Original Research Article

Phenotypic Detection and Genotypic Characterization of Metallo-Beta-Lactamases in Gram Negative *Bacilli* isolated from Mansoura University Hospital in Egypt

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A B S T R A C T

Metallo-β-lactamases (MβLs) production in gram negative bacilli is an emerging health problem worldwide. We aimed to determine the occurrence of MβLs among gram negative bacilli (GNB) by phenotypic and genotypic methods in Mansoura University hospital, Egypt. We conducted a prospective study on 107 GNB isolates recovered from different clinical specimens collected from different patients. Species identification and antibiotic susceptibility profile was determined by MicroScan Wa1kAway system. GNB which showed resistance to imipenem and/or meropenem were screened for MBL production by double-disk synergy test (DDST). Phenotypically detected MBLs isolates were further studied for the presence of three genes; *IMP, GIM* and *VIM* by multiplex PCR. Out of 107 GNB isolates, 62 (57.9%) strains showed resistance to imipenem and/or meropenem. DDST detected MBLs in 46.8% of carbapenem resistant isolates.65.5% of phenotypically detected MβLs isolates were carrying genes for MβLs production. *IMP* was the most frequent detected gene (36.8%) followed by *VIM,* and *GIM* genes (31.6% for each). MβLs production were more frequent in *E.coli*, *Pseudomonas* species, *Acintobacter* species (26.3% for each) followed by *Klebsiella* species (15.8%) and *Moraxella* species (5.3%). Our findings showed production of MβLs in a considerable number of GNB which is alarming and need continuous surveillance to detect such resistant strains. DDST is simple method and could be routinely applied for early detection ofthis resistance to establish the proper antibiotic therapy and infection control measures to combat their spread. Future studies are needed for extensive molecular characterization of MβLsamong GNB in our locality.

Introduction

Worldwide antimicrobial resistance is an increasing problem which occur more in those hospitals where antibiotic is used frequently and the patients are in critical condition (Wadekar *et al*., 2013). β-lactamases are the most important mechanism responsible for antibiotic resistance in gram negative bacilli (GNB) because of their wide range of substrate specificity and their ability to hydrolyze many of β-lactams antibiotics (Fam *et al.,* 2006).

Based on conserved and distinguishing amino acid motifs, β-lactamases enzymes are classified into four molecular classes. A, C, and D classes of β-lactamases contain enzymes that hydrolyze their substrates by formation of an acyl-enzyme through an

active site serine, but class B β-lactamases calledmetallo-beta-lactamases (MβLs) as it needs zinc ion for its activity (Bush and Jacoby, 2010).

MβLs producing bacteria have the ability to hydrolyze wide spectra of beta-lactam containing antibiotics including penicillins, cephalosporins, cephamycins and carbapenems, but cannot hydrolyze monobactam as aztreonam. Also, MβLscatalytic activities are inhibited by metal-chelating agents such as ethylene-diaminetetraacetic acid (EDTA) and not inactivated by inhibitors of β-lactamase such as tazobactam, clavulanate, and sulbactam (Bebrone, 2007).

Five MβLstypes, on the basis of amino acid sequence homology, have been identified; *imipenemase* (*IMP*), *Verona integron-encoded metallo-β-lactamase* (*VIM*), German imipenemase (GIM), Sao Paulo metallo-β-lactamase (SPM), and Seoul *imipenemase* (*SIM*) types (Lee and Lee, 2006).

Genes for acquired MβLs are carried on highly mobile which facilitate its horizontal transfer to other pathogens in the hospital, permitting easy and unnoticed dissemination within hospital. Therefore, presence of MβLs producing bacteria in a hospital is considered a therapeutic problem as well as a serious concern for infection control management (Pandya *et al.,* 2016). So, the rapid detection of beta lactamase producing bacteria is important not only to decrease mortality rates for patients but also to avoid spread of such strains in the hospital environment (Wadekar *et al.,* 2013).

Several phenotypic methods have been described for detection of MβLsamong clinical isolates as double disc synergy test and combined disk using metal chelating

agent such as EDTA and thiol compounds, inhibitors of MβLs-activity, in combination with β-lactams (Fam *et al.,* 2006). Based on analysis of genetic materials that is unique and variable for each organism, several molecular methods as PCR are currently used for analysis of genetic contex and detection of different types of MβLs (Pitout *et al.,* 2005). However, currently, there are no recommendations available from the CLSI (Clinical and Laboratory Standards Institute) or elsewhere for the detection of MβLs producing organisms**.** Therefore, we aimed to detect and characterize MβL producing GNB in Mansoura University Hospital and to evaluate Imipenem-EDTA double-disk synergy test (DDST) as phenotypic screening method for detection of MβLs.

Materials and Methods

This prospective study was carried out through the period from March 2014 till March 2015 with an approval from the Local Ethical Committee of Faculty of Medicine-Mansoura University (MFM- Institutional Research Board). A total of 107 gram negative bacilli (GNB) isolates were obtained from random clinical samples of patients admitted to Mansoura University Hospital. These samples comprised of blood, urine, stool, wound swab, sputum, bronchoalveolar lavage (BAL), and CSF. Collection of samples were done under strict aseptic precautions according to standard protocols and processed at once (Collee *et al.,* 1996).

Isolation of gram negative bacilli

All clinical samples were cultured directly on MacConkey agar. Blood samples were inoculated into BACTEC aerobes Plus™/F culture bottles (Becton Dickinson Diagnostic systems, USA) then incubated in the BACTEC fluorescent series instrument for periodic reading. Subcultures on MacConkey

agar were done for all BACTEC culture bottles with positive growth. GNB were identified by Gram staining of the organisms grown on MacConkey agar.

Identification and susceptibility testing of GNB isolates

GNB isolates were further identified up to the species level by Microscan Gram Negative Breakpoint Combo panels Type 20 on The Micro Scan Wa1kAway diagnostic microbiology system (Semens Health Car Dagnostics, formerly Dade Behreng, USA) according to the manufacturer's manual. In brief, inocula were prepared using the Prompt™ Inoculation System for preparation of standardized inocula. Panel Rehydration and inoculation were performed using the RENOK system with Inoculators -D (B1013-4). A final well concentration of 3-7 X 10^5 CFU/ml was achieved. Results were recorded on MicroScan instrumentation screen.

Phenotypic detection of MβLs production byImipenem-EDTA double-disk synergy test

A total of 62 GNB isolates with antibiotics resistance towards imipenem and/or meropenem were investigated for MβLs production by double-disk synergy test (DDST) according to Lee *et al.,* 2003. Briefly, suspension of an overnight culture of the tested strain was adjusted the turbidity of 0.5 McFarland, then used to swab Mul1er-Hington agar surface. After drying, an imipenem disc (10 μg) was placed at a distance of 20mm from the center of a blank sterile filter paper. Thereafter, 10 μl of 0.1M EDTA (pH 8) was applied to the blank disc. The plate was incubated at 37ºC, increase of the inhibition zone in the area between the EDTA disk and imipenem disk as compared to inhibition zone on the far side of imipenem disk was interpreted as a positive for MβLs.

Molecular detection of genes encoding MβLs by Multiplex PCR

Detection of the most common genes *(IMP, VIM*, and *GIM*) responsible for MβL activity was performed by Multiplex PCR for the 29 phenotypically detected MβLs isolates according to Ellington *et al.,* (2007).

DNA extraction

DNA of the different isolates was extracted by the DNA extraction kit (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions.

DNA amplification

Multiplex PCR for amplification and detection of the studied 3*MBLs* genes was done in a single reaction. 5ul of the DNA extract was added to 25ul of PCR master mix, 1ul of each forward primer, 1ul of each reverse primer and then completed with nuclease free water to reach a total volume of 50ul. The cycling condition used for amplification was as follows: initial denaturation at 94ºC for 5mins, 36 cycles of denaturation at 94ºC for 30s, annealing at 52ºC for 40 s and extension at 72ºC for 50s, followed by a single cycle for final extension at 72ºC for 5 mins. The produced amplicons were separated on agarose gel (2%) then the particular bands were visualized using UV transilluminator. Primers sequences and the amplification products size are summarized in table 1.

Statistical analysis

Data were analyzed using SPSS statistical package programme (version 16, SPSS, Chicago, IL). Normal distribution of variables was tested with the Kolmogorov-Smirnov test. The results were expressed as percentages for categorical variables, p value

of ≤ 0.05 was considered statistically significant.

Results and Discussion

A total of 107 Gram Negative Bacilli isolates (GNB) were recovered from different clinical specimens. The largest proportion of specimens was blood (28.9%), BAL (24.2%), wound swabs (22.4%), followed by stool samples (6.5%), urine samples (6.5%) and peritoneal fluid (3.7%). Species of the 107 studied GNB isolates are summarized in table (2)

The isolated GNB were most resistant for trimethoprim (75.7%), followed by piperacillin and cefepime (72.9%), tetracyclin (69.1%), cefuroxime and ampicillin (63.5% for each), tobramycin and ampicillin/ sulbactam (61.6%), and cefazolin (57.9%). Imipenem and meropenem resistance (as a screening for carbapenem resistance) was detected in 43 (40.2%) and 30 (28%) of isolates respectively, table (3).

Double disc synergy test detected MβLs in 29 (46.8%) of 62 GNB isolates with resistance to imipenem and/or meropenem. Multiplex PCR revealed that out of these 29 phenotypically detected MβLs, the three genes (*IMP*, *VIM* and *GIM*) responsible for MBL activity were detected only in 19 (65.5%) isolates, table (4).

The most common gene responsible for MβLs among GNB as identified by multiplex PCR was *IMP*; 7/19(36.8%) followed by *VIM*, and *GIM* genes; 6/19 (31.6% for each), figure (1). Genes for MβLswere detected more frequently among *E. coli*, *Pseudomonas* and *Acinatobacter* species (26.3% for each) followed by *Klebsiella* species (15.8%) and *Moraxella* species (5.3%) (Table 5).

Gram-negative bacilli (GNB) are one of the most important reasons of sever hospital acquired and community-onset bacterial infections in humans (Pitout, 2008). Emerging antimicrobial resistance is a growing problem in many pathogens throughout the globe despite the discovery of newer antibiotics (Shlaes *et al.,* 1997; Pfaller *et al.,* 1998). Several parts of the world have been reported MβLs producing GNB which is the most widespread and clinically significant mechanism of carbapenem resistance (Rasheed *et al*., 2013). Therefore, we tried to detect the presence of MβL producing GNB phenotypically and genotypically in our Mansoura University Hospital.

We investigated 107 isolates of GNB recovered most frequently from blood, BAL, wound and stool samples. Frequent isolation of GNB from nosocomial infections could be linked to many risk factors such as long hospitalization more than 8 days, previous use of antibiotic, trauma and mechanical ventilation which may contribute to the mortality (Kumar *et al.,* 2012).

Among these 107 GNB isolates, the most common species identified were *E-coli*, *Pseudomonas*, *Klebsiella*, followed by *Acinobacter*, *Moraxella*, *Citrobacter*. The least identified species were *Enterobacter*, *Salmonella, Kluyvera, Shigella, Aeromonas, Bordetella, Edwardsiella, Plesiomonas* and *Proteus*. This distribution of GNB coincided with a study carried out in our locality, Zagazig University Hospitals, Egypt which reported *Escherichia coli, Klebsiella pneumoniae* and *P. aeruginosa*as the commonest species among GNB (Mohammed *et al.,* 2016).

Pattern of resistance among nosocomial bacterial organisms may differ widely from country to country at any time and even within the same country over time (Prashanth and Badrinath, 2004). In the Middle East, the occurrence of carbapenem-resistant GNB is alarmingly increased. In our study, 43 (40.2%) and 30 (28%) of the isolates were

resistant to imipenem and meropenem, respectively. Similarly, Mohammed *et al.,* (2016) and Mohamed and Raafat (2011) showed a high level of imipenem resistance among GNB (50.8% and 52.2% respectively). This increase in prevalence of carbapenem-resistant GNB could be due to extensive use of carbapenems which has likely generated a selective antibiotic pressure (Mohammed *et*

al., 2016). Prevalence of MβLs among the carbapenem resistant isolates across the world as shown in various studies varied from 44.5% to 96.3 % (Kaleem *et al*., 2016; Pandya *et al*., 2011; Chaudhary *et al*., 2008). We found that out of the 62 imipenem and or meropenem resistant isolates screened for MβLs production, only 29 (46.8%) were MBLs producers.

Table.1 Sequence of primers used in amplification of metallobeta-lactamases genes and their amplification products (9)

Gene	Primer sequence	Amplicon size
IMP	F (5'-GGA ATA GAG TGG CTT AAY TCT C-3') R (5'-CCA AAC YAC TAS GTT ATC T-3')	188 bp
VIM	F (5'-GAT GGT GTT TGG TCG CAT A-3') R (5'-CGA ATG CGC AGC ACC AG-3')	390 bp
GIM	F (5'-TCG ACA CAC CTT GGT CTG AA-3') R (5'-AAC TTC CAA CTT TGC CAT GC-3')	477 bp

Table.2 Bacterial species of the studied isolates

Bacterial species	Number	%
E.coli	48	44.9
Pseudomonas	13	12.1
Klebsiella	10	9.3
Acinobacter	8	7.4
Moraxella	6	5.6
Citrobacter	5	4.6
Enterobacter	4	3.7
Salmonella	4	3.7
Kluyvera	2	1.8
Shigella	2	1.8
Aeromonas	1	0.9
Bordetella	1	0.9
Edwardsiella	1	0.9
Plesiomonas	1	0.9
Proteus	1	0.9
Total bacterial isolates	**107**	**100**

Table.3 Antibiotic profile of isolated bacterial species

ANTIBIOTIC	Resistant No (%)	Intermediate No (%)	Sensitive No (%)
Trimethoprim	81 (75.7)	9 (8.4)	17 (15.9)
Piperacillin	78 (72.9)	7 (6.5)	22 (20.6)
Cefepime	78 (72.9)	2 (1.9)	27 (25.2)
Tetracycline	74 (69.1)	8 (7.5)	25 (23.4)
Cefuroxime	68 (63.5)	4 (3.8)	35 (32.7)
Ampicillin	68 (63.5)	3 (2.9)	36(33.6)
Tobramycin	66 (61.7)	14 (13.1)	27 (25.2)
Ampicillin / sulbactam	66 (61.7)	6 (5.6)	35 (32.7)
Cefazolin	62 (57.9)	2 (1.9)	43 (40.2)
Ceftazidime	61 (57)	6 (5.6)	40(37.4)
Cefoxitin	57 (53.2)	5 (4.7)	45 (42.1)
Levofloxacin	55 (51.4)	10 (9.3)	42 (39.3)
Piperazine	55 (51.4)	9 (8.4)	43 (40.2)
Ceftriaxone	54 (50-.4)	11 (10.3)	42 (39.3)
Amp. / clavulanic acid	50 (46.7)	6 (5.6)	51 (47.7)
Ciprofloxacin	48 (44.8)	4 (3.8)	55 (51.4)
Gentamicin	48 (44.8)	2 (1.9)	57 (53.3)
Imipenem	43 (40.2)	2 (1.9)	62 (57.9)
Amikacin	41 (38.3)	6 (5.6)	60 (56.1)
Meropenem	30 (28)	8 (7.5)	69 (64.5)
Tigecycline	7 (6.5)	4 (3.7)	96 (89.8)
Cefotaxime	81 (75.7)	12 (11.2)	14 (13.1)
Aztreonam	8 (7.4)	4 (3.8)	95(88.8)

Table.4 Detection of metallobeta-lactamases among gram negative bacilli
by phenotypic and genotypic methods

	No	%
MBLs-GNB by synergy test	29/62	46.8
MBLs – GNB by multiplex PCR	19/29	65.5%

GNB: gram negative bacilli

Table.5 Bacterial species positive for metallobeta-lactamases by double disc EDTA testand
Multiplex PCR

Bacterial Species	*double disc EDTA test* No (%)	*Multiplex PCR* No (%)
E.coli species	7 (24.1)	5 (26.3)
Pseudomonas species	7 (24.1)	5 (26.3)
Klebsiella species	5 (17.2)	3 (15.8)
Acinobacter species	7 (24.1)	5 (26.3)
Moraxella species	3 (10.3)	1 (5.3)
Total bacterial isolates	29 (100)	19 (100)

Figure.1 Frequency of genotypes among metallobeta-lactamases producing
Gram negative bacilli by multiplex PCR
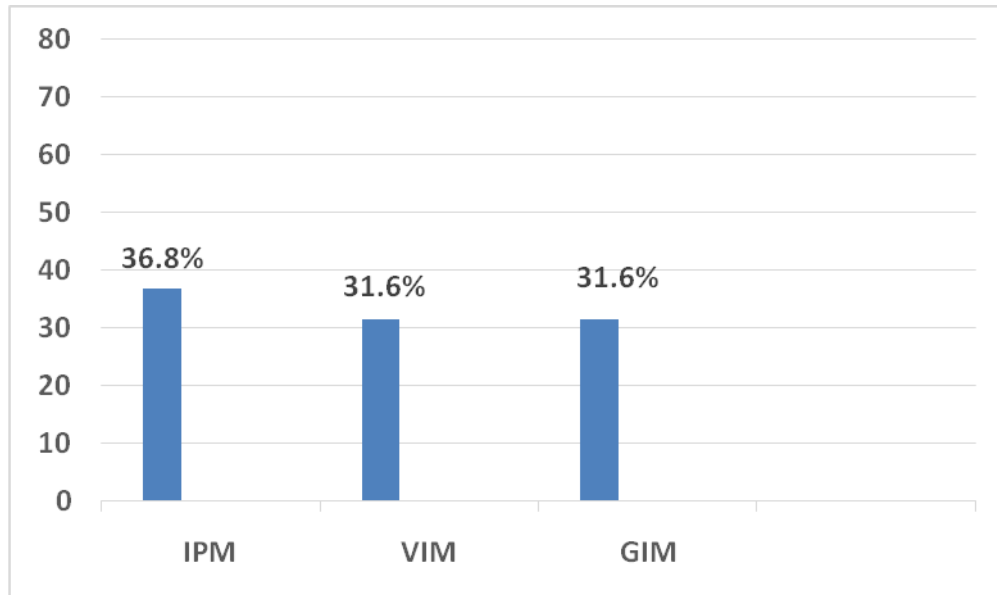
Carbapenems resistance detected in the remaining 33 MBLs non producers could be caused by other mechanisms such as diminished permeability of outer membrane, increased efflux systems or change of penicillin binding proteins (Tellis *et al.,* 2013).

Prevalence rate of MβLs (as detected phenotypically by DDST) in the present study was higher than a previous study in our locality which detected MβLs only in 20% of their studied GNB (El-Kazzaz and Abou El-khier, 2015). Difference in MβLs prevalence could be attributed to many reasons as different age groups, different clinical samples, and different methods for MβLs detection or use of different species in the studies (Pandya *et al.,* 2016). It is known that MβLs are more frequent among *Pseudomonas aeruginosa* species (Lister *et al.,* 2009).

In the present study, Only 19 (65.5%) of the 29 phenotypically detected MβLs producing isolates was found to be positive for the studied MβLs genes. Phenotypically detected MβLs with no genotypic mark of MBLs production might harbor other MβLs genes, such as OXA-type, that were not investigated in this study. Another probable explanation mightbe the susceptibility of some bacterial strains to EDTA, which could affect permeability of bacterial membrane, resulting in false positive results of phenotypic tests used for the MβLs detection (Karthika *et al.,* 2009; Aktas and Kayacan, 2008). In general, phenotypic method, especially DDST is reported to be sensitive for detection of MβLs due to wide genetic varieties among gram negative bacilli isolated from clinical samples (Omair *et al.*, 2012).

Percentage of genes responsible for MβLs among our studied GNB by multiplex PCR, was 36.8% for *IMP* and 31.6% for each of *VIM*, and *GIM* genes. Also Anoar *et al* (2014) reported that *IMP* is the most frequently found MβLs gene among their studied isolates and it recorded a higher percentage of detection than *VIM* genes. On contrary to our findings, Chakraborty *et al.,* (2011), found that most of the studied strains harbor *VIM* genes and not detected *IMP* genes in any of

studied strains. In the beginning, these MβLs were commonly detected in *Klebsiella* species and *E. coli* (Mathur *et al.,* 2002), but now they are produced by all Enterobacteriaceae and other Gram negative bacteria (Kumar *et al.,* 2006).

Our study detected MβLs more frequently among *E. coli*, *Pseudomonas* species, *Acintobacter* species, followed by *Klebsiella* species and *Moraxella* species. Also, Naveenkumar *et al.,* (2014) found that prevalence of MβLs production, is highest in *Pseudomonas* (50%), *E. coli* (34%) and *Acinetobacter* (16%) but not in other gram negative bacilli. In the same context, there are frequent reports of MβLs production in *Pseudomonas aeruginosa* and *Acinetobacter* species from different areas of the world (Nordmann and Poirel, 2002; Noyal *et al.,* 2009; Mishra *et al.,* 2012; Altun *et al.,* 2013). In conclusion, there is a considerable high prevalence of carbapenem resistance among our clinical GNB isolates. Imipenem-EDTA double-disk synergy test identified 46.8% of this carbapenem resistance as MβLs producers. PCR based detection methods are expensive, need experts and identified only 65.5% of these phenotypically detected MβLs.

The most frequent MβLs genes detected in our isolates were *IMP* followed by *VIM&GIM*. Therefore, all GNB isolates with resistant to cabapenem should be screened for MβLs production by simple rapid cost-effective method such as DDST. Moreover, it is important to report beta lactamases production along with routine reports of antibiotic susceptibility test to help the clinician in choosing the proper antibiotics and to initiate effective infection control measures to stop their uncontrolled dissemination in healthcare settings. Further researches are needed to specify the frequency of other important MβLs genes among GNB in our locality.

References

Aktas, Z., Kayacan, C.B. 2008. Investigation of metallo- β-lactamase producing strains of Pseudomonas aeruginosa and Acinetobacter baumanni by E-test, disk synergy, and PCR. *Scand. J. Infect. Dis.,* 40: 320-325.

Altun, S., Tufan, Z.K., Yağcı, S., Önde, U., Bulut, C., Kınıkl, S., Demiroz, A.P. 2013. Extended Spectrum Beta-lactamases, AmpC and Metallo Beta-lactamases in Emerging Multi-drug Resistant Gram-negative Bacteria in Intensive Care Units. *Open. Access. Scientific. Reports,* 2: 4.

Anoar, K.A., Ali, F.A. and Omer, S.A. 2014. Detection of Metallo B- lactamase enzyme in some Gram Negative Bacteria isolated from Burn Patients in Sulaimani City, Iraq. *European. Sci. J.,* 10: 3.

Bebrone, C. 2007. Metallo-beta-lactamases (classification, activity, genetic organization, structure, zinc coordination) and their superfamily. *Biochem. Pharmacol.,* 74: 1686–1701.

Bush, K. and Jacoby, G.A. 2010. Updated functional classifiation of beta-lactamases. *Antimicrob. Agents Chemother.,* 54: 969-76.

Chakraborty, D., Basu, S. and Das, S. 2011. Study on some Gram negative multidrug resistant bacteria and their molecular characterization. *Asian. J. Pharmaceut. and Clin. Res.,* 4(1):108-112.

Chaudhary, U., Bhaskar, H. and Sharma, M. 2008. Imipenem-EDTA disk method for rapid identification of metallo-β-lactamase producing Gram-negative bacteria. *Indian J. Med. Res.,* 127: 406-407.

Collee, J.G., Miles, R.S. and Wan, B. 1996. Tests for the identification of bacteria. In: Collee JG, Fraser AG, Marmion BP, Simmons A (ed) Mackie and McCartney Practical Medical Microbiology, 14th edn. Edinburgh, Churchill Livingstone, pp 131-50.

El-Kazzaz, S.S. and Abou El-khier, N.T. 2015.

AmpC and metallo beta-lactamases producing Gram negative bacteria in patients with hematological malignancy. *African J. Microbiol. Res.,* 9(18): 1247-1254.

Ellington, M.J., Kistler, J., Livermore, D.M. and Woodford, N. 2007. Multiplex PCR for rapid detection of genes encoding acquired MβLs. *J. Antimicrob. Chemother.*, 59: 321-322.

Fam, N., Diab, M., Helmi, H. and El-Dafrawy, I. 2006. Phenotypic detection of metallo-beta-lactamase and extended spectrum beta-lactamases among gram negative bacterial clinical isolates. *Egyptian J. Med. Microbiol.,* 15(4): 719-730.

Kaleem, F,. Usman, J., Hassan, A. and Khan, A. 2010. Frequency and susceptibility pattern of metallo-beta-lactamase producers in a hospital in Pakistan. *J. Infect. Dev. Ctries.,* 4(12):810-13.

Karthika, R.U., Rao, R.S., Sahoo, S., Shashikala, P., Kanungo, R., Jayachandran, S., Prashanth, K. 2009. Phenotypic and genotypic assays for detecting the prevalence of metallo-beta-lactamases in clinical isolates of Acinetobacter baumannii from a South Indian tertiary care hospital. *J. Med. Microbiol.*, 58: 430-435.

Kumar, M.S., Lakshmi, V. and Rajagopalan, R. 2006. Occurrence of extended spectrum β- lactamases among Enterobacteriaceae species isolated at a tertiary care institute. *Indian. J. Med. Microbiol.*, 24(3):208-11.

Kumar, S.H., De, A.S., Baveja, S.M., and Gore, M.A. 2012. Prevalence and risk factors of Metallobetalactamase producing *Pseudomonasaeruginosa* and Acinetobacter species in burns and surgical wards in a tertiary care hospital. *J. Lab. Physicians*, 4(1): 39–42.

Lee, J.H. and Lee, S.H. 2006. Carbapenem resistance in gram-negative pathogens: Emerging Non-metallo-carbapenemases. *Res. J. Microbiol.*, 1: 1-22.

Lister, P.D., Wolter, D.J. and Hanson, N.D. 2009. Antibacterial Resistant Pseudomonas aeruginosa: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clin. Microbiol. Rev.,* 22(4):582-610.

Mathur, P., Kapil, A., Das, B., Dhawan, B. 2002. Prevalence of extended spectrum beta lactamase producing Gram negative bacteria in a tertiary care hospital. *Indian. J. Med. Res.,* 115:153-157.

Mishra, S..K, Acharya, J., Kattel, H.P., Koirala, J., Rijal, B.P. and Pokhrel, B.M. 2012. Metallo-beta-lactamase Producing Gram-negative Bacterial isolates. *J. Nepal. Health. Res. Counc.,* 10(22):208-213.

Mohamed, N.M. and Raafat, D. 2011. Phenotypic and genotypicdetection of metallo-beta-lactamases in imipenem-resistant *Acinetobacter baumannii* isolated from a tertiary hospital in Alexandria, Egypt. *Res. J. Microbiol.,* 6(10): 750–760.

Mohammed, E.H., Fakhr, A.E., Mohammed, E.l. sayed, H.M., Al Johery, S.A.E., Abdel Ghani Hassanein, W.A.G. 2016. Spread of TEM, VIM, SHV, and CTX-M β-Lactamases in Imipenem-Resistant Gram-Negative Bacilli Isolated from Egyptian Hospitals. *Int. J. Microbiol.,* 8382605.

Naveen kumar, C., Swathi, S. and Srikumar, R. 2014. Metallo-Betalatamase (MBL) detection in multidrug resistant gram negative bacilli (MDR) isolates. *J. Innovative Res. Solution,* 1 (1): 1-7.

Nordmann, P. and Poirel, L. 2002. Emerging carbapenemases in Gram negative aerobes. *Clin. Microbiol. Infect.,* 8: 32-31.

Noyal, M.J.C., Menezes, G.A., Harish, B.N., Sujatha, S. and Parija, S.C. 2009. Simple screening tests for detection of carbapenemases in clinical isolates of nonfermentative Gram-negative bacteria. *Indian J. Med. Res.,* 129: 707-712.

Omair, M., Usman, J., Kaleem, .F, Hassa,n A., Khalid, A., Fahim, Q. 2012. Evaluation of Combined Disc Method for the Detection of Metallo-β-Lactamase Producing Gram Negative Bacilli. *Malaysian J. Microbiol.,*

21-25.

Pandya, J., Kamothi, M., Maheta, S., Kikani, K. and Prajapati, S. 2016. The prevalence of Metallo-beta-lactamase (MBL) in gram negative bacilli and their antimicrobial susceptibility pattern at tertiary care hospital, Surendranagar. *Int. J. Res. Med.,* 5(3): 78-83.

Pandya, N.P., Prajapati, S.B., Mehta, S.J., Kikani, K.M. and Joshi, P.J. 2011. Evaluation of various methods for detection of metallo-β-lactamase (MBL) production in gram negative bacilli. *Int. J. Biol. Med. Res.,* 2(3):775-77.

Pfaller, M.A., Jones, R.N., Doern, G.V,. Kugler, K. and the Sentry Participant Group. 1998. Bacterial pathogens isolated from patients with bloodstream infection: frequencies of occurrence and antimicrobial susceptibility patterns from the SENTRY Antimicrobial Surveillance Program (United States and Canada). *Antimicrobial Agents and Chemother*., 42: 1762–70.

Pitout, J., Gregson, D., Poire, l.L. *et al.* 2005. Detection of pseudomonas auroginosaproducing metllo-- beta lactamases in a large centralized laboratory. *J. Clin. Microbiol.,* 43(7): 3129-3135.

Pitout, J.D.D. 2008 Multiresistant enterobacteriaceae: new threat of an old problem. *Expert Review of Anti-Infective Thrapy,* 6 (5): 657–669.

Prashanth, K. and Badrinath, S. 2004. *In vitro* susceptibility pattern of *Acinetobacter* species to commonly used cephalosporins, quinolones, and aminoglycosides. *Indian J. Med. Microbiol.,* 22 (2): 97–103.

Rasheed, J.K., Kitchel, B., Zhu, W., Anderson, K.F., Clark, N.C., Ferraro, M.J., Savard, P., Humphries, R.M., Kallen, A.J. and Limbago, B.M. 2013. New Delhi metallo-β lactamase producing Enterobacteriaceae, United States. *Emerg. Infect. Dis.,* 19: 870–878.

Shlaes, D.M., Gerding, D.N., John, J.F. *et al.,* 1997. Society for Healthcare Epidemiology of America and Infectious Diseases Society of America joint committee on the prevention of antimicrobial resistance. *Clin. Infect. Dis.,* 25: 584–99.

Tellis, R., Muralidharan, S. and Peter, A.I. 2013. Evaluation of three phenotypic methods for the detection of metallo-betalactamase production in non fermenting gram negative bacilli. *Int. J. Biomed. Advance Res.,* 04(05).

Wadekar, M.D., Anuradha, K. and Venkatesha, D. 2013. Phenotypic detection of ESBL and MBL in clinical isolates of Enterobacteriaceae. *Int. J. Curr. Res. Academic Review,* 1(3): 89-95.