefotaxime/Clavulanic Acid (CEC)

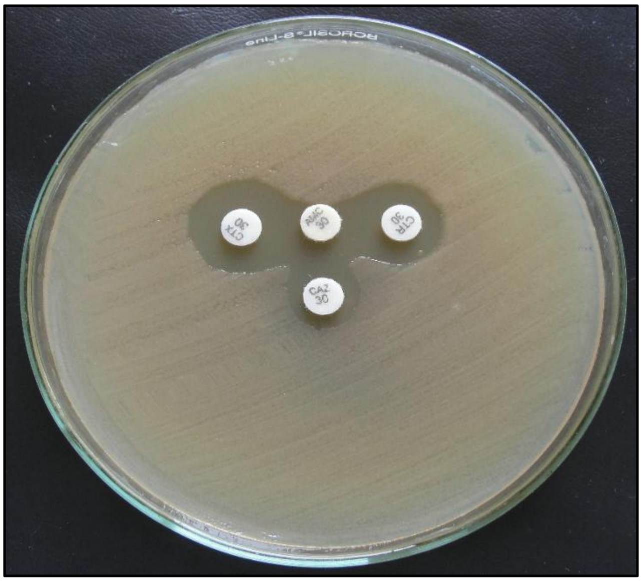

Fig. 2: Double Disc Synergy Test showing the Zone of Inhibition of Ceftazidime (CAZ) Cefotaxime (CTX) and Ceftriaxone (CTR) enhancing towards the Amoxicillin/Clavulanic Acid (AMC) disc Confirming an ESBL Producer

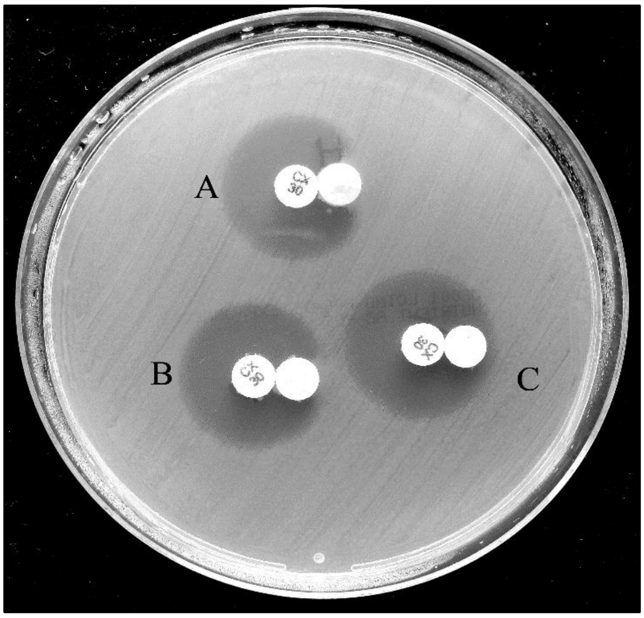

Fig. 3: AmpC Disk Test: Blunting towards Cefoxitin Disc Indicating Positive Test (A), Flattening (Borderline Positive) (B) and Absence of Blunting Indicating Negative Test (C)

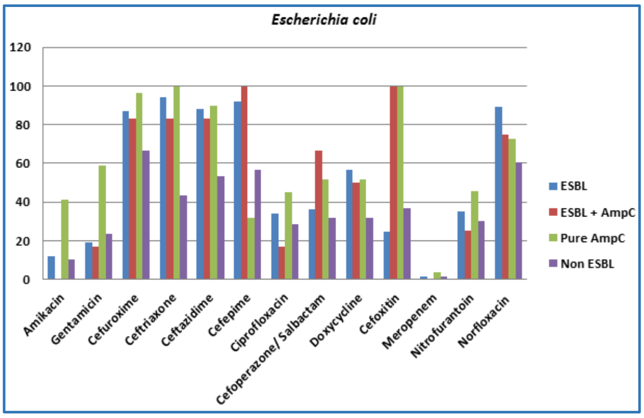

Fig. 4: Antimicrobial Resistance Patterns of Clinical Isolates of β-lactamase and non β-lactamase Producing Escherichia coli

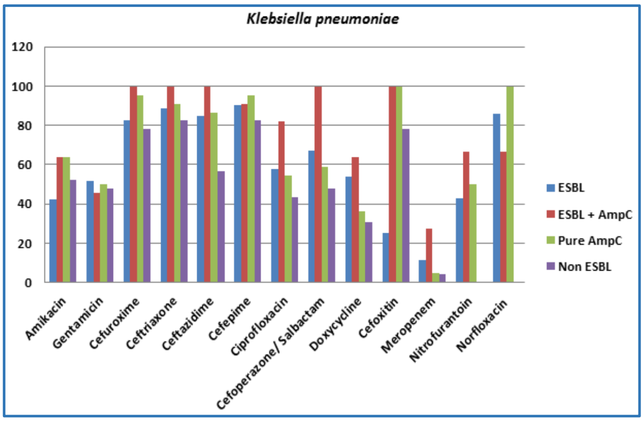

Fig. 5: Antimicrobial Resistance Patterns of Clinical Isolates of β-lactamase and non β-lactamase Producing Klebsiella pneumoniae

DISCUSSION

With the spread of ESBL and AmpC producing strains all over the world, it is necessary to know the prevalence of these strains in hospitals. The overall prevalence of ESBL in the present study was 154/288 (53.5%). ESBL was detected in 58.3% of *K. pneumoniae* and 50.5% of *E. coli* strains.

The prevalence of ESBL among clinical isolates varies greatly worldwide and in geographical areas and is rapidly changing over time. Reports of ESBL detection among clinical isolates of *E. coli* range between 20% and 80.6% and those among *K. pneumoniae* ranges between 20% and 86.7%.[11,12,13] Variation in the detection rates within and across the states could be due to the differences in the methodology used in these studies. Also it may be due to different patterns of antibiotic use and differences in the selection of organisms for the study. The PCDDT which is recommended by CLSI for phenotypic confirmation of ESBL among *E.coli* and *K. pneumoniae* was found to be more sensitive than DDST test. PCDDT detected 154/288 (53.5%) of all the ESBL producers, while DDST detected only 138/288 (47.9%). The DDST lacks sensitivity because of the problem of optimal disc space and the proper storage of clavulanic acid containing discs. Similar observation has been reported by other studies.[14,15]

Techniques to identify AmpC β-lactamase producing isolates are available, but are still evolving and are not yet optimized for the clinical laboratory.[4] Due to lack of reliable detection methods, their exact prevalence is unknown. Various studies have reported prevalence of AmpC between 2.2% 37.5%.[16,17,18] The overall prevalence of AmpC β-lactamases in the present study was 23.6%. Among *E. coli* it was 19.4%, while it was 30.5% among *K. pneumoniae* isolates. High level of AmpC production is typically associated with in vitro resistance to 3 GC's and cephamycins leading to clinical treatment failures with broad spectrum cephalosporins.[19,20]

Co-production of both ESBL and AmpC was observed in (17/288) 5.9% of isolates. It has been stated that AmpC β-lactamases when present along with ESBL can mask the phenotype of the latter.[4] Thus the coexistence of AmpC and ESBL in the same strain may give false negative results for detection of ESBL. When ESBL production is suspected, but the confirmatory test is negative, the strain should be screened for presence of AmpC β-lactamases.

Due to widespread use of antibiotics, MDR *E. coli* and *K. pneumoniae* strains isolated are increasing that poses severe challenges to public health. In the present study, MDR was seen in 79.1% of *E. coli* and 63.5% of *K. pneumoniae* isolates of ESBL producing strains. Resistance of ESBL producing isolates to 3GCs among *E. coli* was found to coexist with resistance to two or more antibiotics such as amikacin ($P=0.03$), gentamicin ($P=0.01$), cefepime ($P=0.00006$), cefoxitin ($P=0.0002$) and doxycycline ($P=0.02$). While in ESBL, *K. pneumoniae* resistance was seen with doxycycline ($P=0.02$) and cefoperazone/sulbactam ($P=0.04$). This coexistence of multi-drug resistance has been reported earlier.[21,22] Mechanisms of co-resistance are not clear, but one possible mechanism is the co-transmission of ESBL and resistance to other antimicrobials within the same conjugative plasmids. The highest drug resistance was observed for cefepime accounting for 90% in *E. coli* and 93.4% in *K. pneumoniae* isolates. Similar high resistance has been observed in other studies in India.[23,24] Resistance to cefepime could be attributed to the high prevalence of CTX-M type ESBLs in these isolates, some of which are capable of hydrolyzing cefepime.[25] Very high drug resistance of 85.9% was seen for norfloxacin in urinary isolates of *E. coli*. Imipenem was found to be the most effective drug against ESBL *E. coli* showing a susceptibility of 98.9 %, whereas 14.3% of ESBL *K. pneumoniae* isolates were resistant to imipenem, which could be because of carriage of carbapenemase genes.

Multi-drug resistance was observed in 28 (96.5%) of AmpC producing *E. coli* and 28/28 (100%) of *K. pneumoniae* isolates. Similar findings have been reported in other studies.[26,27] This emphasizes the need for detecting AmpC β-lactamase in MDR isolates, so as to avoid therapeutic failures and nosocomial outbreaks.

The increased ESBL and AmpC producing isolates are indicative of the ominous trend of more and more isolates acquiring resistance mechanisms, thus rendering the antimicrobial armarium ineffective. The high prevalence of these organisms emphasizes the need for early detection of these β-lactamases, which can help in instituting appropriate antimicrobial therapy and in avoiding the development and dissemination of these multi-drug resistant strains. Every health care institution must develop its own antimicrobial stewardship program, which is based on the local epidemiological data and international guidelines to optimize the antimicrobial use among the hospitalized patients and to improve patient outcomes.[28] Preventive measures like a continuous surveillance and strict implementation of infection control practices can go a long way in containing the menace of drug resistance in health care settings.

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