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Phenotypic Detection of Extended Spectrum Beta Lactamases and Metallo Beta Lactamases in Various Clinical Isolates of Enterobacteriaceae

Dr. Deepali Gupta[1], Dr. Umar Farooq[2*]
[1]JR 3, Department of Microbiology, Teerthanker Mahaveer Medical College and Research Centre, Moradabad, U.P. India
[2]Prof. & Head, Department of Microbiology, Teerthanker Mahaveer Medical College and Research centre, Moradabad, U.P. India

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***Corresponding author**
Dr. Umar Farooq

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Abstract: Enterobacteriaceae group is the main cause of bacterial infection; in this family Escherichia coli and Klebsiella spp. are the most prevalent causes of nosocomial infections. Extended-spectrum β-lactamases (ESBLs) represents a major threat among multidrug-resistant bacteria. Metallo-beta-lactamase (MBL) producing gram negative bacteria are being reported from several parts of the world and have emerged and clinically significant carbapenem resistance mechanisms. MBL producing bacteria can hydrolyze a wide range of beta-lactam antibiotics and lack the ability to hydrolyze aztreonam. This study was conducted in indoor department of Teerthanker Mahaveer Medical College & Research Centre, Moradabad, U.P. which is a tertiary care hospital. This study was conducted during the period from Jan 2015 to June 2016. A total of 200 gram negative Enterobacteriaceae were isolated in 500 no. of samples. They were screened for the β-lactamase production. Among the 500 isolates β-lactamase production was observed in 200 strains. 114 (57%) strains were ESBL producers; followed by 60 (30%) strains were MBL producers. The major ESBL and MBL producer was E.coli. Multidrug resistance to the fluoroquinolones and the aminoglycosides was also observed in the β–lactamase producing organism. The high prevalence of β-lactamases in ICU. The ESBL-producing organisms are a breed of multidrug-resistant pathogens that are increasing rapidly and becoming a major problem in the area of infectious diseases.

Keywords: Extended-spectrum β-lactamases (ESBLs), Metallo-beta-lactamase (MBL), Antibiotic sensitivity pattern.

INTRODUCTION

Enterobacteriaceae group is the main cause of bacterial infection and in this family *Escherichia coli* and *Klebsiella* spp. are the most prevalent causes of nosocomial infections. These pathogens are responsible for a broad spectrum of clinical infections in immune competent and as well as in immuno-compromised people and play a key role in epidemics of nosocomial infection [1].

Extended-spectrum β-lactamases (ESBLs) represents a major threat among multidrug-resistant bacteria isolates. They have risen to prominence among Enterobacteriaceae isolates in nearly all countries, now not only in the nosocomial but also in the community. These ESBL producing pathogens are now recognized globally as major causes of nosocomial and community-acquired infections. The first ESBL was detected in Germany in 1983, among different enterobacterial isolates recovered patients hospitalized at intensive care unit. It was recognized by the producer strains unusual resistance to cefotaxime (CTX) and ceftazidime (CAZ), which was transferable by conjugation to *E. coli*. In 1984, *Klebsiella pneumoniae* isolates with an identical phenotype were detected in different hospitals in France [2].

Carbapenems imipenem (IPM) and meropenem (MEM), are often used to treat infections caused by ESBL producing *E. coli* and *Klebsiella*. However, carbapenemases enzymes recognize almost all hydrolysable β-lactams, and most are resistant to inhibition by all commercially viable β-lactamase inhibitors. Four classes are available in this group: Molecular classes A, C, and D include the β-lactamases with serine at their active site, whereas molecular class B β-lactamases are all metalloenzymes with zinc in active-site. *Klebsiella pneumonia* carbapenemase (KPC) enzymes are belonging to class A carbapenemases that reside on transferable plasmids and can hydrolyze all penicillins, cephalosporins, and carbapenems[3]. Gram negative beta lactamase

producing organisms exhibited resistance to beta lactam antibiotics (e.g. penicillin, cephalosporins, monobactams) were developed during the last 2decades [4].

As a result of continuous point mutations inTEM-1, TEM-2 and SHV-1 genes found among gram negative bacilli, ESBLs emerged which are enzymes first identified in 1983 and mediated resistant to third generation cephalosporins (e.g. ceftazidime, ceftriaxone and cefotaxime) and monobactams (e.g. azetreonam) antibiotics and have been found in a wide range of Gram-negative bacilli, predominantly in *Klebsiella pneumonia* (*K.pneumonia*) and *Escherichia coli* (*E. coli*). ESBLs are carried on plasmids and may be associated with resistance to other types of antibiotics [5].

Extended spectum beta lactamases (esbls)

ESBLs are typically inhibitor-susceptible β-lactamases that hydrolyze penicillins, cephalosporins, and aztreonam and are encoded by mobile genes. The most frequently encountered ESBLs belong to the CTX-M, SHV, and TEM families. ESBL producers are usually multiply drug resistant, including the expanded spectrum (or third generation) cephalosporins (eg.cefotaxime, ceftriaxone, ceftazidime) and monobactams (eg. aztreonam) but not the cephamycins (eg. cefoxitine and cefotean) and carbapenems (eg. imipenem, meropenem, ertapenem).Organism that produce ESBL remain an important reason for therapy failure with cephalosporins and have serious consequences for infection control[6].

Most ESBLs can be divided into three groups: CTX-M, SHV AND TEM types. E.coli and K. pneumonia remain the major ESBL producing organisms isolated worldwide, but these enzymes have also been identified in several other organism of Enterobacteriaceae family and in certain non-fermenters. All of the beta-lactamase enzymes are commonly found in the Pseudomonas areuginosa and E.coli and also detected in K.oxytoca, Proteus mirabilis, Enterobacter, Citrobacter, Salmonella species and other members of Enterobacteriaceae[7].

Metallo-beta lactamases (mbls)

Metallo-beta-lactamase (MBL) producing gram negative bacteria are being reported with increasing frequency from several parts of the world and have emerged as a most widespread and clinically significant carbapenem resistance mechanisms. MBL producing bacteria can hydrolyze a wide range of beta-lactam antibiotics including penicillins, cephalosporins, carbapenems, cephamycins, but lack the ability to hydrolyze aztreonam[8].

Moreover, their catalytic activities are generally not neutralized by commercially available β- lactamase inhibitors such as clavulanate, tazobactam, and sulbactam. These enzymes belong to Ambler class B beta-lactamases based on their amino acid sequence homology and to group 3 according to the Bush classification based on their substrate and inhibitor profiles. MBLs require zinc-ions to catalyze the hydrolysis of beta-lactam antibiotics and due to the dependence on zinc-ions, MBL catalysis is inhibited in presence of metal-chelating agents like ethylenediaminetetraacetic acid (EDTA). MBLs were common in Pseudomonas aeruginosa and Acinetobacter spp, but more recently have emerged at an increasing rate among the members of Enterobacteriaceae[8].

Beta-lactamase inhibitors

The final piece in the penicillin-recognizing protein puzzle is the introduction of beta-lactamase inbibitors. These compounds resemble beta-lactame antibiotics sufficiently well that they can bind to the beta lactamase, either reversibly or irreversibly, protecting the antibiotic from destruction [9].

The three inhibitors of beta-lactamase activity that has found a place in clinical medicine are clavulanic acid, sulbactum and tazobactum. All three inhibitors are effective against staphylococcal penicillinase and have variable effectiveness against the chromosomal enzyme of Gram-negative bacteria. Clavulanate and tazobactum are superior to salbactum in activity against plasmid mediated beta-lactamase of Gram-negative organism including the ESBL[9].

MATERIALS AND METHODS

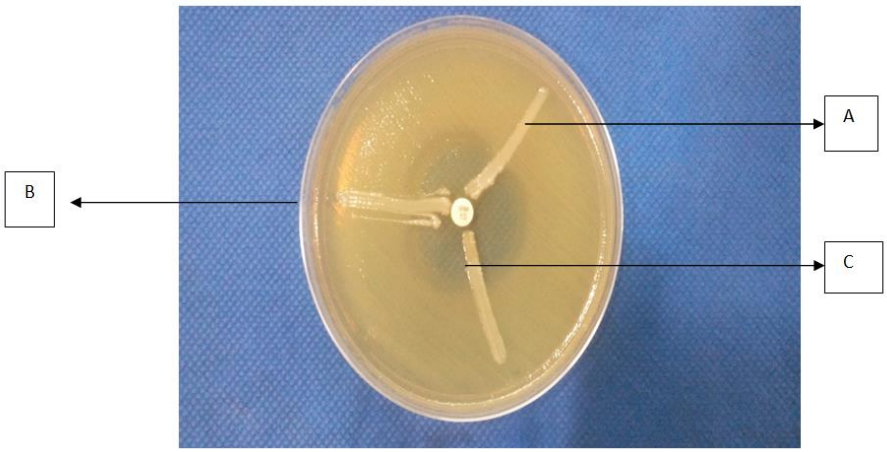
This study was conducted among 200 patients in indoor department of Teerthanker Mahaveer Medical College & Research Centre, Moradabad U. P. which is a tertiary care hospital. A total no. of 200 samples were taken for this study from ICU, PICU, NICU, and various other clinical departments of the hospital during the period from Jan 2015 to June 2016.All the isolates were identified by the standard microbiological tests. The antibiotic sensitivity test was determined by the Kirby Bauer disc diffusion method as per CLSI guidelines.

Phenotypic detection of esbls production

- Extended-spectrum β-lactamases (ESBLs) are Ambler class A penicillinases, which confer resistance to and hydrolyze the expanded-spectrum cephalosporins like ceftazidime, cefotoxime, monobactam-azteronam and related oxyimino β-lactams as well as older penicillins and cephalosporins.
- The Confirmatory test used was double-disk (combined-disk) method.

The disks contained 30 µg of CAZ alone and in combination with 10 µg of clavulanic acid, respectively (Himedia Company,India).

Isolates were examined for their susceptibility to 3rd generation cephalosporins by using ceftazidime (30 µg) and cefotaxime (30 µg) disks. All suspected isolates for ESBLs production were confirmed by the combination disk method on Mueller Hinton agar plates that were inoculated with standardized inoculums (comparable to 0.5 McFarland standards) of the isolates to form a lawn culture. Separate commercial disks containing cefotaxime (30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg) were placed over the lawn culture. The disc was placed centre to centre of the plate and the distance of 25 - 30 mm between the cephalosporin and clavulanate containing discs was observed. An increase in zone size of more than or equal to 5 mm for cefotaxime and ceftazidime with and without clavulanic acid indicated ESBL production [10]



Picture-1: Showing detection of ESBL by Double Disc Method showing Zone of inhibition ≥5mm with Ceftazidime and clavulanic acid than Ceftazidime alone

Identification of mbl

By using Modified Hodge Test and Combined Disc Methods

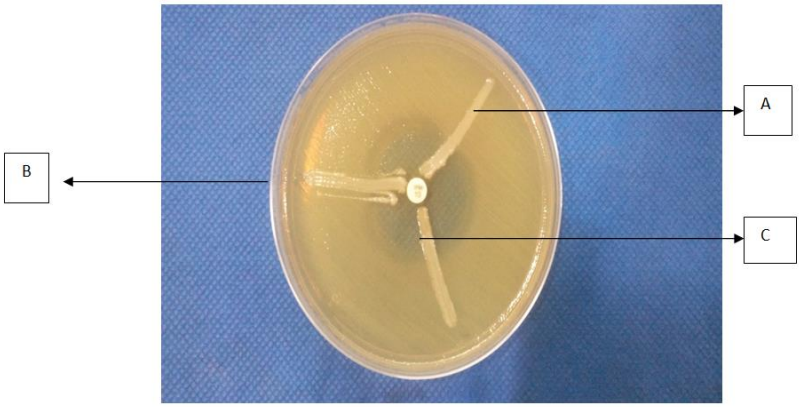
Procedure for modified hodge test

The samples were cultured and the organisms isolated were identified by standard microbiological techniques. The antimicrobial susceptibility to carbapenems was done by disc diffusion method. Zone sizes were measured according to CLSI recommendations. The isolates which showed resistant zones (≤15mm) for imipenem were tested for carbapenemase production by Modified Hodge Test as per CLSI guidelines.

A 0.5 McFarland matched suspension of E.coli ATCC 25922 was diluted 1:10. From this diluted suspension lawn culture was done on Muller Hinton agar (MHA) plate with sterile cotton swabs. The plate was allowed to dry for 3-4 minutes at room temperature. A 10 mcg meropenem disc (Hi media Company) was placed at the centre and test organism was streaked in a straight line from edge of the disc. Plate was incubated at 37ºC for 24 hours. The presence of clover leaf type of indentation at the test organism and ATCC E.coli 25922, within the zone of inhibition of meropenem susceptibility disc was interpreted as positive result as per CLSI guidelines

Quality control of the carbapenem disks were performed according to CLSI guidelines. Quality control of the following organisms MHT Positive K. pneumoniae ATCC 1705 and MHT Negative Klebsiella pneumoniae ATCC 1706 were run with each batch of the test.

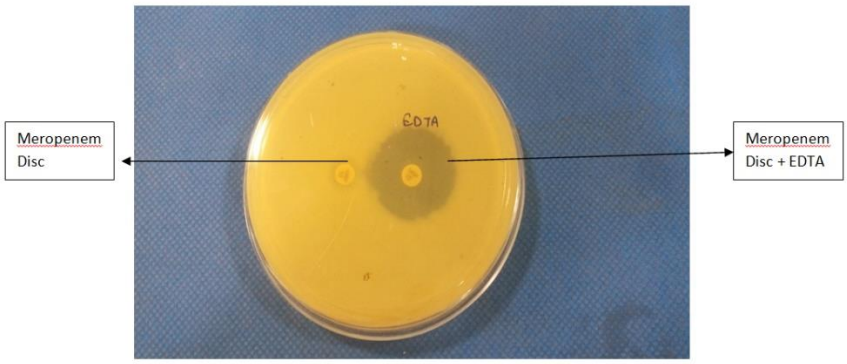
After 24 hrs, MHT Positive test showed a clover leaf-like indentation of the E.coli 25922 growing along the test organism growth streak within the disk diffusion zone. MHT Negative test showed no growth of the E.coli 25922 along the test organism growth streak within the disk diffusion.



Picture-2: Showing Modified Hodge Test
A-Positive test, B-Positive control, C- Negative control

Confirmatory detection of mbl by combined discs method

0.5 M EDTA solution was prepared by dissolving 18.61 gram of disodium EDTA.2H2O in 100 ml of distil water and its pH was adjusted to 8.0 by using NaOH. The mixture was then sterilized by autoclaving. 10µl EDTA solution was poured on meropenem disk to obtain a desired concentration of 1900 µg per disc. Two to three identical colonies of test organism were inoculated in to nutrient broth and incubated at 37ºc for 4-6 hours then turbidity was adjusted to 0.5 Mc Farland. Lawn culture of this suspension of test organism was done on Muller Hinton Agar (MHA) plate with a sterile cotton swab. One 10µg meropenem disk was placed on MHA plate. EDTA impregnated meropenem disc was also placed on the same MHA plate at the distance of 20-25 mm from centre to centre. The plate is incubated at 37ºc for 16-18 hours. An increase in zone size of atleast 7mm around the meropenem –EDTA disc compared to meropenem without EDTA is recorded as an MBL producing strain.



Picture-3: Showing detection of MBL by Combined Disc Method showing increase in Zone of inhibition by ≥7mm with Meropenem and EDTA than Meropenem alone [11,12]

RESULTS

The present study was conducted at Teerthanker Mahaveer Medical College & Research Centre, Moradabad, and U.P. Total no. of 500 isolates were taken for the study. Out of 500, 200 isolates were Enterobacteriaceae group which were taken for the study from January 2015 to June 2016. In 200 isolates 106 (53%) were isolated in female and 94 (47%) were isolated in male. Among 200 clinical isolates majority were E.coli 100 (50%), followed by Klebsiella spp. 46 (23%), Enterobacter spp. 37 (18.5%), Citrobacter spp. 13 (6.5%) and Proteus spp. 4 (2%) in various clinical samples like Pus, followed by Urine, Blood, Sputum, High vaginal swab, Endotracheal secretion, Tracheal secretion, Foley's tip, CSF, Peritoneal fluid, Throat swab, BAL fluid, Thoractomy suction, Intra-uterine pack, Catheter tip, Bronchial washing and Ascitic fluid.

Among various gram negative isolated organism highest ESBL production was observed in E.coli 75(37.5%), followed by Klebsiella spp. 28 (14%), Enterobacter spp. 7 (3.5%), Citrobacter spp. 3 (1.5%) and Proteus spp. 1 (0.5%) were positive by Double disc diffusion test. The total no. of ESBL production was found 114 (57%) were positive and 86 (43%) were negative by Double disc diffusion test.

In the entire isolated organism majority was E.coli 20 (10%) followed by Klebsiella spp. 7 (3.5%), Enterobacter spp. 4 (2%), Citrobacter spp. 2(1%) and Proteus spp. 2 (1%) were suspected to be MBL producers by Modified Hodge Test. The total no. of MBL producers by Modified Hodge Test 35 (17.5%) were positive and 165 (82.5%) were negative. In Combined Disc Test E.coli 31 (15.5%) followed by

Klebsiella spp. 14 (7%), Enterobacter spp. 8 (4%), Citrobacter spp. 4 (2%) and Proteus spp. 3 (1.5%) were found the zone of inhibition was ≥7 mm. So the total no. of MBL producers by Combined Disc Test 60 (30%) were showed the zone of inhibition ≥7mm and 140 (70%) the zone of inhibition was ≤7mm, found to be non-MBL producers.

Table-1: Showing Gender wise distribution of total no. of samples. (n=200)

Sex	Frequency	Percent
Female	106	53.0
Male	94	47.0
Total	200	100.0

The study population consisted of 94 (47.0%) males and 106 (53.0%) females

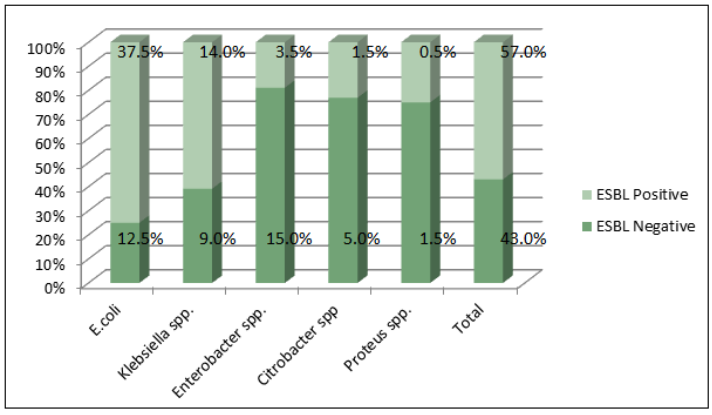
Table-2: Showing frequency of isolated organism in total no. of patients (n=200)

Isolates	Frequency	Percent
E.coli	100	50.0
Klebsiella spp.	46	23.0
Enterobacter spp	37	18.5
Citrobacter spp.	13	6.5
Proteus spp.	4	2.0
Total	200	100.0

E. coli was found from the majority (50%) of the samples followed by Klebseilla spp (23%), Enterobacter spp. (18.5%), Citrobacter spp. (6.5%) and Proteus spp. (2%)

Table-3: This table showing the presence or absence of ESBL was compared among different isolates by Combined Disc Method. A significantly higher number of samples tested positive for ESBL among E. coli (37.5%) and Klebsiella spp (14%) in comparison to Citrobacter spp (1.5%), Enterobacter spp (3.5%) and Proteus spp (0.5%) (n=200)

	ESBL		
	Negative	Positive	Total
E.coli	25	75	100
	12.5%	37.5%	50.0%
Klebsiella spp.	18	28	46
	9.0%	14.0%	23.0%
Enterobacter spp	30	7	37
	15%	3.5%	18.5%
Citrobacter spp	10	3	13
	5.0%	1.5%	6.5%
Proteus spp	3	1	4
	1.5%	0.5%	2.0%
Total	86	114	200
	43.0%	57.0%	100.0%

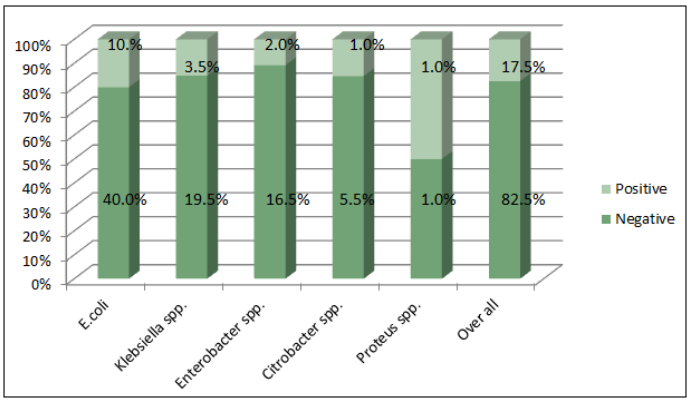


Graph-1: Showing ESBL positive and negative isolates in total no. of samples

Table-4: Showing presence or absence of MBL by MHT (n=200)

	MBL		
	Negative	Positive	Total
E.coli	80	20	100
	40.0%	10.0%	50.0%
Klebsiella spp.	39	7	46
	19.5%	3.5%	23%
Enterobacter spp	33	4	37
	16.5%	2.0%	18.5%
Citrobacter spp	11	2	13
	5.5%	1.0%	6.5%
Proteus spp	2	2	4
	1.0%	1.0%	2.0%
Total	165	35	200
	82.5%	17.5%	100.0%

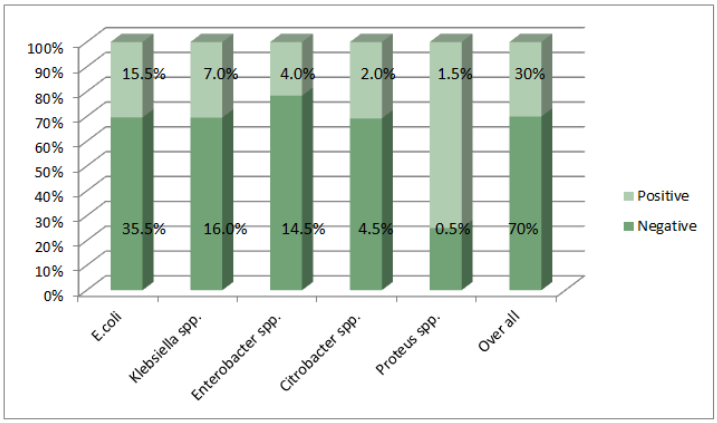
The presence or absence of MBL was compared among different isolates. There was no significant difference in the difference of MBL between different isolateds though the MBL positive cases were more among Proteus spp (50.0%) and E.coli (20.2%) than Citrobacter spp (15.4%), Enterobacter spp (10.3%) and Klebseilla spp (15.6%).



Graph-2: Showing frequency of MBL producing isolates by MHT in total no. of samples

Table-5: Showing MBL production in various isolates by Combined Disc Test among them majority were E.coli 31(15.5%) followed by Klebsiella spp. 14(7%), Enterobacter spp. 8(4%), Citrobacter spp. 4(2%) and in Proteus spp. 3(1.5%) (n=200)

	CDT		
	Negative	Positive	Total
E.coli	69	31	100
	35.5%	15.5%	50.0%
Klebsiella spp.	32	14	46
	16.0%	7.0%	23.0%
Enterobacter spp	29	8	37
	14.5%	4.0%	18.5%
Citrobacter spp	9	4	13
	4.5%	2.0%	6.5%
Proteus spp	1	3	4
	0.5%	1.5%	2.0%
Total	140	60	200
	70.0%	30.0%	100.0%



Graph-3: Showing frequency of MBL Production in various isolates by Combined Disc Diffusion Test

DISCUSSION

The worldwide emergence of multi-drug resistant bacterial strains is a growing concern which is usually found in those hospitals where antibiotic use is frequent and the patients are in critical condition [13]. Therapeutic options for the infections which are caused by the ESBL producers have also become increasingly limited [15]. A study has found ciprofloxacin to be highly effective in treating multi-resistant Gram-negative infections [14]. Recent studies on ESBL production among the members of Enterobacteriaceae which were isolated from clinical specimens, showed an increase in the occurrence of ESBL producers [16].

In the present study, E. coli showed the maximum ESBL production (50.0%) followed by Klebsiella spp. (23.0%), Enterobacter spp (18.5%) and Citrobacter spp (6.5%). This was similar to the studies by Wadekar *et al*. in which, ESBLs were predominantly present among E. coli (50%) compared to Klebsiella spp. (37.5%), Enterobacter spp. (33.3%) and Citrobacter spp. (33.3%),Mathur *et al*. 62% of the E. coli and 73% of the K. pneumoniae isolates were reported to be ESBL producers[17]. These findings have significant implications for empirical management of patients with UTI using third generation cephalosporins.

These findings are also similar to that of Ramesh *et al*. [20] and Kumar *et al*.[21] who reported a high prevalence of ESBLs among E. coli. The findings in our study were also similar to that documented in India, China, Thailand and Pakistan, in which the prevalence of ESBLs ranged between 47% and 70% for *K. pneumoniae* and 37% and 67% for *E. coli*[22-24].

Recent studies on ESBL production among the members of Enterobacteriaceae which were isolated from clinical specimens, showed an increase in the occurrence of ESBL producers [10]. A study from North India on uropathogens such as Klebsiella pneumoniae, Escherichia coli, Enterobacter, Proteus and Citrobacter spp, showed that 26.6% of the isolates were ESBL producers [25].

In intensive care units, where the prevalence and the risk factors which are responsible for the emergence of the ESBL producers is high. Other reasons for the high prevalence of the ESBL producers were indwelling catheters, endotracheal or nasogastric tubes, gastrostomies or tracheostomies, severity of the illness, the excessive use of cephalosporins and a high rate of patient transfer from the peripheral centers[14,26].

The different findings were also recorded in the studies by Wadekar *et al*,[27]. Maximum MBL production was seen in Klebsiella spp. (33.3%) and Enterobacter spp. (16.6%) which was quite different from the present study. A previous study from another tertiary care hospital in Nepal [28] reported comparatively lower incidence of MBL producing gram negative bacteria (1.3%) in lower respiratory tract specimens.

In the study by Bora *et al*. [8] extended spectrum beta-lactamases were found to be prevalent in E. coli and K. pneumoniae isolates and there was a gradual rise in the use of carbapenems, which could be a major cause of MBL mediated resistance. The majority of MBL producing isolates of E. coli (53.56%) and K. pneumoniae (58.97%) were from patients admitted to ICU. The ICU has been described as a factory for creating, disseminating, and amplifying antimicrobial resistance [29]. Among the four different sources of samples (urine, sputum, pus and blood), the highest incidence of MBL producing isolates was in blood samples and lowest incidence was in urine samples for the both pathogens. This observation also indicated the greater use of carbapenems in bloodstream infections.

MBL producing bacterial isolates can confer resistance to carbapenems and all beta-lactam agents except aztreonam although coexistence of other resistance mechanisms such as AmpC type beta-lactamases or ESBLs render them resistant to aztreonam[8]. Whereas in few reports MBL producing Enterobacteriaceae isolates were found to be susceptible to various carbapenems as well as to piperacillin/tazobactam by disc diffusion testing [30].

ESBL producing organisms, being the commonest nosocomial pathogens, it is essential to detect and treat them as early as possible. Since ESBL production is more common among the nosocomial pathogens, early detection will definitely help in controlling hospital infections which are caused by this group of organisms. Enterobacteriaceae are the common isolates in most of the laboratories. Now-a-days, a majority of these isolates are multi-drug resistant. The control of these multidrug resistant organisms is a therapeutic challenge. This difficulty is enhanced further by the co-existence of the resistance to β-lactams, aminoglycosides and fluoroquinolones, as observed in our study. Of all the available antimicrobial agents, carbapenems are the most active and reliable treatment options for infections which are caused by the ESBL producing isolates [10].

CONCLUSION

The ESBL-producing organisms are a breed of multidrug-resistant pathogens that are increasing rapidly and becoming a major problem in the area of infectious diseases. Piperacillin-tazobactam and imipenem are the most active and reliable agents for the treatment of infections which are caused by ESBL producing organisms. MBLs producing Gram-negative bacteria are an increasing public health problem worldwide because of their resistance to all β- lactam except Aztreonam.

MBL genes are typically carried on transferable plasmids or are part of the bacterial chromosome. This enzyme which have been detected primarily in *Pseudomonas aeruginosa* but were also found in other Gram negative bacteria, including non-fermenters and members of the family Enterobacteriaceae. The awareness of the existence of MBL initializes indication for the need for proper use of antibiotics and spread of multi drug resistance bacterial strains within these hospital and communities.

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