Original Article

Prevalence of Extended Spectrum β-Lactamase, Metallo β-Lactamase, and AmpC β-Lactamase among Gram Negative Bacilli Recovered from Burn Wound Infection

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ABSTRACT

Introduction: Infections are the major cause of morbidity and mortality in burn patients. The ESBL, MBL and AmpC are the major mediators of antimicrobial resistance in gram negative bacteria. Early detection of ESBL, MBL and AmpC β-lactamases is crucial to establish appropriate antimicrobial therapy to prevent their inter-hospital dissemination. This study was done to find the prevalence of ESBL, MBL, and AmpC β-lactamases mediated resistance among gram negative isolates.

Methodology: All (160) gram negative bacteria were identified by standard microbiological procedure. Antibiotic susceptibility was performed according to CLSI guidelines. All gram negative isolates were screened for their ability to produce extended spectrum β-lactamases enzyme by using ceftazidime clavulanic acid combination disc method (combined disc diffusion test), AmpC β-lactamases enzyme using AmpC disc test and metallo β-lactamases enzyme by using double disc synergy test (DDST), combined EDTA disc test (CDT), and modified Hodge test (MHT).

Results: Out of 160 gram negative isolates, 113 (70.62%) were β-lactamases producer and 47 (29.37%) were non β-lactamases producer. There were 57 (35.62%) extended spectrum β-lactamases producer, 31(19.37%) metallo β-lactamases, and 61(38.12%) AmpC producer. Highest prevalence of ESBL (76.47%), MBL (35.29%), and AmpC lactamase (64.70%) enzyme producer was observed in *Acinetobacter* species. Many of the isolates produced more than one type of β-lactamase enzymes such as 25 (15.62%) ESBL+MBL, 52 (32.5%) ESBL + AmpC, 31 (19.37%) MBL+AmpC, and 25 (15.62%) ESBL+MBL+AmpC.

Conclusion: A prevalence of 35.62%, 19.37%, and 38.12% were observed for ESBL, MBL, and AmpC β-lactamases, respectively in this study. High prevalence of β-lactamases in burn wound infection emphasized the need for a continuous surveillance to detect resistant strain.

Key words: ESBL, MBL, AmpC, β-lactamase, antibiotic resistance.

INTRODUCTION

Infections are the major cause of morbidity and mortality in burn patients. In the current scenario of rising resistance to various antimicrobial agents, it becomes challenging for microbiologist to detect various mechanisms and formulate alternative antibiotic strategies for their judicious use. The extended spectrum β-lactamases (ESBL), AmpC β-lactamases (AmpC), and Metallo-β-lactamases (MBL) have gained importance as major mediators of antimicrobial resistance in gram negative organism.[1] Burns are the significant public health problem and divesting form of trauma around the world, leading to high morbidity, long term disability, and morality; especially in economically developing countries.[2]

Burnt areas are suitable sites for multiplication of organisms. The organisms which are usually associated with burn infections are aerobic pyogenic organisms like *Staphylococcus, Streptococcus pyogenes*, gram negative organisms like *E.coli, Klebsiella, Pseudomonas, Acinetobacter*, *Proteus* etc., anaerobic organisms like *Bacteroides fragilis*, *Peptostreptococcus, Propionibacterium* species, *Fusobacterium* species, and fungi like *Aspergillus* species, *Candida* species, and *mucormycosis*.[3] *Pseudomonas aeruginosa* and *Acinetobacter* species are also

important nosocomial pathogens that are being increasingly isolated from burn patients.[1]

Now a days, majority of bacteria causing burn infections are multidrug resistant. The major hurdles in treating burn infection are ESBL, AmpC, and MBL producing gram negative bacteria. ESBL are enzyme capable of hydrolysing and inactivating a wide variety of β-lactams including 3rd generation cephalosporins, Penicillin, and Aztreonam. These enzymes are the result of mutation of TEM-I and TEM-2 and SHV-1. These enzymes are plasmid mediated and confer resistance to other microbial also.[4]

AmpC β-lactamases are of major concern as they lead to resistance to a variety of β-lactams, β-lactam/β-lactamase inhibitor combinations, and monobactams. Two types of AmpC lactamases are plasmid mediated and chromosomal or inducible AmpC. The transferable AmpC gene products are commonly called plasmid mediated AmpC β-lactamases. Chromosomal AmpC enzymes are inducible by β-lactam antibiotics such as Cefoxitin and Imipenem, but they are poorly induced by 3rd or 4th generation cephalosporins.[5]

MBL are enzymes that lead to high level resistance to all β-lactams including Carbapenems except Aztreonam. MBL producer also show resistance to aminoglycosides and floroquiolones, further limiting the therapeutic options.[6]

The routine susceptibility tests fail to detect these resistance mechanisms; this may mislead treatment.[3] So the need of the hour is to know the prevalence of these resistant strains in a burn unit so as to formulate a policy of empirical therapy in high risk units where infections due to resistant organisms are much higher. It will help to avoid misuse of extended spectrum cephalosporin and carbapenems which are the mainstay of treatment in hospitalized patients.

The aim of the present study was to find the prevalence of ESBL, MBL, and AmpC mediated resistance among gram negative organisms. Early detection of ESBL, MBL, and AmpC is crucial to establish appropriate antimicrobial therapy to prevent their inter hospital dissemination.

METHODS

The study was conducted in the Department of Microbiology, Dr SN Medical College and associated group of hospitals, Jodhpur, Rajasthan on pus specimens received from the burn unit over a period of one year from May 2017 to May 2018. A total of 160 gram negative bacteria were identified by the standard microbiological tests.[1] The antimicrobial susceptibility testing of the isolates was determined by Kirby Bauer disc diffusion method according to CLSI guidelines 2017.[7] The reference strains, ESBL positive *Klebsiella pneumonia* ATCC-700607 and ESBL negative *Escheri-chia coli* ATCC25922 were included in the study.

β-Lactamases detection test (Screening test): All the gram negative bacteria isolates having reduced sensitivity to Ceftazidime (zone of inhibition of <22mm), Ceftriaxone (zone of inhibition of <25mm), or Cefotaxime as per CLSI guidelines were included in the study. These were further confirmed for ESBL production by using the phenotypic confirmatory disc diffusion test (PCDT) (Figure 1).

Extended spectrum β-Lactamases detection test (ESBL detection): Discs of Ceftazidime + Clavulanic acid (CFC) 30μg /10 μg and Ceftriaxone + Clavulanic acid (CAC) 30 μg /10 μg were used. Organism was considered as ESBL producer if there was a more than or equal to 5 mm increase in zone diameter for Ceftazidime and Ceftriaxone tested in combination with Clavulanic acid versus its zone when tested alone.[7]

Metallo β-Lactamases detection (MBL detection): This was done using phenotypic methods in which 10 μg meropenem antibiotic disc was selected to be used in all phenotypic methods.[8]

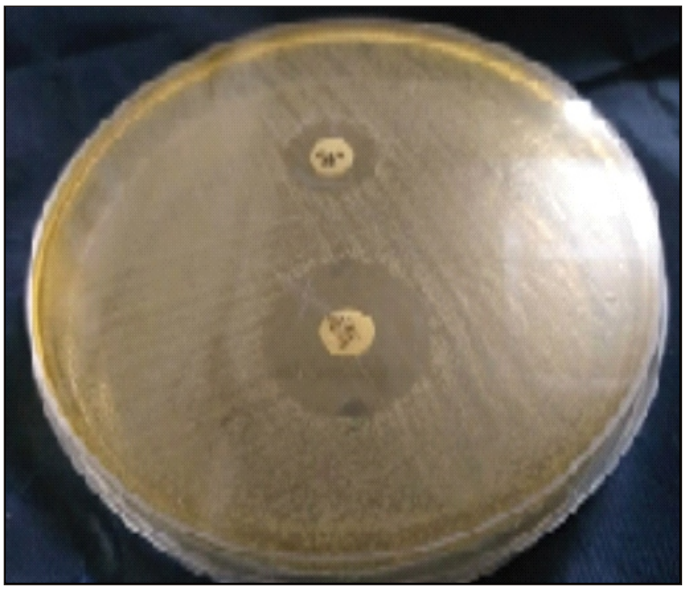


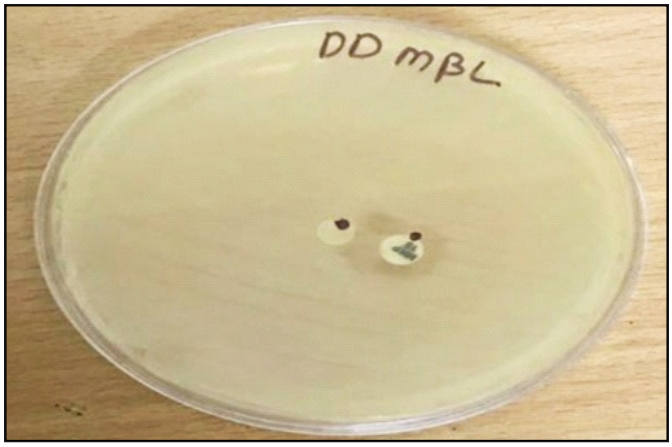
Figure 1: Extended spectrum beta lactamse (ESBL).

MBL screening test: Inoculum was prepared by suspending few colonies of test strain in 0.9% sterile saline and turbidity was adjusted to 0.5% Mc Farland turbidity tube. A lawn culture was made from the inoculum using a sterile cotton swab on the surface of MHA medium and Ceftazidime (30 µg) and Meropenem (10 µg) discs were applied with all sterile precaution. The plates were incubated for 18-24 hours at 37^{0}C. The isolates showing inhibition zone size of ≤16mm with Meropenem (10 µg) and Ceftazidime (30 µg) were identified as MBL producers and shortlisted for confirmation of MBL production.

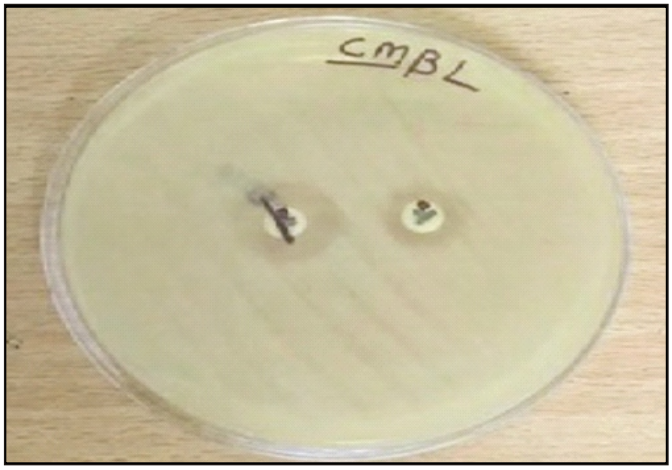
Double disk synergy test (DDST): This test was performed by inoculating the tested organism onto MHA plate as recommended by (CLSI).[8] Inoculum was prepared by suspending few colonies of test strain in 0.9% sterile saline and turbidity was adjusted to 0.5% Mc Farland turbidity tube. A 10 µg Meropenem disk and a blank filter paper disk 6 mm in diameter were placed 10 mm apart from edge to edge, then,10 microliter of 0.5 molar EDTA solution (0.5M EDTA) was prepared by dissolving 186.1g of disodium EDTA $2H_2O$ in 1000 ml of distilled water and adjusting it to pH 8.0 by using NaOH. The mixture was sterilized by autoclaving and applied onto the blank disk. After 18 hours of incubation at 37ºC, the presence of extension of zone towards the impregnated EDTA disk was interpreted as EDTA synergy test positive[9] (Figure 2 A).

Combined EDTA disk test (CMDT): An overnight broth culture of the test strain with an opacity adjusted 0.5 McFarland standards was used to inoculate a plate of Mueller-Hinton agar. 4 µl of the sterilized EDTA solution was added to 10 µg Meropenem disk, then the EDTA impregnated antibiotic disks were dried immediately in an incubator and stored at 20ºC in airtight vials without any desiccants until used. After drying of MHA plate, a 10 µg Meropenem disk and Meropenem disk combined with EDTA was placed 20 mm apart. After 18 hours incubation at 37ºC an increase in the zone size of at least 7 mm around the Meropenem combined EDTA impregnated disk compared to Meropenem disks alone was recorded as MBL producing strains[7] (Figure 2 B).

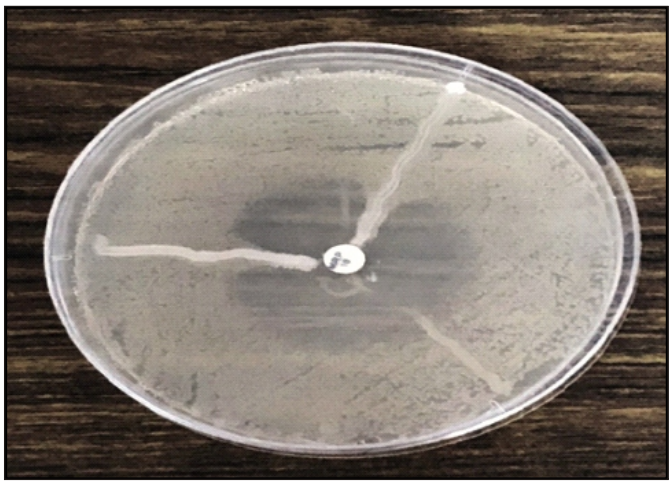
Modified Hodge test (MHT): All the Carbapenemases resistant gram negative bacteria were subjected to MHT test. An overnight culture suspension of pan sensitive strain of *E coli* was prepared by adding two to three isolated colonies of *E coli* strain to 5 ml of normal saline, and the suspension was further diluted by adding 1 ml of suspension to 4 ml of 0.85% NaCl and the mixture was



(A)



(B)



(C)

Figure 2: Metallo-β lactamase (A) Double disk synergy test, (B) Combined EDTA disk test, (C) Modified Hodge test (MHT).

adjusted at 0.5 McFarland's standard. This suspension was streaked across the entire plate of MHA plate. After drying 10 µg of Meropenem disk was placed at the center of the plate and up to 4 different isolates of gram negative bacteria were streaked linearly from the periphery of the plate into the direction of meropenem disk at the center. The test plate was then incubated for 18 hours at 37ºC. The presence of a cloverleaf shaped zone of inhibition around each tested strain is interpreted as carbapenemases producing strain[7] (Figure 2 C).

AmpC disc detection test: The test is based on use of TrisEDTA to permeabilize a bacterial cell and release β-lactamases into the external environment. AmpC discs were prepared in-house by applying 20 microliter of 1:1 mixture of saline and 100x Tris EDTA to sterile filter paper discs, allowing the discs to dry, and storing them at 2 to 8^0 C the surface of a Mueller- Hinton agar plate was inoculated with a lawn of Cefoxitin susceptible *E coli* ATCC 25922 according to the standard disc diffusion method. Immediately prior to use, AmpC disks were rehydrated with 20 microliter of saline and several colonies of each test organism were applied to a disc. A 30 microgram Cefoxitin disc was placed on the inoculated surface of the Mueller-Hinton agar. The inoculated AmpC disc was then placed almost touching the antibiotic disc with the inoculated disc face in contact with the agar surface. The plates were then incubated overnight at 35^0C in ambient air. After incubation, plates were examined for either an indentation or a flattening of zone of inhibition, indicating enzymatic inactivation of Cefoxitin (positive result), or the absence of a distortion, indicating no significant inactivation of Cefoxitin (negative result)[10] (Figure 3).

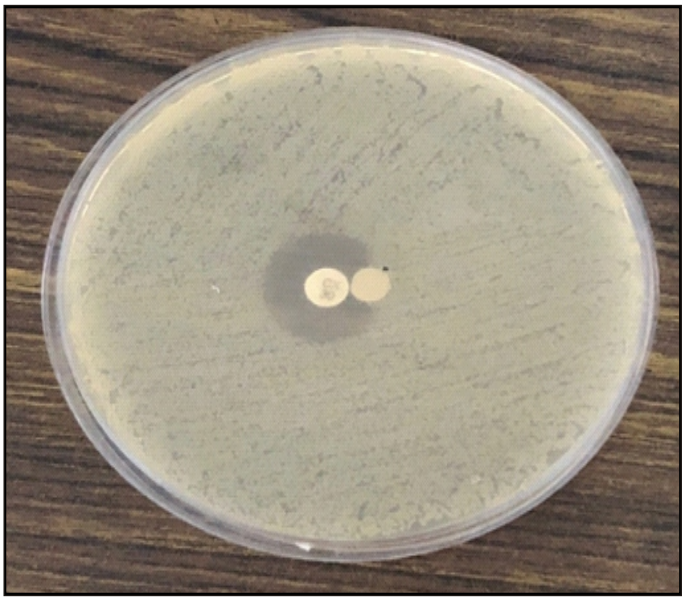


Figure 3: AmpC β-lactamase test.

RESULTS

A total of 160 gram negative organisms were isolated from burn wound infection and further processed as study group. The most common isolate was *Klebsiella* species (43.12%) followed by *E coli* (21.87%), *Pseudomonas aeruginosa* (18.12%), *Acinetobacter* species (10.62%), *Enterobacter cloacae* (3.12%), *Proteus vulgaris* (2.5%), and *Burkholderia cepacia* (0.625%).

Among the 160 gram negative isolates, the β lactamase production was detected in 70.62% isolates. β lactamase production was highest in *Acinetobacter* spp. (100%) followed by *Pseudomonas aeruginosa* (89.65%), *Enterobacter cloacae (80%), Proteus vulgaris (75%), Klebsiella* species (66.66%), and *Escherichia coli (*48.57%) (Table1).

The major ESBL producer was *Acinetobacter* species followed by *Proteus vulgaris*, *Klebsiella* species, and

.Table 1: Prevalence of β lactamase producing isolates in burn unit

Organism	Total no.	β lactamase (n)	%	Non β-lactamases (n)	%
E coli	35	17	48.57	18	51.42
Klebsiella species	69	46	66.66	23	33.33
Pseudomonas aeruginosa	29	26	89.65	3	10.34
Acinetobacter species	17	17	100	0	0
Proteus vulgaris	4	3	75	1	25
Burkholderia cepacia	1	0	0	1	100
Enterobacter cloacae	5	4	80	1	20
Total	160	113	70.62	47	29.37

Escherichia coli. The major MBL production was observed in *Acinetobacter* species, *Pseudomonas aeruginosa,* followed by *Proteus vulgaris and Klebsiella* species. The AmpC production was also maximally seen in *Acinetobacter* species followed by *Pseudomonas aeruginosa* (Table 2).

Various co-production of ESBL+MBL, ESBL+AmpC, MBL+AmpC, and ESBL+MBL+AmpC was observed as per table no 2. The co-production of ESBL+MBL+ AmpC was found to be maximum in *Acinetobacter* species (35.29%) followed by *Proteus vulgaris (25%), Klebsiella* species (20.28%), and *Pseudomonas aeruginosa* (13.79%) (Table 2)**.**

The most common pathogen isolated from burn wound infection in the present study was *Klebsiella* species followed by *E coli*, *Pseudomonas aeruginosa, Acinetobacter* species, *Enterobacter cloacae*, *Proteus vulgaris,* and *Burkholderia cepacia* which is in agreement with the study of Nirmala S et al.[11] However, in another study *Pseudomonas aeruginosa* 34.4% was found to be most common pathogen followed by *Acinetobacter* and *Klebsiella pneumoniae* (22.2%), *E coli* (8.8%) and *Citrobacter* species (3.3%).[9]

35.62% gram negative bacteria were identified as ESBL producer which is in agreement with finding of Bandekar et al[1] whereas S Shweta et al[12] found 39.8% ESBL producer. In the present study, *Acinetobacter* (76.47%) was the predominant ESBL producer followed by *Proteus vulgaris* (75%), *Klebsiella* (36.23%), and E coli (31.42%), but in another study *Klebsiella* (80.9%) was the predominant ESBL producer followed by *Pseudomonas* (51.28%) and *Acinetobacter* (13.3%).[12]

Detection of MBL production in multi drug resistant organism for burn infection has tremendous therapeutic consequences. Treatment option for such isolates are very limited i.e. Polymyxin B and Colistin. In the present study, 19.37% gram negative bacteria were identified as MBL producer which is nearer to the study conducted by Bandekar et al[1] where MBL production was seen in 15.6% of gram negative bacteria. S Shweta et al[12] observed that 16.88% gram negative bacteria were MBL producer. In the present study *Acinetobacter* species (35.29%) was predominant MBL producer followed by *Pseudomonas* (34.48%), *Proteus vulgaris* (25%), and *Klebsiella* (20.28%). This is in accordance with another study by Gupta V et al[9] who showed 25% MBL production by *Acinetobacter* species.

In the present study, 61/160 (38.12%) isolates were AmpC β-lactamases producer which is nearer to the study by Seven H Bakir et al[13] that showed 32.4% AmpC β-lactamases producer; 22.9% in a study conducted by Bandekar et al[1] in burn patients; and a study by Black JA et al[10] showed 31% AmpC β lactamases producer. In the present study, *Acinetobacter* species 11/17 (64.70%) was the most prevalent producer of AmpC β lactamase enzyme followed by *Pseudomonas aeruginosa* (41.37%), *Enterobacter cloacae* **(40%),** and *Klebsiella* species (36.26%).

The coexistence of different classes of β lactamases in a single bacterial isolate may pose diagnostic and treatment challenges. In the present study the co-expresion of ESBL +MBL was reported in 25/160 (15.62%) isolates, whereas the AmpC +ESBL coproduction was shown by 52 (32.5%) isolates and the MBL + AmpC co-production was shown in 31/160 (19.37%). This is in contrast to study done by Khanna et al[14] which showed 1.9%, 5.6%, and 0.6%, respectively. In the present study, the prevalence of various β-lactamases in the gram negative bacteria, which included Enterobacteriaceae and the non-fermenters was 113/160 (70.42%), which was alarmingly high and is concordant with the study of Oberoi L et al[16] who detected 70.69% β-lactamase producing gram negative bacilli. The study reported the ESBL and MBL coproduction in 8.79% isolates, AmpC and ESBL co-production in 6.59% and MBL and AmpC coproduction in 3.67% isolates.

The finding of multiple β-lactamases enzymes producing strains in the present study emphasizes the need for routine detection of these isolates by simple phenotypic methods in order to formulate the proper antibiotic policy. These pathogens are usually found to be multidrug resistant isolates. At this point we need to understand the importance of screening out these multiple enzymes co-existence in order to curb the development and dissemination these multiple drug resistant strains.[1]

CONCLUSION

The early detection of β lactamases producing isolates would be important for the reduction of mortality and morbidity rates for burn patients and also to avoid the

.Tables 2: Distribution of different β-lactamase among gram negative isolates

Organism	Total No.	ESBL N	ESBL %	MBL N	MBL %	AmpC N	AmpC %	ESBL + MBL N	ESBL + MBL %	ESBL+AmpC N	ESBL+AmpC %	MBL+AmpC N	MBL+AmpC %	ESBL+ MBL+AmpC N	ESBL+ MBL+AmpC %
E coli	35	11	31.42	0	0	10	28.57	0	0	10	28.57	0	0	0	0
Klebsiella Species	69	25	36.23	14	20.28	25	36.23	14	20.28	25	36.23	14	20.28	14	20.28
Pseudomonas aeruginosa	29	4	13.79	10	34.48	12	41.37	4	13.79	4	13.79	10	34.48	4	13.79
Acinetobacter species	17	13	76.47	6	35.29	11	64.7	6	35.29	11	64.7	6	35.29	6	35.29
Proteus vulgaris	4	3	75	1	25	1	25	1	25	1	25	1	25	1	25
Burkholderia cepacia	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enterobacter cloacae	5	1	20	0	0	2	40	0	0	1	20	0	0	0	0
Total	160	57	35.62	31	19.37	61	38.12	25	15.62	52	32.5	31	19.37	25	15.62

interhospital dissemination of such strain. To overcome the problem of emergence and the spread of multidrug resistant organisms, there should be routine identification of ESBL, AmpC, and MBL of clinical isolates especially if the patient's conditions merit so.

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