Phenotypic Screening of Clinical Isolates of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* for Metallo-Beta-Lactamase

Ejikeugwu Chika*[1], Duru Carissa[2], Iroha Ifeanyichukwu[1], Ugwu Malachy[2], Nwakaeze Emmanuel[1], Oguejiofor Benigna[1], Esimone Charles[2], Adikwu Michael[3]

[1]Department of Applied Microbiology, Faculty of Sciences, Ebonyi State University, P.M.B. 053, Abakaliki, Ebonyi State, Nigeria.
[2]Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, P.M.B 5025, Awka, Nigeria
[3]University of Abuja, Federal Capital Territory (FCT), Abuja, Nigeria
ejikeugwu_chika@yahoo.com; +2348097684562

Abstract: Metallo-beta-lactamases (MBLs) are carbapenem-hydrolyzing beta-lactamases which have the ability to hydrolyze and confer resistance to carbapenems (imipenem, meropenem, ertapenem) and other beta-lactam antibiotics; and they are an emerging public health problem among clinically important Gram negative organisms including *P. aeruginosa*, *A. baumannii* and the *Enterobacteriaceae*. A total of 99 clinical isolates of *Escherichia coli, Klebsiella pneumoniae* and *P. aeruginosa* from various clinical samples were bacteriologically analyzed in this study using eosin methylene blue (EMB) medium, MacConkey agar and cetrimide selective agar; and the isolates were re-characterized after collection using conventional microbiology techniques. Antimicrobial susceptibility studies were carried out using the Kirby-Bauer disk diffusion technique as per the guideline of Clinical Laboratory Standard Institute (CLSI). The production of metallo beta-lactamase (MBL) was phenotypically confirmed using the inhibitor-based assay. The test Gram negative bacteria showed varying rates of susceptibility to the test antibiotics. However, they were more resistant to sulphamethoxazole-trimethoprim, ciprofloxacin, gentamicin, and ofloxacin. The *P. aeruginosa* isolates were completely susceptible to imipenem and meropenem. But some *E. coli* and *K. pneumoniae* isolates were found to be resistant to imipenem and meropenem. MBL production was phenotypically detected in 5 (12.5 %) isolates of *E. coli,* 6 (15.4 %) isolates of *K. pneumoniae* and 2 (10 %) isolates of *P. aeruginosa* isolates. Prompt and accurate detection of MBL-producing pathogens is crucial as this will help physicians to prescribe the proper therapy for affected patients. Rational use of available antibiotics in both the community and hospital environment is also essential to keeping resistant pathogens at bay, and preventing the emergence and spread of resistance in the hospital and community settings.

[Ejikeugwu Chika, Duru Carissa, Iroha Ifeanyichukwu, Ugwu Malachy, Nwakaeze Emmanuel, Oguejiofor Benigna, Esimone Charles, Adikwu Michael. **Phenotypic Screening of Clinical Isolates of *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa* for Metallo-Beta-Lactamase.** *Life Sci J* 2016;13(10):77-82]. ISSN: 1097-8135 (Print) / ISSN: 2372-613X (Online). http://www.lifesciencesite.com. 11. doi:10.7537/marslsj131016.11.

Keywords: Metallo-Beta-Lactamase, Multidrug Resistance; *Enterobacteriaceae*, Nosocomial Infection; Nigeria

1. Introduction

The increasing prevalence of antibiotic resistant microorganisms, especially those with multidrug resistance mechanisms as metallo-beta-lactamases is of global concern as they are known to make the treatment of bacterial related infections difficult (Jacoby an Munoz-Price, 2005). Pathogenic bacteria including *Klebsiella pneumoniae, Escherichia coli,* and *Pseudomonas aeruginosa* under certain conditions (such as undue exposure to antibiotics) can become resistant to antibiotics of different classes, and these organisms are generally called Multi-Resistant Gram-Negative Bacteria, MRGNB (Walsh *et al*., 2005; NYYPCT, 2008; Saderi *et al*., 2008). Metallo beta-lactamases (MBLs) are beta-lactamases that hydrolyze and confer resistance to carbapenems such as imipenem, meropenem, and ertapenem (Walsh *et al*., 2005; Ejikeugwu *et al*., 2014). They are encoded by genes that have been procured by bacteria either by mutation or horizontally from other organisms, and they can be chromosomally or plasmid-mediated. MBLs were first formally described from serine beta-lactamases in the 1980s (Walsh *et al*., 2005), and they are known to hydrolyze and cause resistance to all carbapenems – which are usually the last line treatment option for infections caused by multidrug resistant organisms. They are mostly found in non-lactose fermenters such as *Pseudomonads* and *Acinetobacter* species; and to a lesser extent in lactose-fermenting *Enterobacteriaceae* (Walsh *et al*., 2005). The presence or occurrence of MBL-producing bacteria in a localized hospital setting poses not only a therapeutic problem but also serious concern for infection control management in the health system. This is due to the fact that organisms producing MBLs are multidrug resistant in nature; and infections with

MBL-positive bacteria can lead to prolonged hospitalization and failure of antibiotic therapy. MBLs are also resistant to serine beta-lactamase inhibitors and they have spread worldwide causing a number of cases in Europe and even in Africa and other parts of the world (Aibinu *et al*., 2007; Libisch *et al*., 2006; Ejikeugwu *et al*., 2016). According to recent reports, MBLs are known to efficiently hydrolyze all beta-lactam drugs except aztreonam (a monobactam) *in vitro* (Walsh *et al*., 2005 and Saderi *et al*., 2008). The Microbiology laboratory should be up and doing in producing and disseminating meaningful local surveillance data with respect to the predominant pathogens and their antibiotic resistance profiles in order to keep the emergence and spread of resistant pathogens at bay. Hospital microbiology laboratories around the globe should also be aware that incorrect or delayed laboratory diagnostic data especially as it relates to antimicrobial susceptibility studies can lead to lengthened empiric antibiotic therapy or blind-treatment which is capable of allowing resistant strains to thrive. It is therefore imperative to detect MBL-producing *Enterobacteriaceae* and *Pseudomonad's* from both the community and the hospital settings in order to forestall the plethora of damage they cause to our therapeutic armamentarium. Early detection of these organisms will help to keep antibiotic resistant bacteria at bay, and infection control strategies that will help to avert future occurrence can be developed; thus the need to undertake this research work.

2. Material and Methods

Collection and processing of bacterial isolates: Ninety nine (99) non-duplicate bacterial isolates of clinical origin were used in this study. The bacterial isolates comprises *Escherichia coli* (n=40), *Klebsiella pneumoniae* (n=39), and *Pseudomonas aeruginosa* (n=20). These isolates were obtained from the culture collection unit of the tertiary hospital under investigation; and they were from clinically relevant specimens of outpatients. The clinical samples from which these isolates were isolated from include urine (n=56), sputum (n=19), ear swab (n=7), pleural aspirate (n=1), conjunctival swab (n=1), wound swab (n=14), and High Vaginal swab, HVS (n=1). After collection, all the bacterial isolates were purified on nutrient agar plates (Oxoid, UK) and re-identified using conventional microbiological techniques (Cheesbrough, 2000).

Antimicrobial susceptibility studies: Antimicrobial susceptibility test was determined by the Kirby and Bauer disk diffusion method as recommended by Clinical Laboratory Standard Institute, CLSI (CLSI, 2004). Briefly, an overnight culture of the test bacteria (adjusted to 0.5 McFarland turbidity standards) was aseptically swabbed on the surface of Mueller-Hinton (MH) agar plate(s) using sterile swab sticks. And commercially available single antibiotic disks comprising sulphamethoxazole-trimethoprim (25 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), ceftazidime (30 µg), cefotaxime (30 µg), amoxicillin-clavulanic acid (20/10 µg), and gentamicin (10 µg) were aseptically placed on the MH agar plate(s). All the antibiotic disks were procured from Oxoid limited, (Oxoid, UK). The plates were incubated at 37^{o}C for 18-24 hrs, and the inhibition zone diameters (IZDs) produced were measured and recorded as recommended by the CLSI criteria (CLSI, 2004; Ejikeugwu *et al*., 2016).

Screening of bacterial isolates for MBL production: To screen the clinical isolates for MBL production, antibiotic disks containing 10 µg of imipenem and 10 µg of meropenem were each placed apart at a distance of 25 mm on Mueller-Hinton agar (Oxoid, UK) plate(s) that was already swabbed with the test bacterium. The plates were incubated at 37^{o}C for 18-24 hrs and the zones of inhibition measured according to the CLSI guidelines. MBL production was suspected if any of the test organism(s) showed reduced susceptibility to any of the carbapenems (imipenem 10 µg and meropenem 10 µg) used for the screening studies (CLSI, 2004, Ejikeugwu *et al*., 2014; Ejikeugwu *et al*., 2016 Walsh *et al*., 2005; Franco *et al*., 2010).

Inhibitor-based assay for phenotypic detection of MBL: Metallo-beta-lactamase (MBL) production in the *E. coli*, *K. pneumoniae* and *P. aeruginosa* clinical isolates was confirmed by the inhibitor-based assay as was previously described (Varaiya *et al*., 2008). Two meropenem disks (10 µg) and two imipenem disks (10 µg) were placed 25 mm apart on Mueller-Hinton (MH) media plate(s) inoculated with the test bacteria (adjusted to 0.5 McFarland turbidity standards). Sterilized EDTA solution (1 µl) was added to one of the imipenem disk and meropenem disk respectively using a micropipette, and the plates were incubated at 37^{o}C for 18-24 hrs. After incubation, the zones of inhibition around the imipenem and imipenem+EDTA disks, and meropenem and meropenem+EDTA disks were measured using a meter rule, recorded and compared. MBL production in the screened bacteria was inferred if the zone of inhibition of imipenem+EDTA disk and meropenem+EDTA disk compared to imipenem and meropenem disks alone respectively is greater than 7 mm (Ejikeugwu *et al*., 2014; Varaiya *et al*., 2008; Ejikeugwu *et al*., 2016).

3. Results

Table 1 show the occurrence of bacteria isolation from the different clinical samples. It was observed that the highest number of bacteria isolates was obtained from urine samples; and this was followed

by sputum and wound swab samples – which are all frequently requested from outpatients visiting the hospital for medical attention. In terms of frequency, *Escherichia coli* (34.3 %) were the most isolated bacterial organism, and this was followed by *Klebsiella pneumoniae* (13.1 %) and *Pseudomonas aeruginosa* (9.1 %). The result of the antimicrobial susceptibility studies conducted on the clinical isolates is shown in Table 2. The clinical isolates showed varied susceptibility pattern to the tested antibiotics. *E. coli* was found to be highly resistant to ciprofloxacin, gentamicin and sulphamethoxazole-trimethoprim while *K. pneumoniae* was also resistant to cefotaxime, gentamicin, ofloxacin and ciprofloxacin. *P. aeruginosa* isolates were resistant to ofloxacin, gentamicin, sulphamethoxazole-trimethoprim and cefotaxime. However, all the *P. aeruginosa* isolates were found to be susceptible to imipenem and meropenem.

Table 1. Frequency of the clinical isolates from different specimen types

Specimens	*K. pneumoniae* n (%)	*E. coli* n (%)	*P. aeruginosa* n (%)
Urine	13 (13.1)	34 (34.3)	9 (9.1)
Ear swab	1 (1.0)	0 (0)	6 (6.1)
Pleural aspirate	1 (1.0)	0 (0)	0 (0)
Sputum	18 (18.2)	0 (0)	1(1.0)
High vaginal swab	0 (0)	1 (1.0)	0 (0)
Wound swab	6 (6.1)	5 (5.1)	3 (3.0)
Conjunctival swab	0 (0)	0 (0)	1 (1.0)
Total	39 (39.4)	40 (40.4)	20 (20.2)

Table 3 show the result of the screening test and phenotypic confirmatory test for the detection of MBL production in the test bacterial isolates. Out of the 99 bacterial isolates used in this study, a total of 27 isolate comprising 11 (27.5 %) *E. coli,* 12 (30.8 %) *K. pneumoniae* and 4 (20 %) *P. aeruginosa* isolates were suspected to produce MBL enzymes. Overall, only 5 isolates of *E. coli*, 6 isolates of *K. pneumoniae* and 2 isolates of *P. aeruginosa* were phenotypically confirmed to be MBL producers by the inhibitor-based assay (Table 3).

Table 2. *In vitro* antibiotic susceptibility patterns of all the clinical isolates

Drugs	P (µg)	***E. coli*** **(n=40)**			***K. pneumoniae*** **(n=39)**			***P. aeruginosa*** **(n=20)**		
		S	**I**	**R**	**S**	**I**	**R**	**S**	**I**	**R**
		n(%)	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**	**n(%)**
CTX	30	18(45)	4(10)	18(45)	10(25.6)	5(12.8)	24(61.5)	5(25)	5(25)	10(50)
CAZ	30	19(47.5)	9(22.5)	12(30)	10(25.6)	14(35.9)	15(38.5)	10(50)	4(20)	6(30)
SXT	25	1(2.5)	0(0)	39(97.5)	5(12.8)	1(2.6)	33(84.6)	0(0)	0(0)	20(100)
CN	10	18(45)	9(22.5)	13(32.5)	18(46.2)	4(10.3)	17(43.6)	11(55)	3(15)	6(30)
OFX	5	10(25)	4(10)	26(65)	16(41)	2(5.1)	21(53.8)	6(30)	1(5)	13(65)
CIP	5	14(35)	1(2.5)	25(62.5)	12(30.8)	4(10.3)	23(59)	9(45)	1(5)	10(50)
IPM	10	38(95)	0(0)	2(5)	34(87.2)	0(0)	5(12.8)	20(100)	0(0)	0(0)
MEM	10	37(92.5)	1(2.5)	2(5)	36(92.3)	0(0)	3(7.7)	20(100)	0(0)	0(0)

Key: P - Potency, S - Susceptible, I - Intermediate, R – Resistant, n – number of isolates(s), CTX-cefotaxime, CAZ-ceftazidime, SXT-sulphamethoxazole-trimethoprim, CN-gentamicin, OFX-ofloxacin, CIP-ciprofloxacin, IPM-imipenem, MEM-meropenem.

Table 3. Prevalence of MBL producers by screening and phenotypic confirmatory test

Clinical isolates	**No**	**Screenedn (%)**	**Inhibitor-based assayn (%)**
E. coli	40	11(27.5)	5(12.5)
K. pneumoniae	39	12(30.8)	6(15.4)
P. aeruginosa	20	4(20)	2(10)
Total	99	27(27.3)	13(13.1)

4. Discussions

One of the major problems of human medicine today is the rapid emergence, spread and increase in the resistance of multidrug resistant (MDR) pathogenic bacteria to some readily available antibiotics (especially the beta-lactams). This growing resistance of pathogens to antibiotics is a challenge to medical health practitioners when it comes to treating and managing most infections caused by MDR bacteria. Our antibiogram results showed varying rates of susceptibility, intermediate and resistance

patterns of the 99 Gram negative bacteria employed in this study. Overall, a high degree of resistance of the isolates to multiple classes of antibiotics used was noted, especially to 3rd-generation cephalosporins where the resistance rates of *E. coli, K. pneumoniae* and *P. aeruginosa* to cefotaxime and ceftazidime were 40 %, 61.5 %, 50 % and 30 %, 38.5 %, 30 % respectively. The resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates to sulphamethoxazole-trimethoprim and gentamicin were 97.5 %, 84.6 %, 100 % and 32.5 %, 43.6 %, 30 % respectively while the resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates to the fluoroquinolones used in our study (ofloxacin and ciprofloxacin) were 65 %, 53.8 %, 65 % and 62.5 %, 59 %, 50 % respectively. The resistance rates of the *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates to imipenem and meropenem were 5 %, 12.8 %, 0 % and 5 %, 7.7 %, 0 % respectively. The susceptibility profile of the Gram negative isolates to the tested bacteria were comparable to a similar study done in Tehran in 2006, in Nigeria in 2008 and in Poland between 2001-2002, which showed a high rate of resistance among *E. coli*, *K. pneumoniae* and *P. aeruginosa* clinical isolates for varying antibiotics including cefotaxime, gentamicin, sulphamethoxazole-trimethoprim, ciprofloxacin, ofloxacin, imipenem and meropenem (Iroha *et al*., 2008; Dzierzanowska-Fangrat *et al*., 2005 and Feizabadi *et al*., 2006). Similar resistance pattern of the Gram negative bacteria used in this study to some commonly available antibiotics were also reported by Aibinu *et al*., (2007) and Pitout *et al*., (2007). Antibiotics as previously reported are the most important risk factors in the development of resistance amongst clinically important pathogens (Jacoby and Munoz-Price, 2005; Walsh *et al*., 2005; Ejikeugwu *et al*., 2016); and this is due in part to the fact that undue exposure of pathogenic bacteria to antibiotics allows the organisms to develop resistance via selective pressure. It is possible that there is a high abuse of antibiotics in the community and/or hospital under study, which warranted the high level of resistance that was envisaged in our study. The natural consequence or phenomenon which allows pathogens to easily adapt to antibiotics (selective pressure imposed on organisms by drugs) is one of the most single reasons why these microorganisms develop antimicrobial resistance as was opine by Livermore and Woodford (2004) and Bush *et al*., (1995). In our study, MBL production in all the 99 clinical isolates was screened by disk diffusion method. Out of a total of 27 Gram negative bacteria that were potential MBL producers in our study, 11 isolates of *E. coli* and 12 isolates of *K. pneumoniae* were suspected to produce MBL while 4 isolates of *P. aeruginosa* isolates were also suspected to produce the enzymes. The result of the confirmation of MBL production showed that 12.5 %, 15.4 % and 10 % of *E. coli*, *K. pneumoniae* and *P. aeruginosa* isolates respectively were positive for MBL production by the disk potentiation method. Comparing the number of potential MBL producers, it was noted in our study that there was a reduction of about 50 % in the actual number of organisms that produced MBLs in relation to those that were suspected initially by the screening test to produce MBLs (as shown in Table 3). The observed prevalence of MBLs in *E. coli* in our study (12.5 %) is lower than studies conducted in India (Chakraborty *et al*., 2010) where the prevalence of MBLs in *E. coli* isolates tested was 28.57 % and in Australia where all 6 *E. coli* isolates tested for MBL production was confirmed to be MBL positive by both the phenotypic and genotypic detection methods (Franklin *et al*., 2006). The prevalence of MBLs in *K. pneumoniae* isolates from our study was 15.4 %. This result however, is in contrasts to the results obtained in India and Australia where the prevalence of MBLs in *K. pneumoniae* isolates were 36.6 % and 100 % respectively (Franklin *et al*., 2006 and Chakraborty *et al*., 2010). MBL production according to reports is found to be more prevalent in *P. aeruginosa* isolates than the *Enterobacteriaceae* (Walsh *et al*., 2005). Despite the fact that our results disputes this findings, imipenem- and meropenem- resistant *P. aeruginosa* isolates that produce MBLs has been reported to be an important cause of hospital-acquired infection, and there emergence and spread poses a challenge to therapeutic use worldwide (Aibinu *et al*., 2007; Deshpande *et al*., 2010; Lolans *et al*., 2005 and Bashir *et al*., 2011). Increase in the prevalence of carbapenem resistance mediated by acquired MBLs has been reported, particularly for *P. aeruginosa* clinical isolates in several countries (Yong *et al*., 2002; Toleman *et al*., 2005; Varaiya *et al*., 2008). This statement however, is not far from the truth since here in Nigeria; reports have shown a prevalence of MBLs in *P. aeruginosa* clinical isolates from Lagos, southwest Nigeria (Aibinu *et al*., 2007). In our study, MBL was detected in 10 % of *P. aeruginosa* isolates. The prevalence of MBL in *P. aeruginosa* isolates (10 %) in our study is noteworthy and it corresponds to similar studies conducted in Mumbai and Kashmir where the prevalence of MBL production in *P. aeruginosa* isolates were 20.8 % and 11.66 % respectively (Varaiya *et al*., 2008 and Bashir *et al*., 2011). However, the prevalence of MBL production in *P. aeruginosa* isolates as shown in our study is lower than the results in Brazil, India and Iran where the prevalence of MBL in *P. aeruginosa* isolates were 22.77 %, 51.28 % and 53.2 % respectively (Saderi *et al*., 2008; Franco *et al*., 2010 and Chakraborty *et al*.,

2010). Conclusively, our study has shown that MBL-producing pathogens are prevalent in Gram negative bacteria. It also shows that these enzymes might be responsible for the spread of nosocomial infections in the hospital under study. Prompt and accurate antimicrobial susceptibility test geared towards detecting multidrug resistant bacteria from clinical specimen is required to contain the emergence and spread of MBL-positive bacteria in hospital environment.

Acknowledgements:

Authors are grateful to the Department of Microbiology, University of Nigeria Teaching Hospital (UNTH), Ituku-Ozalla, Enugu State Nigeria for providing the isolates for this study. We are also grateful to Professor Charles Esimone and Professor Michael Adikwu for their technical assistance.

Corresponding Author:
Ejikeugwu Chika
Department of Applied Microbiology, Faculty of Science, Ebonyi State University, P.M.B. 053, Abakaliki, Ebonyi State, Nigeria
ejikeugwu_chika@yahoo.com
+2348097684562

References

1. Aibinu I., Nwanneka T and Odugbemi T (2007). Occurrence of ESBL and MBL in clinical isolates of *Pseudomonas aeruginosa* from Lagos, Nigeria. *Journal of American Sciences*, 3(4):81-85.
2. Bashir D., Thokar M.A., Fomda B.A., Bashir G., Zahoor D., Ahmad S and Toboli A.S (2011). Detection of metallo – beta – lactamase (MBL) producing *Pseudomonas aeruginosa* at a tertiary care hospital in Kashmir. *African Journal of Microbiology Research*, 5(2):164-172.
3. Bush K., Jacoby G.A and Medeiros A.A (1995). A Functional Classification of Scheme for Beta – Lactamases and its Correlation to Molecular Structure. *Antimicrob. Agents Chemother*, 39:1211-1233.
4. CLSI (2004). Performance standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Information Supplemenet. Updated tables for the NCCLS antimicrobial susceptibility testing standard. M31–A2.
5. Cheesbrough M (2000). District Laboratory Practice in Tropical Countries. 2nd edition. Cambridge University Press, UK.
6. Deshpande P., Rodrigues C., Shetty A., Kapadia F., Hedge A and Soman R (2010). New Delhi Metallo – β – lactamase (NDM-1) in *Enterobacteriaceae*: Treatment options with Carbapenems Compromised. *Japi*, 58:147-149.
7. Dzierzanowska-Fangrat K., Semczuk K., Łopaciuk U., Kurlenda J., Matafiej E., Puacz E., Stalmaska-Dworak E and Dzierzanowska D (2005). Antimicrobial susceptibility of aerobic microorganisms isolated from intra-abdominal infections in pediatric patients in Poland. *Med Sci Moniti,* 11(5):241-245.
8. Ejikeugwu C, Iroha I, Duru C, Ayogu T, Orji O, Eze J, Adikwu M, Esimone C (2016). Occurrence of Metallo-Beta-Lactamase-Producing *Enterobacteriaceae* in Abakaliki, Nigeria. *International Journal of Applied Pharmaceutical Sciences and Research*, 1(2):70-75.
9. Ejikeugwu P.C, Ugwu C.M., Iroha I.R., Eze P, Gugu T.H and Esimone C.O (2014). Phenotypic Detection of Metallo-β-Lactamase Enzyme in Enugu, Southeast Nigeria. *American Journal of Biological, Chemical & Pharmaceutical Science,* 2(2):1-6.
10. Franklin C., Liolios L and Peleg A.Y (2006). Phenotypic Detection of Carbapenem – Susceptible Metallo – β – Lactamase – Producing Gram-Negative Bacilli in the Clinical Laboratory. *Journal of Clinical Microbiology*, 44(9):3139-3144.
11. Franco M.R.G., Caiaffa-Filho H.H., Burattini M.N and Rossi F (2010). Metallo – beta – lactamases among imipenem –resistant *Pseudomonas aeruginosa* in a Brazilian University hospital. *Clinics*, 65(9):825-829.
12. Feizabadi M.M., Etemadi G., Yadegarinia D., Rahmati M., Shabanpoor S and Bokaei S (2006). Antibiotic-resistance patterns and frequency of extended-spectrum β-lactamase – producing isolates of *Klebsiella pneumoniae* in Tehran. *Med Sci Monit*, 12(11):362-365.
13. Iroha I.R., Amadi S.E., Adikwu M.U and Esimone C.O (2008). Detection of Plasmid Borne Extended Spectrum Beta – Lactamase enzymes in Clinical Isolates of *Escherichia coli* from a Community General Hospital. *International Journal of Molecular Medicine and Advance Sciences*, 4(2):46-49.
14. Jacoby G.A and Munoz-Price L.S (2005). Mechanisms of Disease: The New β-Lactamases. *N Engl J Med*, 352:380-91.
15. Lolans K., Queenan A.M., Bush K., Sahud A and Quinn J.P (2005). First Nosocomial Outbreak of *Pseudomonas aeruginosa* Producing and Integron – Borne Metallo-β-Lactamase (VIM-2) in the United States. *Antimicrobial agents Chemotherapy*, 49(8):3538-3540.

16. Livermore D.M and Woodford N (2004). Laboratory Detection and Reporting of Bacteria with Extended – Spectrum β – Lactamases. Health Protection Agency: Guidelines to Diagnostic Laboratories. Pp.1-4.
17. Libisch B., Muzslay M., Gacs M., Minarovits J., Knausz M., Watine J., Ternak G., Kenez E., Kustos I., Rokusz L., Szeles K., Balogh B and Fuzi M (2006). Molecular Epidemiology of VIM-4 Metallo – β – Lactamase – producing *Pseudomonas* spp. isolates in Hungary. *Antimicrobial Agents Chemotherapy*, 50(12):4220-4223.
18. North Yorkshire and York Primary Care Trust, NYYPCT (2008). Multi – Resistant Gram-Negative Bacteria including ESBLs (Extended Spectrum Beta Lactamases). Version 1.00. Pp.1-11.
19. Pitout J.D.D., Chow B.L., Gregson D.B., Laupland K.B., Elsayad S and Church D.L (2007). Molecular Epidemiology of Metallo-β-Lactamase – Producing *Pseudomonas aeruginosa* in the Calgary Health Region: Emergence of VIM-2- Producing Isolates. *Journal of Clinical Microbiology*, 45(2):294-298.
20. Saderi H., Karimi Z., Owlia P., Bahar M.A., Rad S.M (2008). Phenotypic Detection of Metallo – beta-lactamase producing *Pseudomonas aeruginosa* strains isolated from Burned Patients. *Iranian Journal of Pathology*, 3(1):20-24.
21. Toleman M.A., Biedenbach D., Bennett D.M.C., Jones R.N and Walsh T.R (2005). Italian metallo – β – lactamases: a national problem? Report from the SENTRY Antimicrobial Surveillance Programme. *Journal of Antimicrobial Chemotherapy*, 55:61-70.
22. Varaiya A., Kulkarni N., Kulkarni M., Bhalekar P and Dogra J (2008). Incidence of metallo beta lactamases producing *Pseudomonas aeruginosa* in ICU patients. *Indian J Med Res*, 127:398-402.
23. Walsh T.R., Toleman M.A., Poirel L and Nordmann P (2005). Metallo β – Lactamases: the Quiet before the Storm? *Clinical Microbiology Review*, 18(2):306-325.
24. Yong D., Lee K., Yum J.H., Shin H.B., Rossolini G.M and Chong Y (2002). Imipenem – EDTA Disk Method for Differentiation of Metallo-β-Lactamase – Producing Clinical Isolates of *Pseudomonas* spp. and *Acinetobacter* spp. *Journal of Clinical Microbiology*, 40 (10):3798-3801.

10/18/2016