

## Frequency of Class B Carbapenemases (M $\beta$ L) in enterobacteriaceae

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### Abstract

**Objective:** To determine the frequency of Metallo- $\beta$ -lactamase producing Enterobacteriaceae species.

**Method:** The descriptive cross-sectional study was carried out from January to December 2011 in the Department of Microbiology, Basic Medical Sciences Institute, Jinnah Postgraduate Medical Centre, Karachi. A total of 500 specimens were initially collected. The culture positive samples were analysed for further identification, and antimicrobial sensitivity was done according to Clinical and Laboratory Standards Institute guidelines 2009. The Carbapenemases Producing Enterobacteriaceae strains were screened by the Modified Hodge Test, and Metallo- $\beta$ -Lactamase production was confirmed by the EDTA combined disc test.

**Result:** From amongst 402 organisms detected, 200 (49.75%) were Enterobacteriaceae, while *Escherichia coli* was the leading pathogen (65%). Only 6% were identified as Carbapenemases Producing Enterobacteriaceae and 9 (75%) of them were Metallo- $\beta$ -Lactamase producing strains, while 3 (25%) contained other enzymes.

**Conclusion:** Metallo- $\beta$ -Lactamase producing Enterobacteriaceae species are causing problems in tertiary care hospitals.

**Keyword:** Carbapenem, Enterobacteriaceae, Metallobetalactamase. (JPMA 64: 519; 2014)

### Introduction

Carbapenemases are  $\beta$ -lactamases that significantly hydrolyse at least Imipenem and/or Meropenem (Carbapenem) together with other penicillin or cephalosporin antibiotics.<sup>1</sup>

In functional classification scheme of Bush-Jacoby, carbapenemases are found in Group 2f, 3 and 2df, whereas in molecular (Ambler) classification scheme, these are found in classes A, B (sub-group B1 and B2) and D.

Metallo- $\beta$ -Lactamases (M $\beta$ L) constitute the molecular class B (sub-group B1 and B2) of Ambler classification and Group 3 according to the Bush-Jacoby-Medeiros functional classification. Most are broad-spectrum and hydrolyse a variety of penicillins and cephalosporins<sup>2</sup> but not Aztreonam.<sup>1</sup>

Class B  $\beta$ -lactamases are metalloprotein of about 25 kDa molecular weight, which require one or more divalent cations (cofactors), usually zinc, for their activity. These were initially thought to be clinically unimportant, and different from those  $\beta$ -lactamases having serine at the active site.<sup>3</sup> These are inhibited by metal chelators [ethylenediaminetetraacetic (EDTA), cupric chloride

(CuCl<sub>2</sub>), ferrous chloride (FeCl<sub>2</sub>), o-phenanthroline], and thiol compounds that are competitive inhibitors (2-omega-phenylalkyl-3-mercaptopropionic acid and N-2-mercaptoethyl-2-phenylacetamide). The M $\beta$ Ls are not inhibited by clavulanic acid, sulbactam and tazobactam.<sup>4,5</sup>

Most common M $\beta$ L families are the Verona integron-encoded metallo- $\beta$ -lactamases (VIM), Active on Imipenem (IMP), German Imipenemase (GIM), and Sao Paulo metallo- $\beta$ -lactamases (SPM), which are located within a variety of integrons, where they are incorporated as gene cassettes. Their presence on these mobile genetic elements is a greater clinical threat than chromosomally encoded enzymes, as these genes have the ability to transfer from one bacterium to another intergenerally.<sup>6</sup>

Enterobacteriaceae contain more than 100 species that are inhabitants of human and animal intestine. Pathogenic species cause pneumonia, cystitis, pyelonephritis, septicaemia, peritonitis, meningitis and device-associated infections.<sup>7,8</sup> These are estimated to be responsible for approximately 100,000 deaths each year in the US alone, and account for about half of all the clinically significant bacteria isolated by hospital laboratories.<sup>7</sup> Enterobacteriaceae members have the tendency to spread easily between humans (hand carriage, contaminated food and water) and to acquire genetic material through horizontal gene transfer, mediated by plasmids and transposons.<sup>8</sup>

Unfortunately, the reliability of the "big guns"

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(Meropenem, Imipenem, Doripenem, Ertapenem) is compromised by a number of  $\beta$ -lactamases. In the past several years, it appears that the "target" met the challenge and new pathogenic strains carrying carbapenemase genes have been documented from India, Pakistan, Bangladesh and other countries.<sup>5</sup> A study from Italy, during 2007 and 2008, showed 24.1% M $\beta$ Ls (VIM-1) producing strains of *Enterobacter*.<sup>9</sup> At Barnes-Jewish Hospital, 2.9% carbapenemases producing Enterobacteriaceae (CPE) were recovered from blood culture isolates.<sup>10</sup>

Screening of CPE strain is based on detection of reduced susceptibility to carbapenems. The Clinical and Laboratory Standards Institute (CLSI) in 2009 issued recommendations for phenotypic screening of carbapenemase producers among species of Enterobacteriaceae. It has been shown that the sensitivity of the Imipenem zone diameter screening breakpoint  $\leq 21$ mm was 100%.<sup>11</sup>

Phenotypic confirmation is performed by using one or two methods, the modified Hodge test (MHT) and the carbapenemase inhibition tests. The MHT is used for the detection of diffusible carbapenemases, and the inhibition tests are used to distinguish between the different classes of carbapenemases.<sup>11</sup> The MHT uses *Escherichia coli* ATCC 25922 and 10  $\mu$ g Imipenem disc instead of *Staphylococcus aureus* ATCC 25923 and a 10 $\mu$ g penicillin disc, respectively.<sup>12</sup>

For detection of M $\beta$ Ls, EDTA or dipicolinic acid (DA) can be used as an inhibitor. It is recommended that these tests be carried out using either combination disks or an E test strip that contains both a carbapenem and an inhibitor.<sup>11</sup>

## Material and Method

The descriptive cross-sectional study was conducted from January to December 2011 at the Department of Microbiology, Basic Medical Sciences Institute (BMSI), Jinnah Postgraduate Medical Centre (JPMC), Karachi. Prior approval was in hand from the institutional review committee and informed written consent was obtained from each patient/guardian.

The sample size for frequency in a population was calculated by open EPI calculator, and the confidence level was set at 80%. On the basis of the reference study,<sup>13</sup> the calculated sample size was 495.

Irrespective of age or gender 500 culture samples were collected by simple random method from the patients showing signs and symptoms of bacterial infection, and isolates were screened for primary selection according to

CLSI criteria. The clinical samples were collected from the patients admitted in various units of JPMC, a tertiary care hospital with bed strength of 1500, belonging to Pakistan's Federal Ministry of Health. After taking necessary aseptic measures, the samples were collected and necessary data was filled accordingly.

The 500 specimens were divided into four groups: (a) 200 samples of urine were taken from urinary tract infection (UTI) suspected patients; (b) 150 samples of pus were taken from wounds, irrespective of site; (c) 100 samples were taken from the respiratory tract (tracheal aspirates and sputum); and (d) 50 samples of blood were taken from suspected patients of septicemia.

The pus and respiratory secretions were inoculated on Blood agar and MacConkey agar. The respiratory secretions were additionally inoculated on Chocolate agar and Sabouraud Dextrose Agar (SDA). The urine was inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar. These plates were incubated aerobically at  $35 \pm 2^\circ\text{C}$  for 24 hours. Inoculated blood culture bottles were incubated for 24 to 48 hours (and even up to one week where needed) at  $37^\circ\text{C}$  and then examined for turbidity indicative of positive growth. After overnight incubation, established microbiological methods, which include colonial morphology, Gram's staining and biochemical characteristics, were used for identification.<sup>12</sup>

Phenotypically  $\beta$ -lactamase-negative *E. coli* ATCC 25922 was used as negative control.

Overall, 200 isolates of family Enterobacteriaceae *K. pneumoniae*, *Escherichia coli*, *Enterobacter* spp., *Proteus mirabilis*, *Proteus vulgaris*, *Citrobacter freundii*, *Providencia* spp. and *Serratia* spp. were preceded.

Antimicrobial susceptibility testing of the isolated organisms was performed by the disk diffusion technique according to the recommendations of the CLSI.<sup>14</sup>

According to CLSI<sup>14</sup> guidelines, isolates showing following inhibition zone size (Meropenem and/or Imipenem  $\leq 21$ mm) of antimicrobial agents were identified as carbapenem resistant strains. For the confirmation of carbapenemase production, MHT was done<sup>12</sup> (Figure-1).

The 0.1M EDTA stock solution was prepared by dissolving 292.24g of anhydrous EDTA (Sigma-Aldrich, Steinheim, Germany) in 10ml distilled water and its pH was adjusted at 8 by using 10% sodium hydroxide (NaOH). The mixture was then sterilised by autoclaving at  $121^\circ\text{C}$  and 15 lb/inch<sup>2</sup> pressure for 15 minutes.

The combined-disc test (Imipenem/Meropenem 10 $\mu$ g +

EDTA 292µg) was performed as recommended previously with few modifications.<sup>15</sup> Test strains were inoculated on to Muller Hilton Agar (MHA) plates (OXOID, UK) using a 1:10 dilution of 0.5 McFarland standard inoculum with the help of sterile cotton swab. Two Imipenem (IPM 10µg) and two Meropenem discs (MEM 10µg) were placed on a single MHA plate (190mm) 25mm apart (centre to centre). Immediately from the stock solution of EDTA (0.1M) 10µl (containing 292µg of EDTA) was dispensed onto one of each disc. The plates were incubated overnight at 37°C. The results were recorded as an increase in zone of diameter of ≥5mm around the Imipenem and Meropenem discs containing EDTA which were compared to that of Imipenem and Meropenem discs without EDTA and were considered positive for MβL.

Data was analysed using SPSS version 16. The data was initially prepared on Microsoft Excel and then imported to SPSS. Frequencies were worked out and expressed along with percentages. The t test was applied to determine significant difference in antibiotic resistance between CPE and non-CPE bacteria.

**Results**

Of the 500 samples collected, 332 (66.4%) were culture positive 402 micro-organisms were isolated. The isolates were Enterobacteriaceae 200(49.75%), other Gram-negative rods 76(18.9%), Gram-positive cocci 116(28.86%), and yeast 10(2.49%) (Figure-1). Escherichia coli was the leading pathogen 131(65.5%) among the 200 Enterobacteriaceae.

Out of the 200 isolates, 12(6%) were MHT positive and these were E. coli 6(4.58%) out of 131, K.pneumoniae 4(9.52%) out of 42, E. cloacae 01(9%) out of 11 and P. mirabilis 01(11%) out of 9 (Table-1).

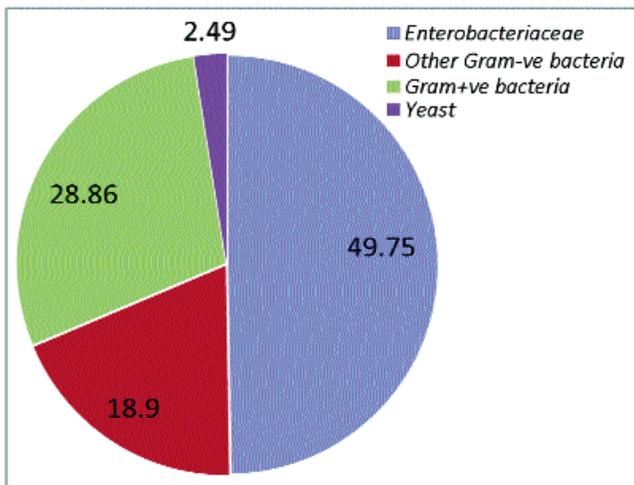


Figure-1: Distribution of different groups of micro-organisms (n=402).

Table-1: Frequency of carbapenemases producing Enterobacteriaceae species (n=200).

Organisms	Number	Per cent
Escherichia coli (131)	6	4.58
K. pneumoniae (42)	4	9.52
E. cloacae (11)	01	9.0
Proteus mirabilis (9)	01	11.0

Table-2: Distribution of confirmed carbapenemases producing enterobacteriaceae (n=11).

Isolate	Specimen				Total
	Urine	Pus	Respiratory secretions	Blood	
Escherichia coli	02	02	02	00	06
Klebsiella pneumoniae	01	01	02	00	04
Enterobacter cloacae	00	00	00	01	01
Total (%)	03(27.27%)	03(27.27%)	04(36.37%)	01(9.0%)	11(100)

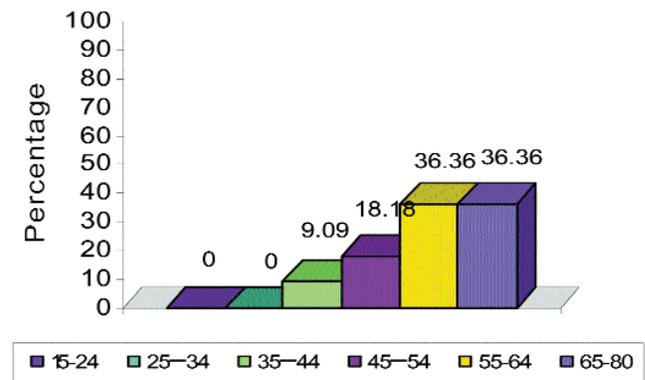
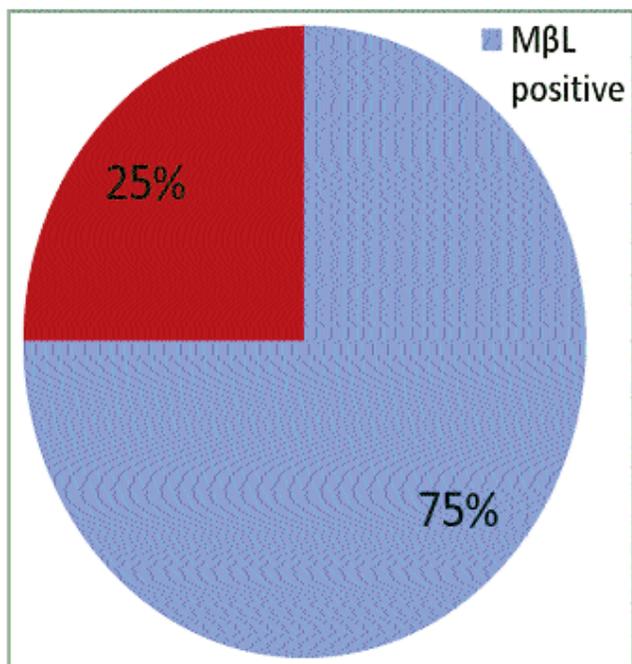


Figure-2: Distribution of confirmed carbapenemases producing enterobacteriaceae according to age.

In accordance with the nature of specimens, overall percentage of CPE was 04(36.37%), 03(27.27%), 03 (27.27%), and 01(9%) for respiratory secretions, urine, pus and blood respectively (Table-2).

CPE were frequently seen in age groups 55-64 years and 65-80 years, 4 (36.36%) each, while the 45-54 age group had 2 (18.18%) (Figure-2).

According to confirmatory tests, out of the total 12 CPE cases, 9(75%) were positive for class B MβL and 03(25%) were negative Figure-3. Escherichia coli were 5(83.34%) positive and 01(16.66%) negative, K. pneumonia 03(75%) positive and 01(25%) negative, E. cloacae 01 (100%) positive for MβL. The P. mirabilis was negative for MβL, indicating that they had some other mechanisms. MHT was the most sensitive phenotypic tool for the



**Figure-3:** Distribution of carbapenemases producing enterobacteriaceae Isolates according to Metallo-β-lactamase confirmatory test (n=12).

**Table-3:** Frequency of confirmed class B Carbapenemases producing Enterobacteriaceae according to confirmatory tests (n=12).

Organisms	Class B (Metallo-β-lactamase)	
	Positive (%)	Negative (%)
Escherichia coli (6)	5 (83.34)	1 (16.66)
K. pneumoniae (4)	3 (75)	1 (25)
E. cloacae (1)	01 (100)	00
Proteus mirabilis (1)	0	1 (100)
Total	9 (75)	3 (25)

confirmation of carbapenemase producing bacteria (Table-3). The sensitivity pattern of non-CPE and CPE to most common antibiotics was highly significant.

### Discussion

The increasing frequency of carbapenem resistant Enterobacteriaceae (CRE) is a major concern to human health, as this significantly limits treatment options for life-threatening infections. Therefore detailed understanding of the molecular basis and epidemiology of carbapenem resistance is needed. The current study was designed to assess the prevalence of carbapenem resistant Enterobacteriaceae.

The prevalence of CRE has got higher from 0.4% (2007) to 9% (2011) and in our study it was 6%. A study from India

reported 27% MβL producing Enterobacteriaceae strains.<sup>16</sup> This shows that carbapenem resistance varies from hospital to hospital and region to region, and that carbapenem resistance is emerging in Pakistan and needs more attention from all concerned.

Male-to-female ratio in our study was 54.55% and 45.5% respectively which is in contrast to a study from Germany which reported 33.33% and 66.66% respectively. The mean age of patients was 59±15 years, which was similar to other studies.<sup>17-19</sup>

In the present study Enterobacteriaceae were 49.75% of the total clinical isolates. Out of these 12 (6.0%) were CRE. The frequency of CRE is lower in the present study compared to the other countries but higher in comparison to the local studies. This may be due to various factors. Firstly, carbapenem is not irrationally used in the country because of its cost. In Pakistan cephalosporins are the first choice for empirical therapy and there is limited use of carbapenem group. Secondly, carbapenem resistance in Pseudomonas is lower in our country (14%) compared to other countries.<sup>20</sup> Lastly, limited work has been done on carbapenem resistance in Pakistan regarding Enterobacteriaceae.

The sensitivity and specificity of MHT was similar as reported earlier by a study that showed all the MHT positive isolates, except one being carbapenemase producing.<sup>21</sup> Out of a total of 12 CRE, 75% were MβL producing strains, 25% were non-MβL producing strains that could be due to Klebsiella pneumonia carbapenemase (KPC), Ampicillin-C gene (AmpC) hyperproduction, porin loss or class D carbapenemases not diagnosed due to the limited resources. These results show that majority of CRE were MβL (75%) producers, while others were less frequent, which is similar to some other Asian studies.<sup>22,23</sup> Escherichia coli, Klebsiella pneumoniae and Enterobacter cloacae were the main MβL producing species, with similar findings from India.<sup>17</sup> The frequency of CPE was 27.27%, 27.27%, 36.37% and 9.0% in urine, pus, respiratory secretions and blood respectively. Similar pattern of CPE was isolated in an earlier study.<sup>22</sup>

Colistin and Tigecycline are the core of therapy for CRE and last available active antimicrobial agents. In our study CPE were 100% sensitive to Colistin which is in strong agreement with the findings from Greece,<sup>18</sup> New York<sup>19</sup> and northeastern United States (US)<sup>24</sup> where it was reported that in vitro susceptibility to Polymyxins among clinical KPC-producing isolates ranged from 90-100%. Carbapenemase-producing isolates are resistant to all antibiotics except Colistin and Tigecycline as reported

from India,<sup>17</sup> which is also in support of our findings.

## Conclusion

There is an alarming increase of infections caused by CRE and most of them are MβL producing.

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