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Research Article

# OCCURRENCE OF VARIOUS BETA-LACTAMASE PRODUCING GRAM NEGATIVE BACILLI IN THE HOSPITAL EFFLUENT.

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**ABSTRACT**

**Aim:** This study was designed to understand the prevalence of antibiotic resistant Gram negative bacilli producing various beta lactamases in hospital effluents.

**Methodology:** A total of 121 Gram negative bacilli were isolated and identified by standard biochemical tests from 31 hospital effluent samples. Antibiotic susceptibility test for isolated bacteria was assessed by Kirby Bauer disc method. Detection of various beta lactamases (ESBL, AmpC and MBL) producing isolates were further carried out by different methods.

**Results:** From the isolated bacteria, *E.coli* was predominant (37.19%) followed by *Pseudomonas* spp. (22.31%), *Klebsiella* spp. (19.83%), Non-fermentative gram negative bacilli (NFGNB) (10.74%), *Enterobacter* spp. (6.61%) and others (3.30 %).

**Conclusion:** The present study suggests that although waste water treatment reduces the number of bacteria however, there is chance of antimicrobial resistant bacteria in the hospital effluent. Hospitals should take sanitary measures to prevent the spread of multi drug resistant bacteria including beta-lactamase producing strains transfer between hospital and the environment. The indiscriminate use of antibiotics in hospitals should be reduced.

**Keywords:** Antibiotic resistance, Beta lactamases, Gram negative bacilli, Hospital effluent.

## INTRODUCTION

Water constitutes a way of dissemination of antibiotic resistant organisms among human and animal populations, the route by which resistance genes are introduced in natural bacterial ecosystems. In such systems, non-pathogenic bacteria could serve as a reservoir of resistance genes and platforms [1]. Water can be a potential source of risk for the consumers, due to the presence of bacteria with not only virulence properties but also with antibiotic resistance and recontamination of water with these strains may influence the spread of pathogenic strains [2]. The presence of antibiotic resistant bacteria in water sources throughout the world has been documented [3-6]. Waste effluent from hospitals contains high numbers of resistant bacterial strains and antibiotic residues at a concentration able to inhibit the growth of susceptible bacteria [7]. Hospital waste effluent could increase the numbers of resistant bacteria in the recipient sewers by both mechanisms of introduction and selection for resistant bacteria. Although sewage treatment, reduces the number of bacteria in wastewater but the effluent generally contains large number of both resistant and susceptible bacteria [8]. Several studies have evaluated the microbiological content of hospital and household waste quantitatively and qualitatively and found that general hospital waste contains bacteria with pathogenic potentials for humans compared to household waste [9]. Studies on antibiotic residues in hospital effluent and in other environmental niches have been conducted mostly in high-income countries, while studies in low and middle income settings are few and sparsely distributed [10]. If the hospital effluents are not treated, concentrated forms of infectious agents and antibiotic resistant microbes are shed into communities resulting in water borne diseases such as cholera, typhoid fever, dysentery and gastroenteritis [11]. The pathogens present in the sewage wastes can reach out and contaminate ground water and surface water [12]. We conducted a prospective study to enlighten the possibility of environmental contamination by Gram-negative bacteria with beta lactamase production coming out from hospital drains in Chittoor district, Andhra Pradesh, Southern India.

## MATERIALS AND METHODS

### Sampling

A total of 31hospital effluent samples were collected from Chittoor district, during August 2012 to December 2012. Effluent samples were collected from the outlet of hospital sewers before the effluent flows into municipal sewage. The samples were collected in 250 ml sterile containers and transported to the laboratory in cold conditions.

### Sample Processing

A loopful of inoculum was inoculated on MacConkey agar plates and incubated at 370C for 18–24 hrs. Colonies were classified as lactose fermenters (LF) and non lactose fermenters (NLF) based on pigmentation [13]. Five colonies from each plate were selected with different colony morphologies by using five-colony method and further purified twice [14]. These were stab inoculated on semisolid media, incubated at 37°C for 24 hrs and stored at 4°C until use. Pure cultures were characterized by colony morphology and biochemical characteristics as described in Bergey´s Manual of Determinative Bacteriology [15].

### Antibiotic Sensitivity Test

The antibiotic resistance profiles of the selected isolates were then assessed by Kirby Bauer's disk diffusion method [16]. The peptone water was inoculated with test culture and incubated at 37 0C for overnight. After incubation, bacterial suspension was adjusted to 0.5 McFarland standards. The test organism was spread on Muller-Hinton agar plates by using swab. The following commercially available antibiotic discs (Hi-media, Mumbai) were placed on agar surface using sterile forceps -Amikacin (AMK) 30 µg; Amoxy-clavanic acid (AMX) 30 µg; Ampicillin (AMP)10µg; Cefoperazone/Sulbactum (CFS) 75/10 µg; Cefotaxime (CTX) 30 µg; Ciprofloxacin (CIP) 5 µg ; Co-trimoxazole (COT) 25 µg; Gentamicin

(GEN) 30 µg; Imipenem (IPM) 10 µg; Piperacillin /Tazobactam (PIT) 100/10 µg; Aztreonam (AZT) 30 µg; Cefepime (CPM) 30 µg; Cefoxitin (CX) 30 µg, Ceftazidime (CAZ) 30 µg, Chloramphenicol (C) 30 µg, Netilmicin (NET) 30 µg, Tetracycline (TE) 30 µg, Tegecyclin (TGC) 15 µg. The plates were incubated at 370C for 14-16 hrs. The zone of inhibition was measured in millimetres using a calliper. Strains were classified as resistant or susceptible according to the criteria recommended by CLSI guidelines [17].

**Detection of Extended spectrum β-lactamases**

The isolates showing resistance to 3rd generation cephalosporins (3GCs) were screened for the presence of ESBL and was confirmed by double disk-diffusion test (DDDT). A 0.5 McFarland of test culture was swabbed on Mueller Hinton Agar plates. Four discs namely Ceftazidime (CAZ-30µg), and Ceftazidime + Clavulanic acid (CAC-30/10 µg), Cefotaxime (CTX-30 µg), and Cefotaxime + Clavulanic acid (CEC-30/10 µg) were placed at a distance of 20 mm (centre to centre) on Muller-Hinton agar plates containing the inoculum. The plates were incubated for 24 hrs at 370C, a > 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested cephalosporin alone confirms ESBL producers (Figure1). K. pneumoniae ATCC 700603 (positive control) and E. coli ATCC 25922 (negative control) were used as quality control of ESBLs [17].

**Detection of AmpC β-lactamases**

Isolates showing resistance (<18mm) to 2nd generation cephalosporin (Cefoxitin 30µg) were screened for the presence of AmpC beta lactamases and the boronic acid double disk-diffusion test was performed to confirm the AmpC [18]. Disks containing boronic acid were prepared by dissolving 120 mg of phenylboronic acid (benzeneboronic acid: Sigma-Al-drich, Bangalore) in 3 ml of dimethyl sulfoxide (DMSO). Three millilitres of sterile distilled water was added to this solution. Twenty micro litres of the stock solution was dispensed onto cefoxitin disk containing 30 µg. Disks were allowed to dry for 30 min and used immediately or stored in airtight vials with desiccant at 40C. A 0.5 McFarland test inoculum was swabbed on Muller-Hinton agar plates. Two disks cefoxitin (30 µg) and cefoxitin + boronic acid (30/400µg) were placed at a distance of 20 mm (centre to centre) on Muller-Hinton agar plates and incubated for 24 hrs at 370 C. AmpC production was indicated if the diameter of the inhibition zone around the cefoxitin + boronic acid disk was > 5 mm greater than the diameter of the inhibition zone around the cefoxitin disk alone (Figure 2).

Modified three-dimensional enzyme extract method was also performed to confirm the AmpC producing isolates. Briefly, 10-15 mg of fresh overnight culture from the Mueller Hinton agar was transferred into a sterile micro centrifuge tube. Inoculum was suspended in peptone water and centrifuged at 3000rpm for 15 minutes. The pellet was subjected to repeated freeze-thawing for seven times and crude enzyme was extracted. Cefoxitin (30 µg) disk was placed at centre on Muller-Hinton agar plates containing 0.5 McFarland of E.coli ATCC 25922 culture. Linear slits (3 cm) were cut using sterile surgical blade, 3mm away from cefoxitin disk. A total of 20-30 µl of enzyme extract was loaded in the slit. The plates were kept upright for 5 to 10 minutes until the liquid dried and were incubated at 370C for 24 hrs. Distortion of inhibition zone was considered a strong positive, minimal distortion was considered as a weak positive and no distortion was considered as negative for the presence of AmpC beta-lactamase [19] (Figure 3).

**Detection of Metallo β-lactamases**

The isolates showing resistance to carbapenems (Imipenem 10µg) were screened for presence of MBL and was confirmed by the IMP-EDTA double disk-diffusion test (DDDT). Disk containing EDTA was prepared by dissolving 186.1 g of EDTA (Ethylene diamine tetra acetic acid) in 1000 ml of distilled water; pH was adjusted to 8.0 by using NaOH and sterilized by autoclaving. 10 µl from stock solution was added on imipenem disk (10 µg) and allowed to dry for 30 min and used immediately or stored in airtight vials with desiccant at 40C. A 0.5 McFarland test culture was swabbed on Muller-Hinton agar plates and disks Imipenem (10 µg) and Imipenem + EDTA (10 µg/750 µg) were placed at a distance of 20 mm (centre to centre) on agar surface and incubated for 24 hrs at 370C. MBL production was indicated if the diameter of the inhibition zone around the Imipenem+ EDTA disk was 5 mm greater than the diameter of the inhibition zone around the Imipenem disk alone [20]. (Figure 4)

**RESULTS AND DISCUSSION**

In our prospective study, we collected hospital effluent before it was released into corresponding municipal sewage. Gram-negative bacteria are of particular concern because these organisms are inherently resistant to many hydrophobic antibiotics [21-23]. Gram negative bacteria are the most common causes of hospital and community acquired infections [24]. So we made an attempt to isolate gram negative bacilli, in which E.coli 45 (37.19%) was predominant organisms followed by Pseudomonas sp. 27 (22.31%), Klebsiella sp. 24 (19.83%), NFGNB13 (10.74%), Enterobacter sp. 08 (6.61%) and others 04 (3.30%) respectively. The isolated strains are probable pathogens and they can cause many infectious diseases to humans. Further Gram negative bacilli strains were assayed for antibiotic sensitivity pattern. Antibiotics used in hospitals and private households are released into effluent and municipal sewage indicates a selection pressure on bacteria [25]. The tested strains showed high level resistance to aztreonam (65.38%), ceftazidime (57.69%), cefotaxime and co-trimoxazole (45.45%), 39.74% of isolates were resistant to cefepime and low level resistance was observed towards imipenem (16.66%) and the resistance to tegecyclin was nil (Table 1). This result showed that these organisms have been well exposed to the tested antibiotics and they have developed resistance mechanisms to them. Thus rendering above drugs ineffective as treatment of choice for infections caused by these pathogens. Bacterial resistance to antimicrobial agents has become a significant problem worldwide [26]. MDR strains are increasing in an alarming rate, most of them are either MBL or ESBL producers and spread of these beta lactamase strains may be happen by hospital nursing stuff to water source finally into waste effluent [27].

**Table 1: Antibiogram of 18 different antibiotics against the Gram-negative bacilli.**

| S. No | Antibiotics/ concentration* | Sensitive (%) | Intermediate (%) | Resistance (%) |
|---|---|---|---|---|
| 1 | Amikacin (30µg) (N¶= 121) | 88 (72.72) | 01 (0.82) | 32 (26.44) |
| 2 | Amoxy clav(20/10µg) (N=121) | 82 (67.76) | 09 (7.43) | 30 (24.79) |
| 3 | Ampicillin (10µg) (N=121 ) | 71 (58.67) | 07 (5.78) | 43 (35.53) |
| 4 | Aztreonam(30µg) (N=78) | 20 (25.64) | 07 (8.97) | 51 (65.38) |
| 5 | Cefoperazone-sulbactum (75/10µg) (N=121) | 81 (66.94) | 02 (1.65) | 38 (31.40) |
| 6 | Cefepime(30µg) (N=78) | 40 (51.28) | 07 (8.97) | 31 (39.74) |
| 7 | Cefotaxime (30 µg) (N=121) | 58 ( 47.93) | 8 (6.61) | 55(45.45) |
| 8 | Cefoxitin (30µg) (N=78) | 40 (51.28) | 10 (12.82) | 28 (35.89) |
| 9 | Ceftazidime (30 µg) (N=78) | 20 (25.64) | 13 (16.66) | 45 (57.69) |
| 10 | Chloramphenicol (30µg) (N=78) | 48 (61.53) | 10 (12.82) | 20 (25.64) |
| 11 | Ciprofloxacin (5 µg) (N=121) | 65 (53.71) | 04 (3.30) | 52 (42.97) |
| 12 | Co-trimoxazole (25µg) (N=121) | 66 (54.54) | 0 (0) | 55 (45.45) |
| 13 | Gentamicin (30µg) (N=121) | 84 (69.42) | 04 (3.30) | 33 (27.27) |

| | | | | |
|---|---|---|---|---|
| 14 | Imipenem (10µg) (N=78) | 63 (80.76) | 02 (2.56) | 13 (16.66) |
| 15 | Nitilmicin (30µg) (N=78) | 45 (57.69) | 10 (12.82) | 23 (29.48) |
| 16 | Piperacillin-tazobactum (100/10 µg) (N=121) | 80 (66.11) | 03 (2.47) | 38 (31.40) |
| 17 | Tegecyclin(15 µg) (N=78) | 78 (100) | 00(0) | 0(0) |
| 18 | Tetracycline (20µg) (N=78) | 50 (64.10) | 02 (2.56) | 26 (33.33) |

***Drug concentration in µg/ disc mentioned in parameters; ¶ Number of samples tested.**

**Table 2: ESBL Produced by different species of gram negative bacilli**

| Microorganisms | No. of 3rd GC resistant isolates (N=58) | ESBL by DDDT* (%) | |
|---|---|---|---|
| | | Positive (N=29) | Negative(N=29) |
| ***E.coli*** | 32 | 20 (62.50) | 12 (37.50) |
| ***Pseudomonas sp.,*** | 06 | 02 ( 33.33) | 04 (66.66) |
| ***Klebsiella sp.,*** | 07 | 04 (57.14) | 03 (42.85) |
| **NFGNB** | 07 | 01 (14.28) | 06 (85.71) |
| ***Enterobacter sp.,*** | 05 | 02 (40.00) | 03 (60.00) |
| ***Others*** | 01 | 00 (00.00) | 01 (100.00) |

*** DDDT-Double disk-diffusion test**

Beta-lactams are the most widely used antibiotics all over the world, and resistance to this antibiotic has resulted in a major clinical crisis [28]. The newer β-lactamases, including extended-spectrum β-lactamases (ESBLs), AmpC β-lactamases (AmpC) and metallo-β-lactamases (MBLs), have emerged worldwide as a cause of antimicrobial resistance in gram-negative bacteria (GNB). Genes for all these enzymes are often carried on plasmids, facilitating rapid spread between microorganisms [29]. The presence of ESBLs and AmpC β-lactamases in a single isolate reduces the effectiveness of the β-lactam-β-lactamase inhibitor combinations, while MBLs and AmpC β-lactamases confer resistance to carbapenems. Often these enzymes are co-expressed in the same isolate [30]. So we conducted a study to detect all the three β-lactamases in the strains of effluent sample. Among 32 third generation Cephalosporin resistant isolates, E.coli, 20 (62.50%) were positive and 12(37.50%) were negative for ESBL production and for remaining isolates tabulated (Table 2).

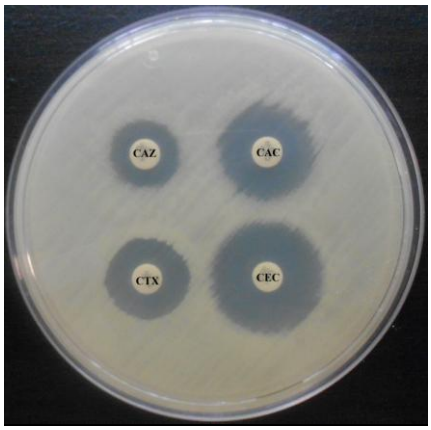


**Figure 1: ESBL detection by DDDT**

**CAZ: ceftazidime; CAC: ceftazidime +clavulanic acid; CTX: cefotaxime; CEC: cefotaxime + clavulanic acid.**

From the 26 isolates of E.coli, 11 (42.30%) were considered as strong positive, 04 (15.38%) intermediate and 11 (42.30) were considered as negative for AmpC production by Modified three dimensional method and only 07 (26.92%) were confirmed by AmpC production with boronic acid disk diffusion method. Of the 6 isolates of Pseudomonas sp. 01(16.66%) and 01 (16.66%) were considered as strong and intermediate positives respectively in the Modified three dimensional method and 02 (33.33%) were positive by boronic acid disk method and for the remaining isolates results were shown in Table 3.

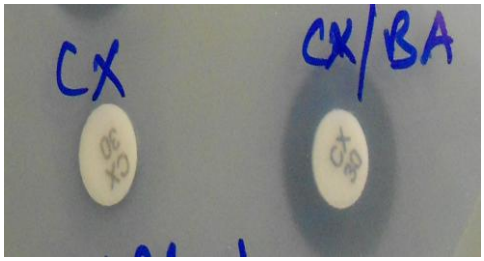


**Figure 2: detection of AmpC by BADDT**

**CX: cefoxitin; CX/BA: cefoxitin/ boronic acid**

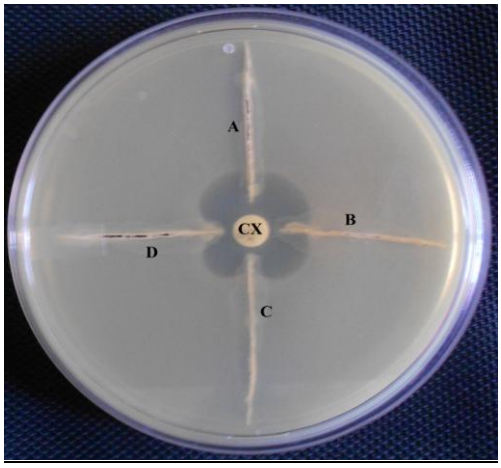


**Figure 3: Detection of AmpC by Modified three-dimensional enzyme extract method**

**CX: Cefoxitin; A: Negative (No distortion); B: Intermediate (minimal distortion); C: Positive (distortion); D: Positive control.**

Among the 13 imipenem resistant isolates (13) 100% were positive for the MBL production by the IMP+EDTA method, but only 6 (46.15%) positive by the Modified Hodge test and results for each organisms tabulated (Table 4).

**Table 4: Detection of metallo beta lactamases**

| Microorganisms (N*=13) | IE-DDDT ¶ (%) |
|---|---|

| | | Positive (N= 13) | Negative (N=0) |
|---|---|---|---|
| ***E.coli*** | 04 | 04 (100)* | 00 (00.00) |
| ***Pseudomonas sp.,*** | 01 | 01 (100) | 00 (00.00) |
| ***Klebsiella sp.,*** | 03 | 03 (100) | 00 (00.00) |
| **NFGNB** | 02 | 02 (100) | 00 (00.00) |
| ***Enterobacter sp.,*** | 03 | 03 (100) | 00 (00.00) |
| ***Others*** | 00 | 00 (100) | 00 (00.00) |

*** Number of organisms screened; ¶Imipenem - EDTA Double disk-diffusion**

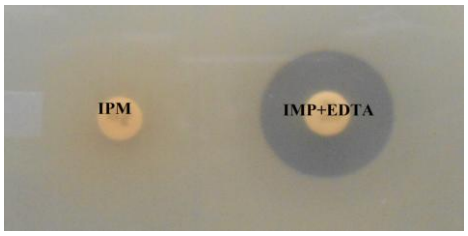


**Figure 4: Detection of MBL by IMP+EDTA diffusion test**

**IMP: Imipenem; IMP+EDTA: Imipenem+Ethylenediaminetetraacetic acid**

The indiscriminate use of antibiotics in medicine, veterinary and agriculture fields has lead to incidence and spread of antibiotic resistance among bacterial populations by gene transfer mechanism. Low concentrations of antibiotics in the environment may select for resistant bacteria [31]. These resistant bacteria from environments may be transmitted to humans, in whom they cause disease that cannot be treated by conventional antibiotics [32].

## CONCLUSION

Finally we concluded that the presence of gram negative bacilli from the hospital sewage is sensible to high and also multi drug resistance was also reported. The isolated strains were showing various beta lactamase resistance mechanisms and this drug resistant strains may cause infections to the healthy living things. To minimize the spread of drug resistant isolates from hospital to environment is crucial, so good safety sterilization methods to be adopted before release of waste materials to the environment or sewage.

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