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Detection of ESBL, Amp C and Metallo Beta Lactamases in Clinical Isolates of Enterobacteriacae from a Tertiary Care Hospital Emergence of Carbapenamase and Other Beta Lactamases among Enterobacteriacae from a Tertiary Care Hospital.

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Enterobacteriacae producing ESBL, Amp C & Metallo beta lactamases have been increasingly reported worldwide. These organisms usually exhibit multidrug resistance that is not always detected in routine susceptibility tests. This leads to uncontrolled spread of ESBL & Amp C producing organisms and related treatment failures. Hence, detection of ESBL, Amp C & MBL is important in the routine clinical laboratory. A total of 100 consecutive Enterobacteriacae i.e. E.coli, Klebsiella spp, Citrobacter spp, Proteus spp isolates from various clinical samples were included in this study. Detection of ESBL production was done by phenotypic confirmatory test as per CLSI guidelines. Amp C production was detected by Amp C disk test as described by Black et al.MBL was detected by EDTA disc potentiation test. Among the 100 clinical isolates tested, ESBL production was seen in 34 (34%), Amp C production in 36 (36%), ESBL & Amp C coproduction in 24 (24%) of the isolates, MBL production in 8 (8%) isolates. The study emphasizes the high prevalence of multidrug resistant enterobacteriacae producing beta-lactamase enzymes of diverse mechanisms. Thus proper antibiotic policy and measures to restrict the indiscriminative use of cephalosporins and carbapenems should be taken to minimize the emergence of this multiple beta-lactamase producing pathogens.

Keywords: AmpC β-lactamases, extended spectrum β-lactamases, coexistence, prevalence, Gram negative bacteria.

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INTRODUCTION

The members of the Enterobacteriaceae are gram-negative, fermentative bacilli and have an important role in nosocomial and acquired infections. The predominant mechanism for resistance to β-lactam antibiotics in Gram-negative bacteria is by the synthesis of β-lactamases. β-lactamases are enzymes produced by some bacteria and are responsible for their resistance to β-lactam antibiotics like penicillins, cephamycin and carbapenem [1,2]. β-lactamase deactivates the molecular antibacterial properties of β-lactam antibiotics there by breaking and opening the common element in their molecular structure β-lactam. Some of these enzymes include extended spectrum β-lactamase (ESBL), AmpC and carbapenemase [2,3].

ESBLs are plasmid-mediated β-lactamase that are capable of efficiently hydrolyzing penicillin, narrow and broad spectrum cephalosporins and monobactams (Aztreonam),but they do not hydrolyze cephamycin or carbapenems (imipenem, meropenem). β-Lactamase, inhibitors such as clavulanic acid, sulbactam and tazobactam generally inhibit ESBL producing strains [3,6]. ESBL producing isolates are most commonly found in Klebsiella pneumoniae and Escherichia coli [4,5].

AmpC β-lactamase is primarily chromosomal and plasmid-mediated and are resistant to β-lactamase inhibitors such as clavulanic acid but can hydrolyze cephamycin. Carbapenems are one of the antibiotics of last resort for many bacterial infections such as E. coli and K. pneumoniae producing AmpC and extended spectrum β-lactamase but the emergence of carbapenamase which have versatile hydrolytic capacities have the ability to hydrolyze pencillins, cephalosporins, monobactams and carbapenems [6-8].

Infection caused by organisms producing such enzymes have resulted in poor outcomes, reduced rate of clinical and microbiological responses, longer hospital stays and greater hospital expenses [9]. Physical contact is the most likely mode of transmission and the gastrointestinal tract of colonized or infected patients is the most frequent reservoir while transient carriage of bacteria on the hands of healthcare workers may lead to transmission to patients [9,10].

The spread of these resistant bacteria in hospitals all over the world, conferring multiple antibiotic resistances in the treatment and management of life threatening infections necessitate this study. With the increase in occurrence and types of these multiple β-lactamase enzymes, early detection is crucial, the benefits of which include implementation of proper antibiotic therapy and infection control policy. Hence the present study was designed to investigate the presence of different classes of β-lactamase enzymes in clinical isolates of Enterobacteriacae.

MATERIALS AND METHODS

A total of 100 consecutive , non-repetitive clinical isolates of enterobacteriacae isolated from various clinical samples such as pus(39), urine(28) sputum(25), Ear swab(6), body fluid (4), blood (3) were included in this study . All the isolates were identified biochemically by the standard methods and were stored at4°C in 0.2% semisolid agar until used.

Antimicrobial susceptibility testing

The antibiogram of the isolates were determined by the standard Kirby Bauer's disc diffusion method (3). The following antibiotics discs (Hi-Media, India) were used such as, ampicillin (10 µg), amikacin (30 µg), gentamicin (10 µg),co-trimoxazole (25 µg), ciprofloxacin (5 µg), cefotaxime (30µg), ceftazidime (30 µg), ceftriaxone (30 µg), cefoxitin (30 µg) and imipenem (10 µg). The zone diameters were interpreted as per Clinical Laboratory Standards Institute (CLSI) recommendations (9). *Escherichia coli* ATCC 25922 strain was used for quality control.

Detection of ESBL production

Isolates which were resistant to third generation cephalosporins were tested for ESBL production by combination disk method using cefotaxime (30 µg), cefotaxime/clavulanic acid (10 µg), ceftazidime (30 µg) and ceftazidime/clavulanic acid (10 µg). A ≥5mm increase in diameter of inhibition zone of

cephalosporin+clavulanate disc when compared to cephalosporin disc alone was interpreted as evidence of ESBL production [4].

Detection of AmpC production

AmpC disk test

Isolates that yielded a cefoxitin zone diameter less than 18 mm and resistant to 3GC (screen positive) were tested for AmpC enzyme production by AmpC disk test (7). Briefly, 0.5 McFarland suspensions of ATCC E. coli 25922 was inoculated on the surface of Mueller-Hinton agar plate. A 30 g cefoxitin disc was placed on the inoculated surface of the agar. A sterile plain disc inoculated with several colonies of the test organism was placed beside the cefoxitin disc almost touching it, with the inoculated disk face in contact with the agar surface.

After overnight incubation at 37°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 no significant inactivation of cefoxitin (negative result) [8].

Detection of MBL production

Metallo β-lactamase production was detected by Meropenam-EDTA disk test. Two 10 µg Meropenam disks were placed on the plate, and appropriate amounts of 10 µl of 0.5M EDTA solution were added to one of them to obtain the described concentration (750 µg). The inhibition zones of Meropenam and Meropenam-EDTA disks were compared after 16 to18 hours of incubation in air at 35˚C. If the increase in inhibition zone with Meropenam and EDTA disk was ≥ 5 mm, then the Meropenam disk alone was considered to be the MBL producer. Carbapenemase production was further confirmed by modified Hodge test (MHT) [4,9].

RESULTS

Antimicrobial susceptibility testing

Out of the 100 total isolates tested, 57(57%) were resistant to 3GC (cefotaxime, ceftazidime, ceftriaxone), while 43 (43%) strains were susceptible. Majority of the Klebsiella, E.coli and Enterobacter isolates showed multidrug resistance. They were resistant to at least one non-lactam antibiotic (amikacin, gentamicin, co-trimoxazole, and tetracycline. (Table1)

Table 1: Antibiotic susceptibility pattern of Enterobacteriacae

Antimicrobial agents	% susceptibility
Ampicillin	7(7%)
Amikacin	72 (72%)
Ampicillin-sulbactum	38(38%)
Cefotaxime	43(43%)
Cefoxitin	21(21%)
Ceftazidime	35(35%)
cefepime	22(22%)
Ceftriaxone	25(25%)
Co-trimoxazole	58(58%)
Ciprofloxacin	64(64%)
Gentamicin	79(79%)
Imipenem	90(90%)
Nitrofurantion	55(55%)
Nalidixic acid	61(61%)
Tetracycline	34(34%)

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ESBL, AmpC and carbapenemase producing isolates

Out of the 100 isolates screened for ESBL production, 34 were confirmed to produce ESBL giving an overall prevalence of 34%. The highest prevalence of ESBLs was found in *E. coli* (17%), followed by *K. pneumoniae* (12%) *Enterobacter spp* (2%), *P. mirabilis* (1%) *P.vulgaris* (1%) and had the least ESBL prevalence of 34% (Table 2) (figure 1)

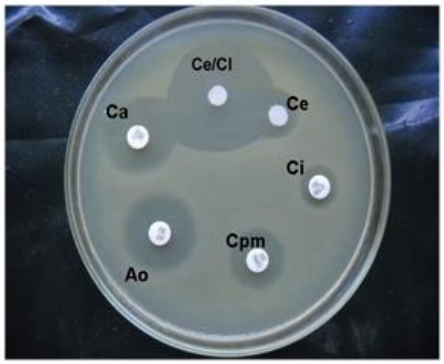

Figure 1: ESBL Detection

The susceptibility of the isolates to cefoxitin disc showed that 21 isolates equivalent to (46.3%) were found to be either resistant or showed reduced susceptibility to cefoxitin. The overall prevalence of AmpC β-lactamases was 16%. Similar to ESBL, *E.coli* had the highest prevalence of (8%), followed by *K. pneumoniae* (5%), *Proteus spp* (2%), and *Enterobacter* (1%).

Among the AmpC producers, 10% showed indentation (high production of AmpC enzyme) while 6 % showed flattening (low production of AmpC enzyme). (Figure 2)

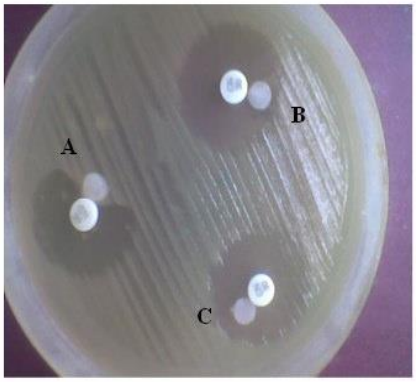

Figure 2: AmpC disk test: Presence of blunting towards cefoxitin disk indicates test positive (A) absence of blunting indicates test negative (B and C).

Furthermore, ten out of the hundred isolates (10%) produces carbapenemase. The highest prevalence of carbapenemase producers was in K. pneumoniae (6%) and E. coli (2%) (Table 2). (figure3)

Table 2: Prevalence of ESBL, AmpC and carbapenemase producers among Enterobacteriacae.

SN	Bacterial Species	No of isolates screened	ESBL Positive (%)	AmpC positive (%)	MBL positive (%)
1	*E. coli*	48	17	8	2
2	*K. pneumoniae*	30	12	5	6
3	*Enterobacter*	6	2	1	-
4	*Citrobacter*	9	1	-	-
5	*P. mirabilis*	5	1	1	-
6	*P. vulgaris*	2	1	1	-
total		100	34	16	8

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Figure 3: MBL detection

Co-Production of ESBL, AmpC, MBL and carbapenemase

The co-production of ESBL, AmpC and MBL was also observed among the isolates. Various combinations of different types of enzymes were found particularly in E. coli and K. pneumoniae (Table3) (figure 4)

Table 3: Different β-lactamase mediated resistance mechanism in AmpC producing *Enterobacteriacae* (n = 100).

ESBL+MBL (%)	AmpC +ESBL (%)	AmpC +MBL (%)
16	24	5

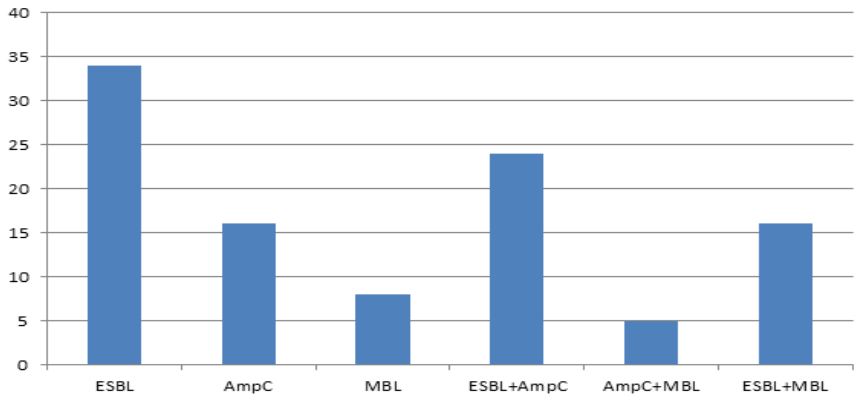

Figure 4: -Distribution of ß- lactamases

DISCUSSION

The infections which are caused by multidrug-resistant gram negative bacilli that produce various β lactamase enzymes have been reported with an increasing frequency and they are associated with a significant morbidity and mortality [9]. The numerous β- lactamases are encoded either by the chromosomal genes or by the transferable genes which are located on the plasmids or the transposons [10]. Initially, these enzymes were commonly found in the Klebsiella species and in E.coli but now, these enzymes are produced by all members of Enterobacteriaceae and other gram negative bacilli [12]. The growing increase in the rate of antibiotic resistance of these isolates is a major cause of concern. β-lactam have been the mainstay of treatment for serious infections, the most active of these being carbapenems, which are advocated for use in treatment of infections caused by ESBL producing Enterobacteriaceae [11,13], particularly Escherichia coli and Klebsiella pneumoniae. Pathogens that produce ESBL or AmpC β lactamases along with carbapenemases are particularly challenging for clinicians and are a major threat worldwide [14,16].

In our study, the prevalence of various β lactamases in the gram negative bacteria, which included the Enterobacteriaceae, was 69 %, which was alarmingly high. The ESBL production was (34%) found to be maximum as compared to the other β lactamases. According to the mentioned studies, it seems that the prevalence of beta-lactamases producing Enterobacteriaceae in different parts of the world can be varied

from 0% to over 70%. This difference could be due to the factors such as differences in the type and mode of antibiotic consumption that cause genetic mutations in bacteria and producing the mentioned enzymes [17]. In addition, cultural, nutritional and ethnic differences in various populations caused variations in the normal flora [18]. Different phenotypic methods in various studies could also be another reason.

Out of the 21 (21%) of the isolates showing resistance to cefoxitin in the present study, only 16 (16%) were AmpC producers. Cefoxitin resistance in this type of AmpC negative isolates could be due to a decreased permeability of porins. It was 17.3% in Kolkata [16] and 22.9% in a study which was done by Bandekar et al., [13] in burn patients, whereas a study which was done by Bhattacharjee et al showed 22% AmpC producing *Pseudomonas aeruginosa* [17].

In our study, 8% of the isolates were MBL producers. Several studies from India have shown a prevalence rate of 8-10% of *enterobacteriaceae* isolates being carbapenemase producers [20].

The coexistence of ESBL and MBL was reported in 16% isolates, whereas the AmpC and the MBL co production was shown by 5% isolates and the AmpC and the ESBL co production was shown in 24% isolates. A study which was done by Arora et al reported the AmpC and MBL coproduction in 46.6% isolates and the ESBL and AmpC co production in 3.3% isolates [16].

The increase in the prevalence of the AmpC, MBL and the ESBL producing isolates may be indicative of the ominous trend of more and more isolates acquiring the resistance mechanisms, thus rendering the antimicrobial armamarium ineffective. In our study, the multidrug resistant strains showed co resistance to the fluoroquinolones and the aminoglycosides, but they were moderately susceptible to imipenam and the ampicillin-sulbactum combination, which was in concordance with the findings of other studies [18,19].

CONCLUSION

Microbiology laboratories must be able to detect resistant pathogens in a timely manner, especially those that are falsely susceptible in vitro to drugs that may be considered for therapy of infected patients. Microbiological excellence is needed more than ever, and ESBLs, AmpC β lactamases and carbapenemases production should be detect accurately. In addition, there should be good communication between the microbiologist and the health care worker to make better patient outcomes, facilitating effective infection control, reducing spread of resistant pathogens and helping hospitals to meet accreditation standards. This will help in the fight against multidrug resistance pathogens and if corrective measures are not taken, in the absence of novel agents in the near future, the spread of MDR isolates may lead to therapeutic dead ends.

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