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Isolation and Antimicrobial Susceptibility Paradigm of Carbapenem Resistant Metallo-Beta-Lactamase Producing Gram Negative Rods

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ABSTRACT

Rapid spread of acquired Metallo-beta-lactamases (MBLs) among major Gram negative pathogens is an emerging threat and a matter of great concern worldwide. Especially increasing resistance to 3rd and 4th generation cephalosporin and carbapenems is an issue of high importance. Present study was aimed to determine the frequency and antimicrobial susceptibility patterns of MBL producing carbapenem-resistant Gram-negative rods (GNRs) isolated from clinical specimens. A clinical descriptive study was conducted in Faisalabad, Pakistan from June 2016 to January 2017. A total of 152 clinical samples were processed according to standard microbiological methods. Isolated GNRs were subjected to susceptibility testing against various antibiotics as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Carbapenem-resistant isolates were subjected to detection of Metallo-beta-lactamase production by Double Disc Diffusion test (DDDT) and Modified Hodge's test. In 152 specimens, different species identified in order of their frequency were; *Klebsiella pneumoniae* (59; 38.8%), *Pseudomonas aeruginosa* (46; 30.3%), *Escherichia coli* (36; 23.7%), *Klebsiella oxytoca* (4; 2.6%), *Serratia marcescens* (3; 2%), *Enterobacter agglomerans* (2; 1.3%) and *Enterobacter cloacae* (2; 1.3%). Out of 103 carbapenem-resistant isolates, 58 (55.1%) were positive by DDDT and 49 (46.7%) by MHT. Most of the carbapenems MBL resistant isolates were resistant towards drugs such as cephalosporin, ceftazidime but majority of them were sensitive to polymyxins, tazocin and fusidic acid. Proper screening of MBLs producing GNRs should be done from clinical specimens along with antimicrobial susceptibility testing for developing an effective treatment strategy and thus better control of infections caused by these bugs in future.

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Authors' Contribution
MHR gave the idea and supervised the research work. MZ collected samples and performed culturing. MFH helped to perform antimicrobial susceptibility testing. MUQ analyzed the data. MS helped in practical work and manuscript write up.

Key words
Frequency, Metallo-beta-lactamases, Gram negative rods, Clinical specimens, Antimicrobial susceptibility.

INTRODUCTION

The emergence of antibiotic resistant organisms is a major public health concern, particularly in hospitals and other health care settings. They seem to be biologically fit and are capable of inflicting serious life threatening infections that are difficult to manage because treatment options are too limited. Probably, antibiotic resistance in bacteria is a demonstration of the survival of the fittest with a serious outcome as a treatment failure. With every passing day, the number of untreatable infections is increasing. It was less than a decade after the introduction of carbapenem and or cephalosporin in the clinical practice, in 1980's that Gram negative bacteria have become resistant to these agents (Datta and Wattal, 2010).

Over the previous couple of years, Metallo-beta-lactamase producing Gram Negative Rods are being notifiable with the increasing frequency from various parts of the world and they seems as a most generally circulated and have carbapenem resistance mechanism. MBL producing *Pseudomonas aeruginosa* was first rumored in 1991 in Japan and since then has been supposed from various parts of the world including Asia, Europe, Australia, South America, and North. Lactamase producing bacteria are increasing in number and inflicting additional severe infections, as a result of their continuous mutation (Bora *et al*., 2014). In recent years, MBLs have been identified from clinical isolates with increasing frequency from several parts of the world and strains that produce these enzymes have been responsible for prolonged treatment and acute infections (Pitout and Chow, 2008).

Genetic coding for beta-lactamase enzymes have mutated consistently in response to heavy pressure of antibiotic usage, ultimately causing to the development

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of newer broad spectrum β-lactamases. Carbapenemases represent the most versatile family of β-lactamases that hydrolyze not only carbapenem but other beta-lactams as well, with the sporadic exception of monobactams. The majority of them resist inhibition by all commercially viable beta-lactamase inhibitor (Maltezou, 2009). Gram-negative organisms are generally determined as normal flora in healthy individuals, as they hardly cause serious infection in healthy individual, but become great concern in hospitalized, immunocompromised, and in intensive health care unit patients, where they causes severe invasive infection and responsible for nosocomial outbreak due to several resistance mechanisms (Nordmann and Poirel, 2002).

There are several mechanisms by which bacteria acquired resistance to β-lactam antibiotics, most clinically and recently identified as efflux, reduced permeability, alteration of transpeptidase and by production of β-lactamases. Five variants of MBLs have been reported (IMP and VIM most dominant followed by SPM, GIM, and SIM), and their prevalence are increasing rapidly (Peleg *et al*., 2004). The spread of resistance determinants is facilitated by a range of factors, including existence on genetic mobile elements, antibiotic misuse, poor infection control practices, and increased international travel (Yong *et al*., 2002). In Gram-negative pathogens, lactamase production remains the most significant contributing factor to β-lactam resistance. Penicillins, cephalosporins, monobactams, and carbapenems can all be hydrolyzed by many members of the β-lactamase family of enzymes, which turn out into microbiologically toothless compounds.

Keeping in view the importance of the carbapenem and increasing trend of resistance against it, the present study was conducted with the objectives to determine the frequency of Metallo-beta-lactamase producing carbapenem-resistant bacteria among Gram Negative Rods isolated from clinical specimens in Pakistan, to determine antimicrobial susceptibility pattern of MBL producers and the comparative evaluation of two phenotypic methods to detect MBL producing bacteria.

MATERIALS AND METHODS

Study design and setting

A clinical descriptive study was conducted in Postgraduate Research Laboratory of Department Microbiology Government College University Faisalabad Pakistan.

Collection of clinical specimens

Clinical specimens were collected from Chughtai's Lahore Lab, including Naso bronchial lavage, pus swabs, blood, sputum, catheter tips and urine. Consent for sampling was obtained from Ethical Review Committee (ERC) Government College University Faisalabad and from the patients.

Isolation and identification of bacteria

Gram negative rods (GNRs) were isolated and identified from these clinical specimens following standard microbial techniques. Samples including naso bronchial lavage, pus swabs, blood, sputum, catheter tips, and urine were cultured using routine bacteriological media, including MacConkey, Blood, Chocolate and CLED medium. Cultures were processed according to standard operating procedure of CLSI (2016). After obtaining the pure colonies of the selective strains, along with characteristic staining and morphological patterns, they were subjected for the detection of Metallo-β-lactamases (MBLs) production.

Antimicrobial susceptibility testing

Disc diffusion method was used according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2016). Inoculum size was made equivalent to the 0.5 McFarland standards whose absorbance range was equal to 0.08-0.13 at 625 nm. Commonly used antibiotics selected for anti-microbial susceptibity testing from different groups were: β-lactams/β-lactamases: Amoxicillin and Ampicillin. Cephems and Cephalosporins: Cefepime, Cefoperazone, Cefotaxime, Cefuroxime, Ceftazidime, Ceftriaxone, Cephalexin, Cephradine, Cefaclor, Cefixime. Carbapenems: Imipenem, Meropenem. Fluoroquinolones: Ciprofloxacin, Ofloxacin, Moxifloxacin, Levofloxacin. Miscellaneous: Sulphamethoxazole, Polymyxins, Fusidic acid.

Screening for MBL production

Carbapenem-resistant isolates were subjected to detection of Metallo-beta-lactamase (MBL) production using following two phenotypic tests.

Double disc diffusion test

Isolates showing resistance to carbapenems (Imipenem 10µg) were screened for presence of MBL by the IMP-EDTA double disk-diffusion test (DDDT). The test inoculum with turbidity according to 0.5 McFarland standards was spread onto Muller Hinton agar with a sterile cotton swab. A set of drugs including Imipenem+EDTA disc and Imipenem disc alone were used. MBL production was taken as positive if the diameter of the inhibition zone around the Imipenem+EDTA disc found to be 5 mm greater than the diameter of the inhibition zone around the Imipenem disc alone (Yong *et al*., 2002).

Modified Hodge's test

Modified Hodge test (MHT) is a simple phenotypic test for detection of presence of carbapenemase enzyme in bacteria. The surface of a 150 mm Modified Hodge's agar (MHA) plates were inoculated overnight with a suspension of *Escherichia coli* ATCC 25922, adjusted to a 0.5 McFarland standard. After a brief drying period, a 10 µg imipenem disc was placed at the centre of the plate and the test isolates were streaked heavily from the edge of the disc toward the edge of the plate. The MHT was considered positive, if *E. coli* growth was observed within the inhibition zone of the imipenem disc, giving a distorted zone and interpreted as carbapenemase production. *P. aeruginosa* ATCC 25853 was used as MBL positive control (Lee *et al*., 2003).

Statistical analysis

Descriptive statistics was used to represent the frequency of carbapenems GNRs isolated from clinical specimen and for their antimicrobial susceptibility pattern against different classes of antibiotics. Level of significance was set as ($p<0.05$). All analysis was done by using SPSS Version 20.

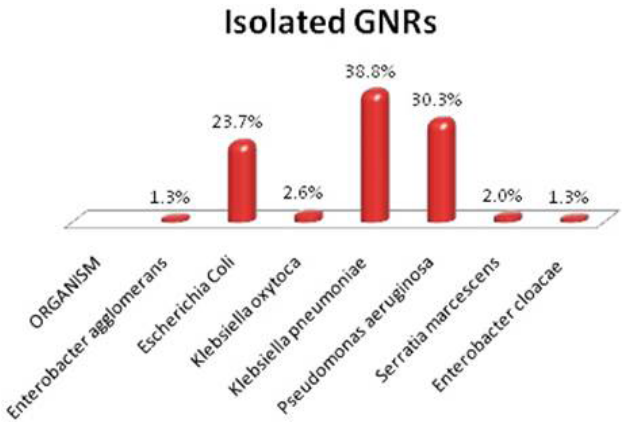

Fig. 1. Frequency of different bacterial species isolated from clinical specimens.

RESULTS

A total of 152 samples were included in present study. These were collected from Chughtai's Lahore Lab (Pvt.) Ltd., Pakistan. Clinical specimens analyzed for further identification were; pus swabs (83; 54.6%), sputum (32; 21.1%) and urine (37; 24.3%) samples. Out of 152 specimens different species identified in order of their frequency were; *Klebsiella pneumoniae* (59; 38.8%), *Pseudomonas aeruginosa* (46; 30.3%), *Escherichia coli* (36; 23.7%), *Klebsiella oxytoca* (4; 2.6%), *Serratia marcescens* (3; 2%), *Enterobacter agglomerans* (2; 1.3%) and *Enterobacter cloacae* (2; 1.3%) as shown in Figure 1.

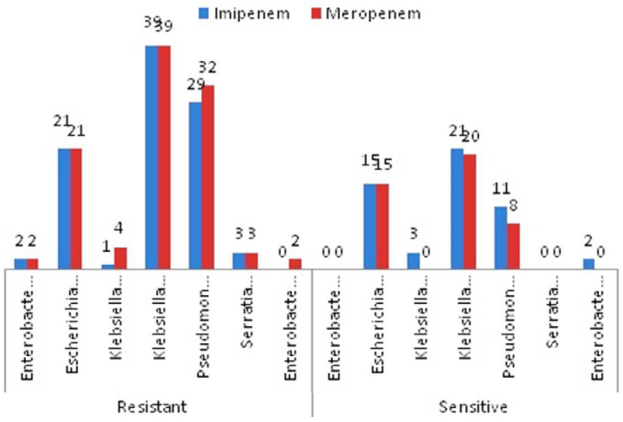

Fig. 2. Carbapenem resistant and sensitive isolates.

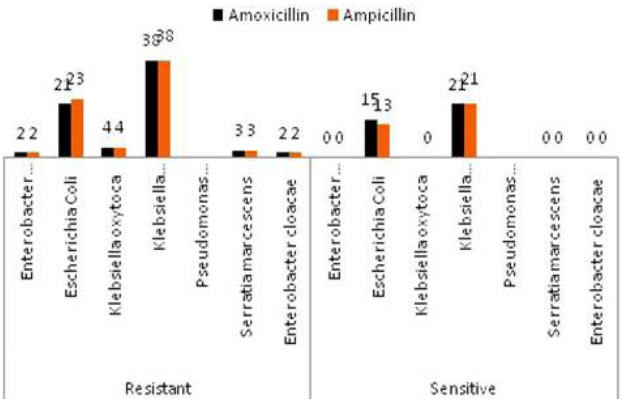

Fig. 3. β-lactamases resistant and sensitive isolates.

Antimicrobial susceptibility testing was done on Muller Hinton agar plates. Among 152, 103 (68%) isolates were resistant to Meropenem and 95 (63%) isolates were resistant to imipenem. It was observed that *Klebsiella pneumoniae* 39 (26%), *Pseudomonas aeruginosa* 29 (19%) and *Escherichia coli* 21 (14%) were predominant carbapenem-resistant isolates followed by *Serratia marcescens* 3 (3%), *Klebsiella oxytoca* 2 (1%) and *Enterobacter agglomerans* 2 (1%) as shown in Figure 2. Among other antibiotics, it was observed that isolates resistant to carbapenems were also resistant to β-lactams/β-lactamases as shown in Figure 3. Among Fluoroquinolones only Ciprofloxacin showed antimicrobial activity against predominant resistant isolates as shown Figure 4. *E coli* and *Klebsiella pneumonia* which were resistant to other antibiotics like Cephems and Cephalosporins were most sensitive to Cefoperazone (Fig. 5). Most of the multidrug resistant isolates were sensitive to Polymyxins B, Tazocin

and Fusidic acid (Fig. 6).

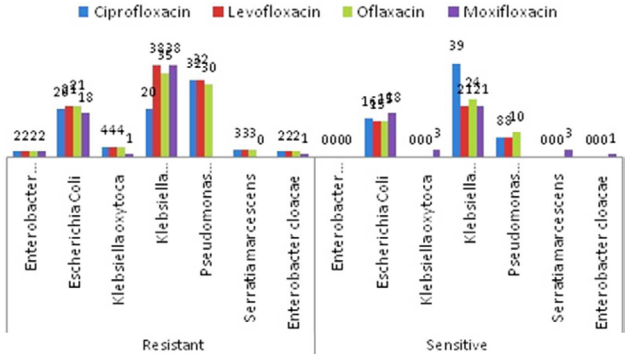

Fig. 4. Fluoroquinolones resistant and sensitive isolates.

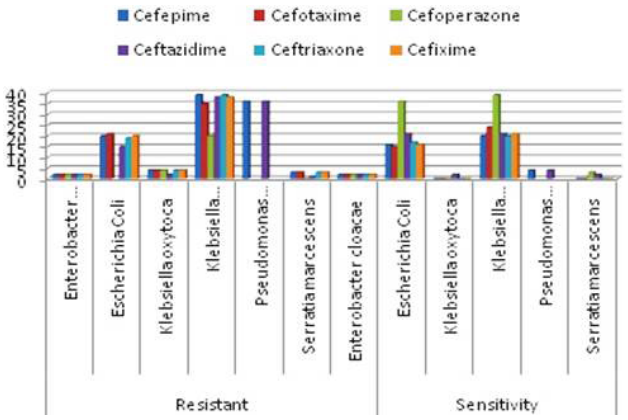

Fig. 5. Cephems and Cephalosporins resistance and sensitive isolates.

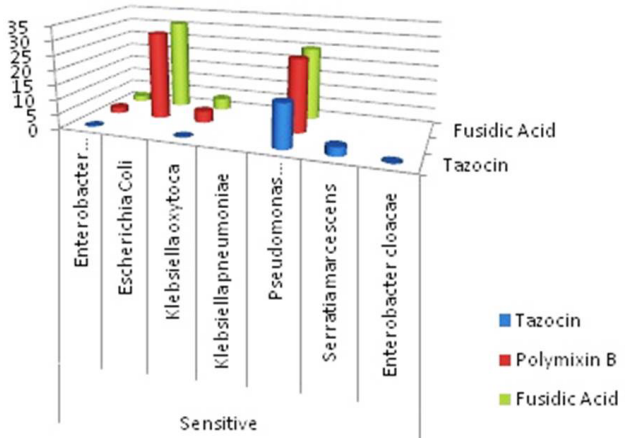

Fig. 6. Sensitivity against miscellaneous antibiotics.

103 carbapenem-resistant isolates were further subjected to two phenotypic screening tests (Figs. 7, 8). Results showed that 58 (55.1%) isolates were positive by DDDT and 49 (46.7%) were positive by MHT as shown in Table I.

Table I.- Percentages of positive and negative results through DDDT and MHT.

	DDDT (%)	MHT (%)
Negative	45 (44)	54 (52)
Positive	58 (56)	49 (48)
Total	103 (100)	103 (100)

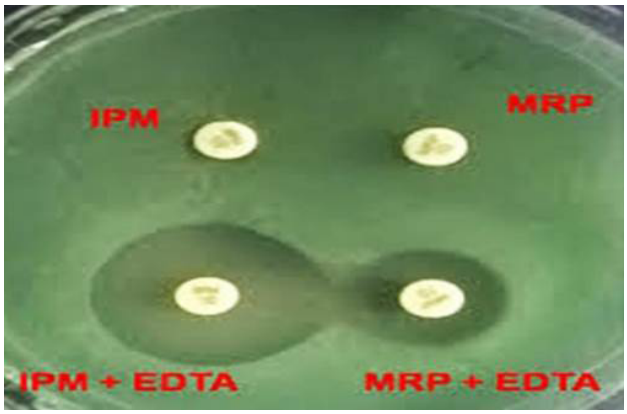

Fig. 7. Double disc diffusion test (DDDT).

Fig. 8. Modified Hodge's test (MHT).

DISCUSSION

The emergence of antibiotic resistant organisms could be a major public health concern, particularly in hospitals and alternative health care settings. The rapid spread of acquired Metallo-beta-lactamases (MBLs) among major Gram-negative pathogens is an emerging threat and a matter of great concern worldwide. Especially the increasing resistance to 3rd and 4th generation cephalosporin

and carbapenems is an issue of high importance. In recent years, MBLs have been identified from clinical isolates with increasing frequency from several parts of the world and strains that produce these enzymes have been responsible for prolonged treatment and acute infections. The increase in number of MBL producing GNBs is alarming in Pakistan, where we are already facing problem of higher level of antibiotics resistance. Therefore detection of metallo-β-lactamase is an important tool for control of the spread of resistance.

Present study was carried out to determine the frequency and antimicrobial susceptibility patterns of MBL producing carbapenem-resistant Gram-negative rods (GNRs) from clinical isolates. All clinical samples were processed according to standard microbiological methods. Isolated GNRs were subjected to susceptibility testing against various antibiotics by disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. After obtaining the purified colonies of the selective strains, along with characteristic staining and morphological patterns, they were further selected for Metallo-β-lactamases (MBLs) production. Carbapenem-resistant isolates was subjected to the detection of Metallo-beta-lactamase production by double disc diffusion test (DDDT) and Modified Hodge's test.

A total of 152 samples were included in present study. Clinical specimens analyzed for further identification were; pus swabs (83; 54.6%), sputum (32; 21.1%) and urine (37; 24.3%) samples. Out of 152 specimens different species identified in order of their frequency were; *Klebsiella pneumoniae* (59; 38.8%), *Pseudomonas aeruginosa* (46; 30.3%), *Escherichia coli* (36; 23.7%), *Klebsiella oxytoca* (4; 2.6%), *Serratia marcescens* (3; 2%), *Enterobacter agglomerans* (2; 1.3%) and *Enterobacter cloacae.*

Among the 152 strains included in our study, 103 (68%) isolates were resistant to Meropenem and 95 (63%) isolates were resistant to imipenem. Among other antibiotics, it was observed that isolates resistant to carbapenems were also resistant to β-lactams/β-lactamases. Among Fluoroquinolones only Ciprofloxacin showed antimicrobial activity against predominant resistant isolates. *E. coli* and *Klebsiella pneumoniae* which were resistant to other antibiotics of Cephems and Cephalosporins group were most sensitive to Cefoperazone. 103 carbapenem-resistant isolates were further subjected to two phenotypic screening tests and results showed that 58 (55.1%) isolates were positive by DDST and 49 (46.7%) were positive by MHT. Most of the multidrug resistant isolates were sensitive to Polymyxins B, Tazocin and Fusidic acid.

Out of 152, 66% of Imipenem and Meropenem resistant species were Carbapenemase producers, only 38% were confirmed positive by DDDT and 32% by MHT, which is similar (36.5%) to the study reported by Nagdeo *et al*. (2012). In a study by Marie and Krishnappa (2013), 56% of the Meropenem resistant isolates were MBL producers. But our study reports a very high rate (38%) maximum of carbapenemase production among isolated resistant species, which is alarming, as these isolates are also resistant to Cephalosporins, Aminoglycosides and Quinolones, making the treatment option very narrow with only Polymyxins B and Fusidic Acid.

In the present study out of 103 isolates, 46.7%, 55.1% were positive for MBL by MHT and DDST, respectively. Our findings are in accordance with Bora *et al*. (2008), who found 55% isolates positive for MBL by CDT and 57.14% isolates positive for MBL by DDDT. In other studies done by Franklin (2006) showed 49% positive isolates by DDST and 43% by MHT. In the present study maximum MBL production was seen in all carbapenem resistant isolates by both methods. In other study done by Mirsalehian *et al*. (2010) showed 65% prevalence of MBL in *P. aeruginosa* while in *Acinetobacter* spp., whereas MBLs prevalence was found to be 50% by Nasrin *et al*. (2015). In other study done by Rajput and Naik (2015), MBL was produced by 20% of *Pseudomonas* isolates and 80% of *Acinetobacter* isolates. In a recent study conducted at Lahore, Pakistan, Akhtar *et al*. (2018) reported that out of 100 carbapenem resistant isolates, 93 and 89 isolates were positive for carbapenemase and beta-lactamase production, respectively.

CONCLUSION

Based on these results, it was concluded that the increase in Carbapenem resistance among MBLs producing GNRs is alarming in the country. Therefore proper screening of these bacteria should be done in routine from clinical specimens along with antimicrobial susceptibility testing for developing an effective treatment strategy and thus better control of infections caused by these bugs in future.

Statement of conflict of interest

All authors declared that no conflict of interest.

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