STUDY OF CARBAPENEM RESISTANCE IN NON-FERMENTING GRAM NEGATIVE ISOLATES
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HOW TO CITE THIS ARTICLE:
Syed Haroon Hussain, Sukesh Kumar B. Y, Kondal Rao. “Study of Carbapenem Resistance in Non-Fermenting Gram Negative Isolates”. Journal of Evidence based Medicine and Healthcare; Volume 2, Issue 34, August 24, 2015; Page: 5114-5123, DOI: 10.18410/jebmh/2015/713

ABSTRACT: BACKGROUND: The introduction of Carbapenems (Meropenem, Imipenem) into clinical practice represented a major advance in the treatment of serious bacterial infections caused by beta-lactam resistant bacteria. However, carbapenem resistance has been frequently observed in non-fermenting bacilli like Pseudomonas aeruginosa and Acinetobacter species, probably due to lack of drug penetration and/or carbapenem-hydrolyzing β-lactamases.

OBJECTIVES: The objectives of the present study are to isolate nonfermenters from various clinical samples, determine their antibiogram and to detect any carbapenem resistance by the application of routine phenotypic methods. MATERIALS AND METHODS: A total of 132 samples were collected from 132 patients samples includes pus, urine, sputum, blood, endotracheal tubes, intravenous cannula isolated non-fermenters from various clinical samples and identifying the organism their antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method. Metallo-β-lactamase (MBL) detection was done by the Imipenem-EDTA Combined Disc Method and the Imipenem-EDTA Double Disc Synergy Method. RESULTS: Among 132 isolates, Pseudomonas aeruginosa constituted 115 of the total isolates, Imipenem resistance in the present study in Ps. aeruginosa was 41.73%. Among the 57 isolates resistant to imipenem, 50(87.71%) showed a ≥7mm zone enhancement by Imipenem-EDTA Combined Disc Method, 35 (61.4%) showed zone enhancement by the Imipenem-EDTA Double Disc Synergy Method. CONCLUSION: Among the two tests used in the present study, the Imipenem-EDTA Combined Disc Method was found to be more sensitive (p<0.05). MBL E test can be used to increase the sensitivity of detection

KEYWORDS: Imipenem, Carbapenem, Combined disc test.

INTRODUCTION: Carbapenems belong to the class of beta-lactam antibiotics which have a broad spectrum anti-bacterial activity. Their structure renders them highly resistant to beta-lactamases. This group originally developed from theinamycin, a naturally derived product from Streptomyces cattleya. Imipenem, Meropenem and Ertapenem are members of this class. Imipenem is produced from a compound produced by Streptomyces cattleya, Meropenem is derived from theinamycin and Ertapenem is a semi synthetic derivative.

Carbapenems have a broad spectrum with excellent activity against organisms which are cephalosporin resistant by virtue of expression of chromosomal or plasmid Extended Spectrum β-Lactamases. They are especially important in their activity against nosocomial infections caused by non-fermenters. Carbapenems are indicated in a wide variety of nosocomial infections like urinary tract infections, intra-abdominal, gynaecological, skin and soft tissue, bone and joint and lower respiratory tract infections.
The introduction of Carbapenems (Meropenem, Imipenem) into clinical practice represented a major advance in the treatment of serious bacterial infections caused by beta-lactam resistant bacteria. However, carbapenem resistance has been frequently observed in non-fermenting bacilli like Pseudomonas aeruginosa and Acinetobacter species, probably due to lack of drug penetration and/or carbapenem-hydrolyzing β-lactamases.2

Critically ill patients receiving lifesaving therapy in Intensive Care Units (ICU) often require broad spectrum antibiotics, insertion of various indwelling devices, nutritional support and immunosuppressive therapy.3 Though ICU patients occupy only 5-10% of hospital beds, they contribute to over 20% of hospital acquired infections. It is in this modern context of intensive care with various invasive interventions breaking the natural anatomical and physiological barriers, the importance of non-fermenters as nosocomial pathogens increased considerably.4

The term “Non-fermenters” refers to a group of aerobic, non-spore forming Gram negative bacilli that are either incapable of utilizing carbohydrate as a source of energy or degrade them via “oxidative” rather than fermentative pathways.5 In addition, they possess cytochrome oxidase activity and fail to grow or grow poorly on MacConkey.6

Although frequently considered to be commensals or contaminants, the pathogenic potential of non-fermenters has been established beyond doubt by their recurrent isolation from clinical samples and their association with human disease.7 They also have innate resistance to many antibiotics. The common forms of resistance are inability of the drug’s penetration into the cell, inactivation or modification of the antibiotic and production of Amp-C and metallo β-lactamases.8

Metallo β-lactamases (MBL) production is one of the mechanisms of carbapenem resistance in non-fermenters. So, rapid detection of MBL production is necessary to modify treatment and initiate efficient infection control which prevents the dissemination of plasmid mediated MBL genes to other species of gram negative bacilli.9 Treatment alternatives in such cases are unavailable, if available expensive/toxic and the outcome is generally poor.10

As there were no pre-existing studies on Carbapenem resistance pertaining to non-fermenters in this region, the present study is undertaken inpatient admissions range from 350 to 400 per day. The incidence of hospital acquired infections during the study period is 12%.

The objectives of the present study are to isolate non-fermenters from various clinical samples, determine their antibiogram and to detect any carbapenem resistance by the application of routine phenotypic methods.

MATERIALS AND METHODS: The present study was conducted in the Department of Microbiology for a period was from June 2011 to November 2011. After obtaining approval from the Institutional Ethics Committee, samples were collected from in-patients in different wards of the hospital. Written informed consent from these inpatients was obtained.

Inclusion Criteria: All patients admitted to ICU developing infective symptoms were included.

The patients were closely monitored for any evidence of clinical manifestations suggestive of infections viz, fever, leukocytosis, leucopenia, purulent endotracheal secretions, copious wound discharge, evidence of radiological infiltrates in chest X-ray etc. Non-fermenters isolated from two consecutive samples from the same patient.
Exclusion Criteria: Patients with ≤24 hours stay in the hospital after admission, admitted in the ICU following more than 24hrs of stay in neighboring hospitals, Non-fermenters not isolated from two consecutive samples from same patient were excluded from the study.

Appropriate clinical sample based on the clinical manifestations like pus, sputum, blood, urine, endotracheal aspirates were collected taking adequate aseptic precautions. A total of 132 samples were collected from 132 patients samples includes – pus (55), urine (32), sputum (21), blood (19), endotracheal tubes (4), intravenous cannula (1).

Basic approach for identification of non-fermenters is that all non-lactose fermenting colonies on MacConkey with smooth colonies on blood agar plate were considered significant. Tiny colonies on blood agar plate with poor or no growth on MacConkey were considered significant.

These isolates were then subjected to Gram stain and Inoculated in Triple sugar iron agar (TSI). All GNB which lack acid production in TSI manifested as alkaline slant and an alkaline deep reaction K/K or alkaline slant and no change reaction K/- considered as NFGNB and preserved for further identification tests.

Once ten isolates of NFGNB were isolated, they were subjected to various biochemical tests for speciation. The tested for Motility, Oxidase, Citrate Utilization, Urease Production, Lysine, Arginine, Ornithine decarboxylase test (LAO), Hugh-Leifson Oxidation-Fermentation test, Nitrate Reduction and Pigment Production. The pathogenicity and significance of all the NFs isolated from the patients was confirmed by repeat isolation of the organism by obtaining a second sample from the same patient.

After isolating and identifying the organism their antimicrobial susceptibility testing was performed by Kirby-Bauer disc diffusion method. The test was performed on Mueller-Hinton agar (MHA) using commercially available discs. Turbidity of actively growing broth culture was adjusted so that it is optically comparable to that of 0.5 McFarland standards (CLSI, 2014).

A sterile cotton swab was dipped into the suspension and was pressed firmly on the side wall of test tube above the fluid level to drain the excess fluid. The dried surface of MHA was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times rotating the plate approximately 60 degrees each time to ensure an even distribution of inoculum.

The predetermined battery of antimicrobial discs which included Amikacin (30µg), Gentamycin (10µg), Ciprofloxacin (5µg), Piperacillin (100µg), Cefotaxime (30µg), Ceftazidime (30 µg), Cefepime (30µg), Polymyxin-B (300 units), Piperacillin-Tazobactam (100/10µg), Aztreonam (30µg) and Imipenem (10µg) was dispensed onto the surface of inoculated agar plate. The discs were evenly distributed so that they were not closer than 24mm from center to center. The plates were incubated aerobically at 37°C for 16 to 18 hours.

After this period, each plate was examined and if the plate was satisfactorily streaked and the inoculum was correct, the resulting zones of inhibition will be uniformly circular and there will be confluent lawn of growth. The diameter of zones of inhibition was measured including the diameter of disc. The zone size were interpreted by referring to CLSI standards and reported as susceptible, intermediate or resistant.
MIC of all the isolates was determined by the E test Imipenem and interpreted by referring to the CLSI standards. Controls used with each batch- Pseudomonas aeruginosa ATCC 27853.

RESULTS:

| Specimen     | P. aeruginosa | P. fluorescens | A. baumanii | A. Iwoffi | Al. faecalis |
|--------------|---------------|----------------|-------------|-----------|-------------|
| Pus          | 46            | 5              | 2           | -         | 2           |
| Urine        | 28            | 2              | 1           | 1         | -           |
| Sputum       | 19            | 1              | -           | -         | 1           |
| Blood        | 17            | -              | 1           | 1         | -           |
| Endotracheal tube | 4  | -           | -           | -         | -           |
| IV Cannula   | 1             | -              | -           | -         | -           |

Table 1: Specimen/Organism wise Distribution

Table 3 shows the distribution of isolated strains in different clinical samples. Maximum number of isolates was from pus and Ps. aeruginosa was the most frequent isolate (46/55) followed by 5 strains of P. fluorescens and 2 each of Acinetobacter baumanii and Alcaligenes faecalis. Out of 32 samples, 28 isolates were of Ps. aeruginosa.

| Age Group | Isolates (%) | Males | Females |
|-----------|--------------|-------|---------|
| ≤ 20      | 11(8.33%)    | 6(54.54%) | 5(45.45%) |
| 21 – 30   | 34(25.75%)   | 20(58.82%) | 14(41.17%) |
| 31 – 40   | 8(6.06%)     | 3(37.5%)   | 5(62.5%) |
| 41 – 50   | 28(21.21%)   | 18(64.28%) | 10(35.71%) |
| ≥50       | 51(38.63%)   | 27(52.94%) | 24(47.05%) |

Table 2: Age & Sex Distribution of Samples

Table 4 depicts the age and sex distribution of samples. Maximum numbers of samples 51 were from the age group of ≥50 years and 52.94% were from males and 47.05% were from females. 28 samples were from the age group 41-50 years, males comprising 64.28% of the samples and females 35.71% of samples. 34 samples were isolated from the age group of 21-30 years, among them 58.82% of samples were from males and 41.17% were from females.

| Antibiotic  | P. aeruginosa | P. fluorescens | A. baumanii | A. Iwoffi | Al. faecalis |
|-------------|---------------|----------------|-------------|-----------|-------------|
|             | S  | R  | S  | R  | S  | R  | S  | R  | S  | R  | S  | R  | S  | R  |
| Amikacin    | 51 | 64 | 4  | 4  | 1  | 3  | 1  | 1  | 2  | 1  |     |     |     |     |
| Gentamycin  | 14 | 101| 2  | 6  | -  | 4  | -  | 2  | 1  | 2  |     |     |     |     |
| Ciprofloxacin| 45 | 70 | 2  | 6  | 1  | 3  | -  | 2  | 1  | 2  |     |     |     |     |
| Piperacillin| 25 | 90 | 1  | 7  | -  | 4  | -  | 2  | 1  | 2  |     |     |     |     |
Table 3: Isolates Resistant to Antibiotics Tested

*Pip-Taz – Piperacillin-Tazobactam

Table 3 shows sensitivity and resistance to different antibiotics in the various isolates. All the isolates were sensitive to Polymyxin-B.

| Antibiotic     | No. of Isolates | Sensitive ≤ 4µg | Intermediate 8µg | Resistant ≥ 16µg |
|----------------|-----------------|-----------------|------------------|------------------|
| Cefotaxime     | 27              | 88              | 2                | 6                |
| Ceftazidime    | 59              | 56              | 3                | 5                |
| Cefepime       | 65              | 50              | 3                | 5                |
| Polymyxin-B    | 115             | -               | 8                | -                |
| Pip-Taz*       | 65              | 50              | 4                | 4                |
| Aztreonam      | 60              | 55              | 5                | 3                |
| Imipenem       | 67              | 48              | 3                | 5                |

Table 4: Mic Determination by E Test (Imipenem)

Table 8 depicts MIC of isolates tested by the E test Imipenem. Of the 132 isolates, 67 (50.75%) were sensitive, 57(43.18%) were resistant and 8(6.06%) isolates fall in intermediate category.

| No. of Resistant Isolates | Zone enhancement by Combined Disc Method | Zone enhancement by DDST |
|---------------------------|------------------------------------------|---------------------------|
| 57                        | 50(87.71%)                               | 35(61.40%)                |

Table 5: Comparison of the two Methods

Table 11 gives the comparison of the two methods used for MBL detection. Of the 57 isolates resistant to Imipenem, 50(87.71%) showed zone enhancement by Imipenem-EDTA Combined Disc Method and 35(61.40%) showed zone enhancement by Imipenem-EDTA Double Disc Synergy Test.

| Isolate                  | MBL Producers | Non MBL Producers |
|--------------------------|---------------|-------------------|
| Pseudomonas aeruginosa   | 48(41.73%)    | 67(58.26%)        |
| Pseudomonas fluorescens  | 5(62.5%)      | 3(37.5%)          |
| Acinetobacterbaumannii   | 3(75%)        | 1(25%)            |
| Acinetobacterlwoffi      | 1(50%)        | 1(50%)            |
| Alcaligenesfaecalis      | -             | 3(100%)           |

Table 6: Mbl Producers in Isolates

Table 12 depicts MBL producers among various isolates. 48(41.73%) strains of Ps. aeruginosa were MBL producers. 5(62.5%) strains of P. fluorescens showed MBL production.
3(75%) strains of Acinetobacter baumanii and one strain of Acinetobacter lwoffi were MBL producers. Among the 3 isolates of Alcaligenes faecalis, none showed MBL production.

| No. of isolates (n=57) | MIC(µg/ml) |
|------------------------|------------|
| 40(70.17%)             | 2          |
| 11(19.29%)             | 4          |
| 6(10.52%)              | 6          |

Table 7: Mic Determination by Broth Dilution Method

Table 13 shows MIC determination of Imipenem resistant isolates by the broth dilution method using Imipenem and EDTA. Of the 95 isolates, 67(70.52%) showed MIC of 2 µg/ml, 19 (20%) showed MIC of 4µg/ml and 9(9.47%) showed MIC of 6µg/ml.

DISCUSSION: Carbapenems are β-lactam antibiotics used for the treatment of multidrug resistant gram negative bacterial infections. This is due to the stability of these agents against the majority of β-lactamases and their high rate of permeability through the outer membrane.

During the last decade, there have been several reports of resistance to carbapenems. Detecting resistance to carbapenems is important as the resistant bacteria, especially nonfermenting gram negative bacteria can cause outbreaks in ICU’s increasing the morbidity and mortality. This may be due to the underlying disease or limited therapeutic options due to high degree of multiple drug resistance. This drug resistance may spread to other organisms through horizontal gene transfer,

Resistance to carbapenems is due to decreased permeability of the outer membrane, efflux pumps, alteration of the penicillin binding proteins and carbapenem hydrolyzing enzymes - the carbapenemases. These enzymes may be Class B – MBL (IMP, VIM) or Class D – Oxacillinases (OXA-23 to OXA-27) or Class A – Clavulanic acid inhibitory enzymes (SME, NMC, IMI, KPC). These enzymes may be plamid or chromosomally mediated. The spread of resistance among other gram negative bacteria by gene transfer is a real threat.

As carbapenem resistance is mediated by several mechanisms, cross resistance is seen among related antibiotics. There are several methods the underlying mechanism of carbapenem resistance. Kirby-Bauer disc diffusion test is a simple, easy and cost effective test that can be performed conveniently to screen carbapenem resistance.

| Year | Author             | Organism     | % of Resistance |
|------|--------------------|--------------|-----------------|
| 2002 | Navaneeth et al.   | Pseudomonas  | 12%             |
| 2003 | Taneja et al.      | Acinetobacter| 18.5%           |
|      |                    | Pseudomonas  | 42%             |
| 2005 | Hemalatha et al.   | Pseudomonas  | 16%             |
| 2005 | Jesudason et al.   | Pseudomonas* |                 |
| 2007 | Sinha et al.       | Acinetobacter| 14%             |
2008  Valenstein P et al.\textsuperscript{16}  Pseudomonas  26%  \\
2008  Behera et al.\textsuperscript{17}  Pseudomonas  69%  \\
\hline
\textbf{Table 8: Various Studies In India}

*- All isolates in the study were known MBL producers.

In the present study, among the 132 isolates of nonfermenters, 57(43.18%) were resistant to imipenem. This is the first attempt to find carbapenem resistance considering the disease burden, both in outpatient as well as inpatient units of, it is a matter of considerable concern demanding an urgent “re-vision” of antibiotic policy.

The predominant isolate in the present study was Pseudomonas aeruginosa, constituting 115 out of the total 132 samples. Imipenem resistance in the present study in Ps. aeruginosa was 41.73%. It correlates well with the work of Gladstone et al,\textsuperscript{18} (2005). They reported a resistance of 42.8% in Ps. aeruginosa. However, Navaneeth et al,\textsuperscript{11} (2002), reported a resistance of 12%. Valenstein Pet al,\textsuperscript{16} (2008), reported a resistance of 26%. Behera et al.\textsuperscript{17} (2008), reported a resistance of 69%.

In studies done abroad, Irfan et al,\textsuperscript{19} (2008), reported a resistance of 59.52%.

A total of 6 isolates were Acinetobacter species in the present study. 4 out of the 6 isolates of Acinetobacter were found to be imipenem resistant i.e. 66.66%. The sample size (of Acinetobacter) is too low to comment on the antibiogram or resistance pattern. Similar studies gave varied results. Taneja et al,\textsuperscript{12} (2003), reported a resistance of 18.5% in Acinetobacter. Sinha et al,\textsuperscript{15} (2007), reported a resistance of 14%. In studies done abroad, Corbella et al,\textsuperscript{20} (2000), reported 36% resistance and 50% resistance was reported by Manikal et al,\textsuperscript{21} (2000). Irfan et al,\textsuperscript{19} (2008), reported a resistance of 96.6% in Acinetobacter spp.

The percentage of MBL producing isolates in India ranges from 12% to 69%. The percentage was lowest in studies conducted in 2002, which may probably be due to the emerging phase of MBL production. Similar study in present times in the same place may well show a significant rise in statistical parlance.

Metallo-β-lactamase (MBL) detection was done by two methods in the present study. They are the Imipenem-EDTA Combined Disc Method and the Imipenem-EDTA Double Disc Synergy Method.

The results of the Imipenem-EDTA Combined Disc Method are shown in the table 9. Among the 57 isolates resistant to imipenem, 50(87.71%) showed a ≥7mm zone enhancement.

This correlates well with the study of Hemalatha et al,\textsuperscript{13} (2005) reported a 87.5% increase in zones in Pseudomonas. Behera et al,\textsuperscript{17} (2008), recorded a zone enhancement in 85.71% of isolates. Irfan et al,\textsuperscript{19} (2008) reported a 100% increase in the zone of inhibition with the Imipenem-EDTA Combined Disc Method for Pseudomonas and 96.6% for Acinetobacter.

The results of Imipenem-EDTA Double Disc Synergy Method among the 57 isolates resistant to imipenem, 35(61.4%) showed zone enhancement. The present study correlates with Behera et al, 2008, who recorded a 64.28% zone enhancement. Jesudason et al, 2005, reported 72% zone enhancement.
Among the two tests used in the present study, the Imipenem-EDTA Combined Disc Method was found to be more sensitive. It detected MBL production in 87.71% of isolates as opposed to 61.4% by the Imipenem-EDTA Double Disc Synergy Method. The p value is significant on comparison of the two methods (p<0.05).

The MBL E test can be added to increase the sensitivity of detection of MBL. This test has the ability to detect MBL, both chromosomally and plasmid mediated (Walsh et al, 2002). Given the cost constraints of the E test, a simple screening method like the Imipenem-EDTA Combined Disc Method can be used for routine testing in a Microbiology Laboratory.

MBL genes located on plasmids may carry genes encoding other antibiotic resistance determinants like aminoglycoside resistance genes. Such strains are resistant to β-lactams, aminoglycosides and fluoroquinolones. However, they remained sensitive to Polymyxin-B.

It is unfortunate that MBL’s are seen in nonfermenting gram negative bacterial isolates where therapeutic options are severely limited. Polymyxin-B or Colistin represents the best treatment options, but colistin is very expensive, toxicity of Polymyxin-B and colistin limits their use.

An effort to limit inappropriate use of broad-spectrum antibiotics, efficient hospital antibiotic policies, detection of resistant strains and infection control are needed to control the increasing incidence of carbapenem resistance.

**CONCLUSIONS:** Metallo-β-lactamase (MBL) detection was done in which among the 57 isolates resistant to imipenem, 50(87.71%) showed a ≥7mm zone enhancement by Imipenem-EDTA Combined Disc Method, 35(61.4%) showed zone enhancement by the Imipenem-EDTA Double Disc Synergy Method.

Among the two tests used in the present study, the Imipenem-EDTA Combined Disc Method was found to be more sensitive (p<0.05). MBL E test can be used to increase the sensitivity of detection.

**ACKNOWLEDGEMENTS:** I would like to express my special thanks of gratitude to Dr. M V Ramanamma who gave me the golden opportunity to do this project .I am really thankful to her.

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Date of Submission: 08/08/2015.
Date of Peer Review: 10/08/2015.
Date of Acceptance: 13/08/2015.
Date of Publishing: 20/08/2015.