JOURNAL OF PURE AND APPLIED MICROBIOLOGY, October 2014. Vol. **8**(5), p. 4099-4105

Prevalence of ESBL, MBL and Ampc-β- Lactamases Producing Multidrug Resistance Gram Negative Bacteria in a Tertiary Care Hospital

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(Received: 12 May 2014; accepted: 20 July 2014)

The rapid spread of antibiotic resistance among gram negative bacteria (GNB) is an emerging threat and a matter of apprehension worldwide. Several mechanisms for the resistance of pathogenic organismshave been established, of which established one is the enzymatic hydrolysis of the antibiotic by specific enzymes called β -lactamases. The present study was undertaken to determine the prevalence of various β -lactamases in the multidrug resistance (MDR) gram negative bacilli from a tertiary care hospital. A totalof 596 consecutive, non- duplicate MDR GNB strains were isolated from various clinical samples which were received over a period of one year. The organisms were identified by standard biochemical tests and antibiotic susceptibility was determined by disc – diffusion method using Clinical Laboratory Standard Institute (CLSI) guidelines. They were then screened for the β-lactamase production. statistical analysis was done by using Chi square test. A total of 596 MDR isolates, 503 (84.39%) were β- lactamase producers from which 306 (51.34%) were AmpC producers, followed by 118 (31.54%) ESBL producers and 115 (19.29%) were MBL producers. The ESBL production was maximally seen in *Escherichia coli*, while the AmpC production was mainly observed in *Enterobacter spp*. and major MBL producer was *Acinetobacter spp*. The co production of the ESBL/MBL/ AmpC β- lactamases was observed in 105 (20.87%) strains. The present study revealed the high prevalence of β-lactamases among the MDR gram negative isolates which advocates an urgency of an early detection of β-lactamase producing organisms and thus an indiscriminate use of the higher antibiotics could be restricted as far as possible.

Key words: Multi Drug Resistant, Gram Negative Bacteria, ESBL, MBL, AmpC β- lactamase.

The introduction of β -lactam antibiotics into the health care system in the latter stages of World War II represents one of the most important contributions to the medical science in recent history. Today, β -lactams remain the most widely utilized antibiotics because of their comparatively high effectiveness, low cost, ease of delivery and minimal side effects[1].

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β-lactamases are the enzymesproduced by most of the bacterial strainsconfer significant resistanceto their bacterial hosts by hydrolysis of the amide bond ofthe four-membered β -lactam ring. These enzymes areespecially important in Gram-negative bacteria as theyconstitute the major defence mechanism against β -lactam-based drugs. The spread of β -lactamase genes hasbeen greatly exacerbated by their integration withinmobile genetic elements, such as plasmids or transposons,which aid the rapid transfer of genetic materialamong microbes. Even more intimidating is the organizationof β -lactamase genes within

integrons as part ofmulti-drug resistance gene cassettes whichencodes resistance not only to β - lactams but also to otherantibiotic classes such as aminoglycosides, macrolides,sulphonamides and chloramphenicol[2].

Three major groups of β-lactamase enzymes are usually distinguished such as class C cephalosporinase (AmpC), ESBL and carbapenemases such as MBL, are of great concern in the health care settings[3].

AmpC β- lactamases are clinically important because they confer resistant to narrow spectrum as well as broad spectrum cephalosporins, β-lactum- β- lactamases inhibitor combination and aztreonam. Group 1 AmpC β-lactamases are poorly inhibited by clavulanic acid; however, they are inhibited by cloxacillin[4]. ESBL producing organisms confer resistant to penicillin, cephalosporins and monobactums. They cannot hydrolyzecephamycins and are inhibited by Clavulanic acid[5]. Transferable metallo β-lactamases (MBLs) are most feared because of their ability to hydrolyze almost all drugs including carbapenems[6]. The presence of ESBL and AmpC-β –Lactamase in a single isolate reduces the effectiveness of β –Lactam - β –Lactamase inhibitor combinations, while MBL and AmpC- β – Lactamases confer resistance to carbapenems. Often, these enzymes are co- expressed in the same isolates[7].

The present study was undertaken to determine the prevalence of ESBL, MBL and AmpC producing multidrug resistant gram negative bacteria isolated from a tertiary care hospital and their susceptibility pattern against a number of antimicrobial agents was analysed.

MATERIALS AND METHODS

Isolation and Biochemical identification

A total of 596 consecutive, non- duplicate multidrug resistant gram negative bacterial strains were isolated from various clinical specimens such as blood, urine, stool, pus, sputum, wound swab, tracheal aspiration, cerebrospinal fluid (CSF), high vaginal swab (HVS) etc from outpatient department (OPD), wards, cabins, intensive care unit (ICU) and neonatal intensive care unit (NICU) of IMS &SUM Hospital, Bhubaneswar. All isolates were identified morphologically and biochemically by standard procedures and antimicrobial susceptibility was performed by using Kirby-Bauer's disc-diffusion method as per CLSI guidelines. Of the total 18,756 various clinical samples obtained over a period of one year (July 2012-Aug 2013), 3669 samples show positive growth, and of which only 1767 (48.16%) samples were yielded gram negative bacteria. In this study, we have selected only multi drug resistance gram negative bacterial strains those were resistance to two or more unrelated classes of antibiotics n=596 and excluded the strains of GPC (gram positive cocci) and some GNB (gram negative bacilli) showing higher sensitive pattern from this study.

Antibiotic susceptibility tests

Antibiogram of the isolates was done by Kirby Bauer's Method using antibiotic disks from Himedia, Mumbai. Antibiotics used for Gram-negative bacilli were ceftazidime (30µg) (CAZ), ceftazidime/clavulanic acid (30/10 µg) (CAC), amikacin (30 µg) (AK), amoxyclav(30 µg) (AMC),ofloxacin (5 µg) (OF), norfloxacin (5 µg) (NX), ceftriaxone (30 µg) (CTR), piperacilin/ tazobactun (100/10 µg) (PIT), gentamicin (10µg) (GEN), cefoperazone /sulbactum (75/30 µg) (CFS), netilimicin (30 µg) (NET), imipenem (10 µg) (IPM), meropenem (10 µg) (MRP), co-trimoxazole (25µg) (COT), tigecycline (15 µg) (TGC) and nitrofurantoin (300 µg) (NIT).However, co-trimoxazole(25 µg)(COT) and nitrofurantoin(300 µg)(NIT) were used only in case of urine samples. ESBL positive *Klebsiella pneumonia* ATCC 700603 and ESBL negative *Escherichia coli* ATCC 25922 were used as reference strains in this study.

Gram-negative bacteria with resistance or with decreased susceptibility (intermediate by CLSI criteria) to third generation cephalosporins were tested for ESBL production by following method.

Detection of ESBL

NCCLS confirmatory test

The test strain was cultured overnight and suspended to achieve a 0.5 McFarland standard turbidity and was lawn cultured onto a Muller-Hilton agar plate using a sterile cotton swab. After drying, antibiotic discs of ceftazidime (30 µg) and ceftazidime plus clavulanic acid (30/10 µg) were placed at a distance of 20mm from each other, and incubated overnight. Organism was considered as ESBL producer if there was a ≥ 5mm increase in zone diameter of ceftazidime/clavulanate disc than

that of ceftazidime disc alone[8] (Fig. 1).

Test for MBL production

MBL producing strains were suspected when the isolate was resistant to Carbapenem group of antibiotics (meropenem, imipenem, ertapenemetc).

Double disc synergy test

The test strain was cultured overnight and suspended to achieve a 0.5 McFarland standard turbidity and was lawn cultured onto a Muller-Hilton agar plate using a sterile cotton swab and allowed to dry. 5 µl of the EDTA solution was added to a 6-mm blank filter paper disk (Whatsman no.1 filter paper) which contained approximately 930 µg of EDTA. An imipenem disc (10 µg) was placed on the MHA plate and EDTA filter paper disc was placed at a distance of20mm from centre to centre. After overnight incubation at 37°c, the presence of an enlarged zone of inhibition towards the EDTA disc was interpreted positive for an MBL producer [9](Fig. 2).

Detection of AmpC beta lactamase

Three dimensional tests

AmpCenzyme production was tested by a modified three dimensional extract test described by Manchanda&Singh. Briefly, 10-15 mg fresh overnight growth from MHA was taken in a micro centrifuge tube. Peptone water then added and centrifuged at 800 g for 15 min.Crude enzyme extract will be prepared by repeated freeze thawing for five to seven times. Lawn culture of *E. coli* ATCC 25922 was prepared on MHA plates andcefoxitin (30 mg) discs were placed on the plate.Linear slits will be cut using a sterile surgical blade 3 mm away from the cefoxitin disc; 10 mg enzyme extract will be added to a well made at the inside of the outer edge of the slit.The wells could easily be loaded with the enzyme extract in 10 µL increments until the well was filled to the top. Approximately30–40 µL of extract was loaded in the wells. The plates were kept upright for 5–10 min until the solution dried.The plates will be incubated at 37°C for overnight.The isolates showing clear distortion of zone of inhibition of cefoxitin were taken as AmpC producers. The isolates with no distortion were taken as AmpC non-producers and isolates showing minimal distortion were taken as indeterminate strains[10](Fig. 3).

Statistical analysis

The statistical analysis was performed by using Chi- square test and *p* value of less than 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

The frequency use of antibiotics and even the dose and period of administration vary greatly from country to country, region to region and to some degree even locally. This has led to large differentials in the emergence of resistant strains. It is essential to study and report trends in antimicrobial resistance regularly [11].

The emergence and dissemination of numerous types of β-lactamases as ESBLs, MBLs and AmpC enzymes among gram negative bacterial strains pose a therapeutic challenge to the health care settings. These enzymes collectively can hydrolyze almost all β-lactam drugs which are most frequently used including carbapenems which are called the last resort for the treatment of serious infection [12].

A total of 596 MDR isolates of *E.coli*(n=269), *Klebsiellapneumoniae*(n=83), *Klebsiella oxytoca* (n=20), *Acinetobacter spp.* (n=66), *Pseudomonas spp.* (71), *Enterobacter spp* (n=33), Citrobacter spp.(n=33), *Proteus mirabilis* (n=10), *Proteus vulgaris* (n=6) and one number of *Providencia spp*. were recovered from different

Table 1. Distribution of various beta – lactamases from the isolated organisms. ($P < 0.01$)

Organisms	MBL No.	%	ESBLNo.	%	AmpCNo.	%
Acinetobacter spp. (n=66)	23	34.84	13	19.69	40	60.60
Citrobacter spp. (n=33)	8	24.24	9	27.27	17	51.51
E.coli (n=269)	28	10.4	118	43.86	110	40.89
Enterobacter spp. (n=33)	9	27.27	7	21.21	24	72.72
Klebsiella spp. (n=103)	25	24.27	20	19.41	64	62.13
Pseudomonas spp. (n=71)	21	29.57	11	15.49	41	57.74
Proteous spp. (n=16)	1	6.25	8	50	7	43.75
Providencia spp. (n=1)	0		1	100	1	100

Table 2. Antibiotic susceptibility pattern of MDR gram negative bacilli in various clinical isolates. (P< 0.01)

Antibiotics	Resistant (%)	Sensitive (%)	Intermediate (%)
CAZ	94.45	5.54	0
CAC	61.84	37.81	0.33
AK	41.04	57.77	1.18
AMC	93.16	6.42	0.37
OF	84.64	13.68	0.67
NX	88.94	10.2	0.85
CTR	95.6	4.4	0
PIT	56.89	42.05	1.04
GEN	57.66	40.28	2.04
CFS	55.44	42.57	1.98
NET	44.6	54.6	0.8
IPM	15	80.91	4.05
MRP	49.46	49.89	0.63
COT	73.97	26.02	0
TGC	5.78	92.91	0
NIT	27.06	71.28	1.65

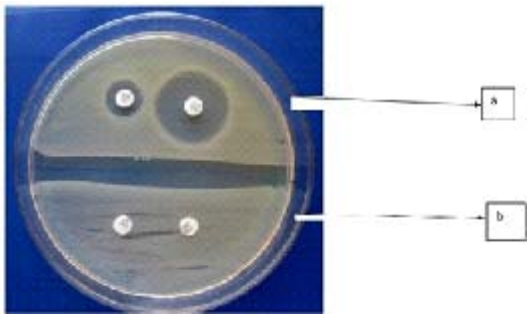

Fig. 1. NCCLS confirmatory test for ESBL. Isolate showing ESBL production, zone of inhibition given by the Ceftazidime+ clavulanic acid (CAC) disk is ≥ 5 mm than those of Ceftazidime (CAZ) disk alone. (a) ESBL Positive (b) ESBL Negative.

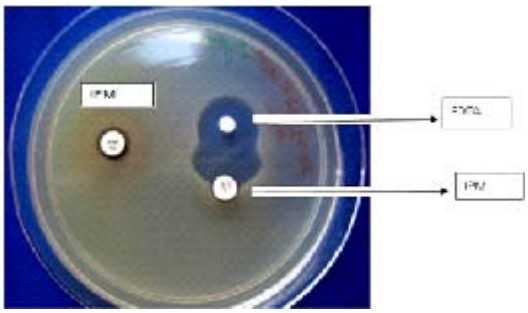

Fig. 2. Double disc synergy test for MBL. Zone of inhibition towards the EDTA disc was interpreted positive for an MBL producer. IPM – imipenem, EDTA- Ethylene diamine tetra acetic acid.

clinical specimens(Table-1).The ESBL production was maximally seen in *Escherichia coli* 43.86%, followed by *Citrobacter spp*. 27.27%, *Klebsiella spp*. 19.41%, *Acinetobacter spp*. 19.69% and *Pseudomonas spp*. 15.49%, while the AmpC production was mainly observed in*Enterobacter spp*. 72.72% followed by *Klebsiella spp*. 62.13%, *Acinetobacter spp*. 60.60% and *Pseudomonas spp*. 57.74%. *Providencia spp*. Shows 100% ESBL and MBL producing strains but we can't consider this because the sample size is very low. The major MBL producer was *Acinetobacter spp*. 34.84% followed by *Pseudomonas aeruginosa* 29.57%, *Enterobacter spp*. 27.27%, having nearly same in case of *Klebsiella spp*. 24.27% and *Citrobacter spp*. 24.24%. This showed a significant correlation (*p* value <0.01)(Table-1).

Statistics have shown that ESBL producing E. coli are found to be the highest in India (60%) followed by Hong Kong (48%) and Singapore (33%) [13]. Oberoi*et al* [14] reported 35.16% were ESBL producer and E. coli was the major ESBL producing organism which was similar to our findings (31.54%). AmpC beta-lactamases are cephalosporinases which are poorly inhibited by clavulanic acid and can be differentiated from other ESBLs by their ability to hydrolyse cephamycins as well as other extended-spectrum cephalosporins[15]. AmpC- β-lactamases are enzymes which demonstrated or presumed to be chromosomally or plasmid mediated, have been described in various gram negative bacteria [16]. AmpC β- lactamase was detected in 51.34%, which was higher when compared with other studies done

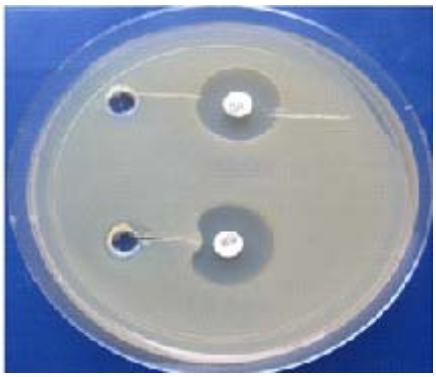

Fig. 3. AmpC β- lactamase production by three dimensional extract test.

by Hemlatha V *et al*[17] 47.3% and two other Indian studies by Shigal S *et al*[18] 8% and Manchanda V *et al* 43% [19].The MBLs hydrolyses all beta lactum groups of antibiotics except for aztreonam *in vitro* [20]. The detection of MBL producing organisms is essential to optimal treatment of patients and to control the spread of resistance [21]. We found 19.29% of clinical isolates were MBL producers and this was low when compared with a study from Karachi, Pakistan by Irfan S *et al*[22].

Of the 596 MDR gram negative isolates, 503 (84.39%) were β- lactamase producers from which 306 (51.34%) were AmpC producers, followed by 118 (31.54%) ESBL producers and 115 (19.29%) were MBL producers. The co-production of the ESBL/MBL/ AmpC β- lactamases was observed in 105 (20.87%) strains (Fig. 4).

In our study, the prevalence of various β-lactamases in the GNB was 84.39% which was found to be maximum as compared to the previous study done by Oberoi*et al* from ICU patients (70.69%). In this study the AmpC producers were isolated maximum (51.54%) followed by ESBL (31.54%), but the study was done by Oberoi*et al* reported 35.16% ESBL producer followed by 10.98% MBL producer which was totally different from our study [14]. Another study done by Dalele G *et al*[23] reported from uropathogens ESBL production was 66.9% followed by AmpC producer 21.1%. In our previous study, we have reported 51.78% and 17.85% were found to be ESBL and MBL producers, respectively from urinary isolates [24]. The coexistence of ESBL and MBL was reported in 5(0.83%) isolates where as the AmpC and MBL co-production was shown by 58(9.73%) isolates and the AmpC and ESBL co-production was shown in 42(7.04%) isolates. Study was done by Oberoi *et al*[14] reported the ESBL and MBL co-production in 8.79%, which was higher than our study, whereas AmpC and MBL co-production in 3.67%, which was lower than our study, and AmpC and ESBL co-production was shown in 6.59% isolates which corroborates with our study. Another study done by Arora*et al*[25] reported that AmpC and MBL production in 46.6% isolates and the ESBL and AmpC production in 3.3% isolates.

The antibiotic sensitivity pattern of the MDR gram negative bacilli revealed that the maximum sensitivity was seen for tigecyclene (92.91%) followed by imipenem (80.91%), nitrofurantoin (71.28%), amikacin (57.77%) and netilimicin (54.6%). The maximum resistance was seen against ceftriaxone (95.6) followed by ceftazidime (94.45%), amoxycillin/clavulanate (93.16%), norfloxacin (88.94%), ofloxacin (84.64%), cotrimoxazole (73.97%),ceftazidime/clavulanic acid (61.84%) and gentamicin (57.66%)(Table 2).This showed a significant correlation (*p* value <0.01).

MBL producing organisms showed highest rate of resistant against almost all antibiotics except tigecycline (6.93% resistant). In case of ESBL producers they shows highest rate of resistant to third generation like CAZ (100%) followed by AMC (87.79%), NX (85.4%), OF (83.78%), COT (73.73%) and CTR (71.02%). Lowest resistant was seen in inhibitor based compounds and carbapenem group of compound- CAC (3.19%), CFS (11.32%), PIT (18.33%) and IPM (1.61%). AmpC producers were highly resistant to

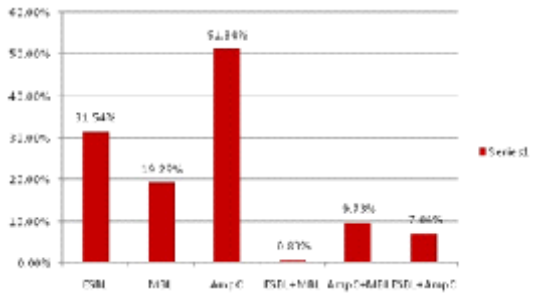

Fig. 4. Distribution of various beta-lactamases and co-producers

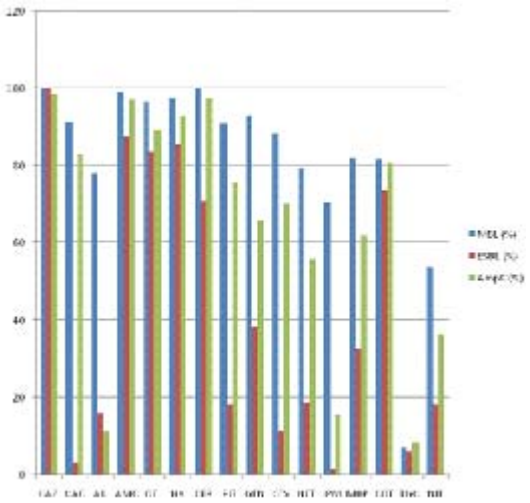

Fig. 5. Antibiotic resistant pattern of the isolated organisms producing different types of beta- lactamases.

CAZ (98.69%) followed by CTR (97.34%), AMC (97.15%), NX (93.09%), OF (89.47%), CAC (83%) and COT (80.83%). Lowest resistant was seen in TGC (8.57%) followed by AK (11.18%) and IPM (15.51%) (Fig. 5).

Imipenem is highly beta-lactamase stable and has an unusual property of causing a post antibiotic effect on Gram-negative bacteria. The resistance to imipenem was found to be 9% reported by Hassan S A *et al*[26]. But our study shows high resistance to imipenem i.e. 15%. In the present study Imipenem showed 98.39% sensitivity to all the ESBL producers, it is because ESBL and MBL co- production was found in the same isolates but studies from India which reported 100% sensitive to Imipenem showed by all ESBL producers [27,28]. But increase use of carbapenems leading to emergence of MBL-mediated resistant [24]. Among beta lactum/ beta lactum inhibitor drugs highest sensitivity was observed in CAC followed by CFS. Similar observations have been reported by Sharma M *et al*[28].

From this study we observed MBL producing organisms were found to be resistant against several antimicrobial agents used; only TGC can active against these organisms. Similar findings have been reported by Kumar E *et al*[29] that MBL producing strains were susceptible to potentially toxic antibiotics such as tigecycline, colistin and polymyxin- B.

One study was done by Dalela G *et al*[23] reported that AmpC producers are highly sensitive to imipenem (6.7%) but from our study we observed AmpC producers are 15.51% sensitive , which is higher due to the co-production of AmpC and MBL in the same isolates.

CONCLUSION

The present study indicates the high prevalence of β-lactamases among the multi drug resistant gram negative isolates which emphasizes the need for an early detection of β-lactamase producing organisms by simple screening methods and in turn can help in providing an appropriate antimicrobial therapy. Strict infection control practices, proper following of antibiotic policies and measures to restrict the indiscriminative use of cephalosporins and carbapenems in the hospital environment should be undertaken to minimize the emergence of this multiple β-lactamase producing pathogen whose spread would leave no other option to treat MDR Gram-negative bacterial infections.

ACKNOWLEDGEMENTS

Theauthors are grateful to Prof (Dr.) D.K Roy, Dean, IMS & SUM Hospital and Prof (Dr.) M.R nayak, President, Siksha 'O' Anusandhan University for providing necessary facility to carry out the laboratory analysis and encouraging throughout and Dr E. VenkataRao for his support in statistical analysis of the generated data.

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