Evaluation of Metallo-Β-Lactamase Enzyme Among Gram Negative Bacterial Isolates From Patients Admitted In A Tertiary Care Hospital, India.

[1*]Dr. Moumita Adhikary, [2 *]Dr. Partha Sarathi Chakrabarty
Assistant Professor, Department of Microbiology, College of Medicine & Sagore Dutta Hospital, Kamarhati, Kolkata
Corresponding Author: Dr. Partha Sarathi Chakrabarty

Abstract: *Resistance of metallo-β-lactamase has emerged worldwide among Gram-negative bacilli. There are several non-molecular techniques which take advantage of the enzyme's zinc dependence by using chelating agents, such as EDTA to inhibit its activity. Our laboratory has chosen some simple and cost-effective phenotypic MBL detection methods which will identify such organisms. This study was carried out from 1stJanuary 2015 to 31stDecember 2016 in a tertiary care hospital, Kolkata, West Bengal, India. Urine, throat swabs and wound swabs were collected from patients after admission. GNB were isolated and antibiotic resistance were evaluated by Kirby Bauer's disc diffusion method. MBL was detected by four different tests i.e., Modified Hodge Test (MHT), Double disc synergy test(DDST), combined disk test(CMDT) and E-test. Out of 590 samples collected from the patients admitted in the hospital, growth were seen in 271 samples out of which GNB were seen in 191 cases, where 48 (25.13%) cases showed MBL. Urine for culture was highest in number 88.47% followed by wound/pus swab 7.1% and least with throat swab 4.40%. MBL in urine were 81.2%, wound swab 1.87% and throat swab had no MBL. Out of a total of 191 GNB most common isolate was Escherichia coli 44% followed by Klebsiella pneumoniae 18%, Pseudomonas aeruginosa 16%, Acinetobacter baumannii 16%, Proteus mirabilis 6% and Proteus vulgaris 5%. Screening for MBL was done by CMDT (most sensitive) 25.13%. DSST showed almost similar prevalence i.e., 23.56%. Highest cases of MBL were found from Klebsiella pneumoniae 42.85% followed by Acinetobacter baumannii 14.28%, Pseudomonas aeruginosa 39.09% and Proteus vulgaris 22.22%. Thus, treatment of the patients infected with MBL producer is formidable and demands challenge with the current limited antibiotic options with Colistin, Polymyxin B and Tigecycline. Therefore, a meticulous epidemiological survey and routine standardization of CMDT is necessary.*
Keywords: *Metallo-β-Lactamase(MBL), Gram negative bacilli(GNB), Combined Disc test(CMDT)*

Date of Submission: 18-12-2017   Date of acceptance: 28-12-2017

I.   Introduction

        Metallo-β-lactamase has emerged worldwide as powerful resistance determinant among Gram-negative bacilli. They hydrolyse virtually all classes of β-lactams including Carbapenems, which often represent the last option for the treatment of infections with multidrug resistant Gram-negative bacteria.(1)(2) So, the clinical utility of carbapenems are under threat with the emergence of acquired carbapenemases, particularly Ambler class B metallo-β-lactamases (MBLs).(2)Ambler and others have classified carbapenemase broadly into two types based on the reactive site of the enzymes. One is Serine carbapenemases and the other is Metallo-β-lactamases. Further, on the basis of amino acid sequences they have been divided in four groups (A-D) out of which A (e.g.; *Klebsiella pneumoniae* carbapenemase (KPC), Guiana extended spectrum(GES) and D (e.g.;OXA), belong to the serine group while group B alone belong to the MBL. While, Ambler Group A and D are sensitive to clavulanic acid and oxacillin respectively, MBL of Ambler class B are clavulanic acid resistant enzymes. They require divalent cation of zinc as co-factor for enzymatic activity and are universally inhibited by EDTA as well as other chelating agents of divalent cation(3)(4). Five such enzyme types have been identified (IMP, VIM, SPM, GIM, and SIM types). Also, a new strain which has recently emerged is NDM (New Delhi MBL), expressing high level resistance to all penicillins, cephalosporins, aztreonam, cefoxitin, carbapenems and ciprofloxacin and was susceptible only to colistin.(4)

        Detection of carbapenemase is difficult. Several non-molecular techniques have been studied, all taking advantage of the enzyme's zinc dependence by using chelating agents, EDTA or 2-mercaptopropionic acid, to inhibit it's activity(4)(5). There are few inhibitor tests which help to identify MBL producers in which the test seeks synergy between Carbapenems and EDTA. The inhibitor used is mostly EDTA which chelates the zinc ions and thus there is loss of Carbapenemase activity(6). Our laboratory has chosen some simple and cost-effective phenotypic MBL detection methods which will identify such organisms. They are the Modified Hodge

test (MHT), double disc synergy test, combined disk test and E-test. We have validated our report by using a broad range of bacterial species using phenotypic methods and also by a statistical analysis.

II.   Materials And Methods

This prospective observational study was carried out in College of Medicine and Sagore Dutta Hospital, Kamarhati, Kolkata, West Bengal, India. Tests for the study were performed in the department of Microbiology, College of Medicine & Sagore Dutta Hospital, Kamarhati, Kolkata from 1stJanuary 2015 to 31stDecember 2016 (2 years). Samples like urine, throat swabs and wound swabs were collected from patients who have developed any type of infection like UTI, pneumonia and wound infections respectively, after admission in this tertiary care hospital. Processing of the clinical samples for identification of the microbial agent causing infection in patients already admitted to the hospital was done routinely. Total number of patient suffering from Gram negative infection was noted. Different culture media such as blood agar, MacConkey's agar and UTI chrome agar to determine colonial morphology and biochemical methods were utilized for phenotypic identification. Antibiotic resistance pattern of these Gram negative bacteria, mainly with respect to carbapenems where evaluated i.e., screening them for carbapenemase enzyme by standard Kirby Bauer's disc diffusion method. Ambler group B metallo β-lactamase enzyme was detected by four different phenotypic tests. The tests being Modified Hodge Test (MHT), Double disc synergy test (DDST), Combined disk test (CMDT) and E-test.

2.1 Double disk synergy test (DDST)

This test is performed by inoculating the tested organism onto MHA plate as recommended by CLSI(7). A 10μg meropenem disk and a blank filter paper disk 6 mm in diameter were placed 10 mm apart from edge to edge, then, 10 μl of 0.5 molar EDTA solution was applied to the blank disk and incubated at 37ºC for 18 hours. The presence of extension of zone towards the impregnated EDTA disk was interpreted as EDTA synergy test positive.

2.2 Combined EDTA disk test (CMDT)

An overnight broth culture of the test strain with an opacity adjusted to 0.5 McFarland standards was used to inoculate a plate of Mueller-Hinton agar as recommended by CLSI(7). After drying of MHA plate, a 10 μg Meropenem disk and meropenem disk combined with EDTA was placed 20 mm apart and incubated at 37 ºC overnight. After 24 hours, an increase in the zone size of at least 7 mm around the Meropenem combined EDTA impregnated disk compared to Meropenem disks alone was recorded as MBL producing strains.

2.3 E-Test

Metallo-β-lactamase enzyme and MIC of Meropenem were tested by using E-test which was a plastic strip impregnated in one half with Meropenem gradient against seven dilution (0.125, 0.19, 0.25, 0.38, 1.0, 1.5, 2, 3, 4, 8 μg/ml) and on the other end with Meropenem overlapped with constant concentration of EDTA ranging from 0.032-2 μg/ml. Tested colonies from overnight culture were suspended with 0.9% of normal saline (NaCl) to match the turbidity of 0.5 McFarland's standard. Test organism was inoculated on Mueller-Hinton agar plate by using a sterile cotton swab to produce a uniform layer and dried for about 15 min. E-test MBL strip was applied and the plate was incubated for 16 to 18 hours at 37ºC. MIC end points were read where the inhibition ellipses intersected the strip. MIC ratio of Meropenem/Meropenem+EDTA (MRP/MRPE) was calculated. A positive MBL test was decided if the value of (MRP/MRPE) is > 8 or if there was a zone of deformation insensitive area or appearance of phantom zone along the strip, according to the manufacturer recommendation(HIMEDIA)(7).

2.4 Modified Hodge Test (MHT)

E. coli ATCC® 25922 (the indicator organism) in saline was prepared and matched with 0.5 McFarland standard suspension. This broth was further diluted in a proportion of 1:10 in saline. Now this E. coli ATCC 25922 solution was inoculated on MHA plate just like the routine disk diffusion procedure and plate is allowed to dry for 3–10 minutes. Meropenem disk 10μg was placed on the centre of the plate. Then a positive control i.e., *K. pneumoniae* ATCC® BAA-1705, a negative control i.e., *K. pneumoniae* ATCC® BAA-1706 and the test organism were streaked linearly as straight line from the periphery of the plate into the direction of Meropenem disk at the centre from 3 different sites and the test plate was incubated for 18 hours at 37ºC according to standard guidelines of CLSI(7). Qualitative data analysis was done by Chi-square test. Null hypothesis was rejected when p value <0.05. Results were presented as follows: Qualitative data – Pie chart and Bar graph. Standard statistical software package SPSS version 18 and Microsoft Excel were used. There was no ethical controversy and conflict of interest.

III. Result

Out of 590 samples collected from the patients admitted in the hospital, growth were seen in 271 samples out of which Gram negative bacilli were seen in 191 cases. Out of which MBL were found in 48 (25.13%) cases.(Figure 1) Samples collected for the test were urine, throat swab and wound/pus swab where urine for culture was highest in number 522/590 (88.47%) followed by wound/pus swab culture 42/590 (7.1%) and least for culture was throat swab 26/590 (4.40%). Out of 223/522(42.7%) growth in urine samples, Gram negative bacilli were seen in 165/223 (74%) cases, out of which MBL were seen in 39/48(81.2%) cases. On the other hand, 42 cases of wound swab showed growth in 39/42(92.85%) cases out of which 22/39(56.41%) showed Gram negative bacilli with 9/48(1.87%) MBL. Among 26 throat swab, growth were seen in 9/26(34.61%) cases out of which 4/9(44.4%) cases were seen to grow Gram negative bacilli with no cases of MBL.(Figure 1) Out of a total of 191 Gram negative cases most common isolate was *Escherichia coli* 84/191(44%) followed by *Klebsiella pneumoniae* 35/191(18%), *Pseudomonas aeruginosa* 31/191(16%), *Acinetobacter baumannii* 21/191(16%), *Proteus mirabilis* 11/191(6%) and *Proteus vulgaris* 9/191(5%). (Figure 2) Screening for MBL was done by CMDT. Among the four tests done i.e., DDST, CMDT, MHT and E-test, most sensitive was found to be the CMDT which was 48/191(25.13%). DDST showed almost similar prevalence i.e., 45/191(23.56%). Least was found with MHT 26/191(13.61%) and E- test 23/191(12.04%). (Figure 4: A, B, C)(Table 1)(p<0.05) Highest percentage of cases of MBL were found from *Klebsiella pneumoniae* 15/45(42.85%) followed by *Acinetobacter baumannii* 12/84(14.28%), *Pseudomonas aeruginosa* 11/31(39.09%) and *Proteus vulgaris* 2/9(22.22%) Least was seen among *Escherichia coli* 12/84(14.28%). *Proteus mirabilis* showed no cases of MBL. (Figure 3)
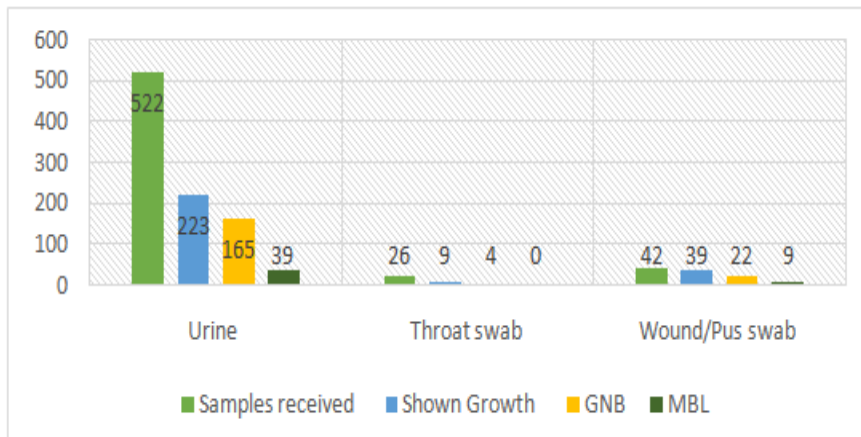

Figure:1. Shows the type and number of samples, their rate of Growth,
Gram Negative bacilli and among them growth of Metallo-β-Lactamase producing strain.
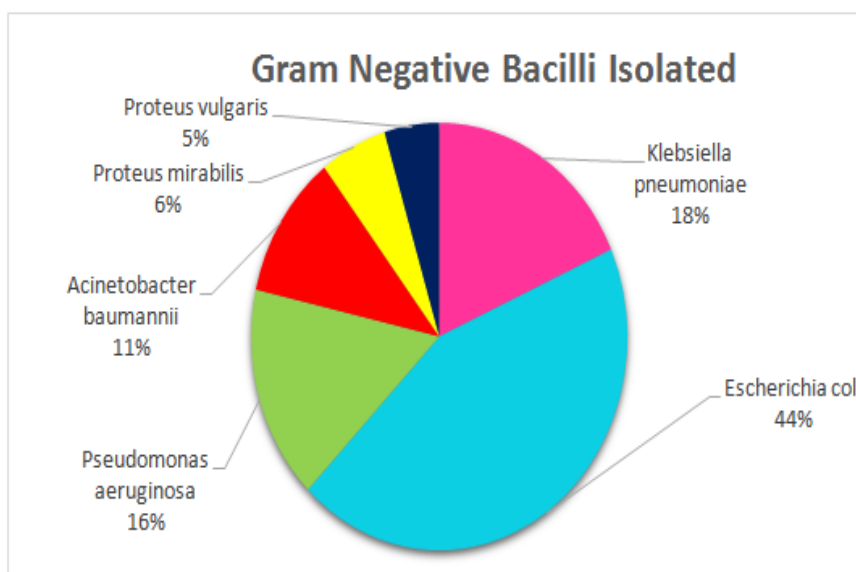

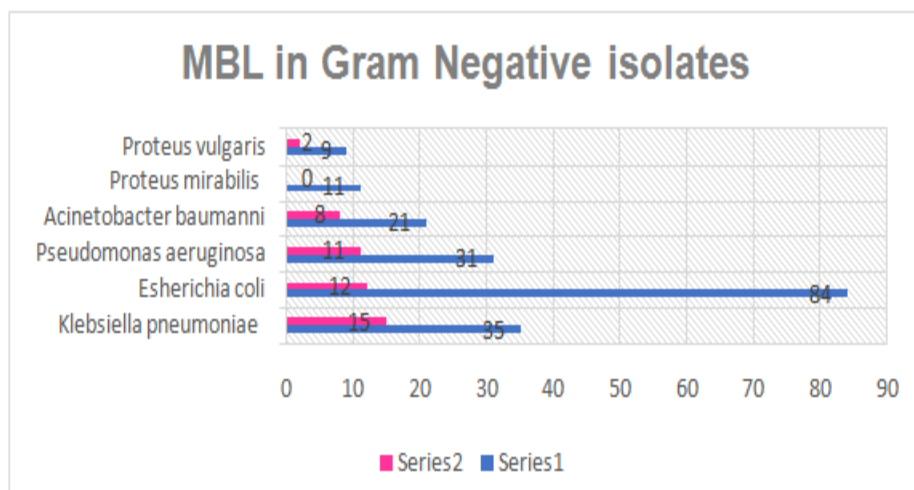**Figure 2:** Organisms found among Gram negative bacilli

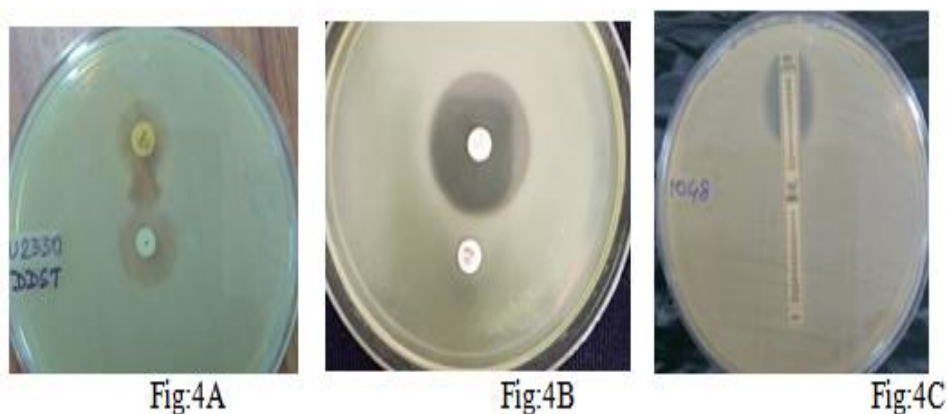**Figure 3:** Shows Metallo-β-Lactamase producing Gram negative organisms

Figure 4: A-DDST, B-CMDT and C-E-test

Table 1: Shows rate of MBL in different Gram negative isolates

Organism	DDST	CMDT	MHT	E test
Klebsiella pneumoniae	14(31.11%)	15(31.25%)	8(30.76%)	7(30. 43%)
Esherichia coli	12(26.66%)	12(27.08%)	7(26.92%)	6(26.08%)
Pseudomonas aeruginosa	10(22.22%)	11(22.91%)	6(23.07%)	5(21.73%)
Acinetobacter baumannii	7(15.55%)	8(16.66%)	4(15.38%)	3(13.04%)
Proteus mirabilis	0(0.00%)	0(0.00%)	0(0.00%)	0(0.00%)
Proteus vulgaris	2(4.44%)	2(4.16%)	1(3.84%)	2(8.69%)
Total MBL	45(23.56%)	48(25.13%)	26(13.61%)	23(12.04%)

IV.   Discussion

The production of MBL among Gram negative isolates among many geographical regions, in recent past years have been intriguing. The worldwide spread of acquired MBL in clinically important pathogens such as members of *Enterobacteriaceae*, *Pseudomonas spp.* and *Acinetobacter spp.* are a great concern(8). Infections with these organism pose a serious therapeutic challenge, implying poor patient prognosis, even death(9)(10)(11). So, detection of MBL producing organisms in the clinical microbiology laboratory has become a major concern for the choice of appropriate therapeutic scheme and implementation of infection control measures(12).Out of 590 samples collected from the patients admitted in the hospital, growth were seen in 271 samples out of which Gram negative bacilli were seen in 191 cases. Out of which MBL were found in 48 (25.13%) cases (Figure 1) which was almost similar to a study done by Khande et al(13). Samples collected for the test were urine, throat swab and wound/pus swab were urine for culture was highest in number 522/590 (88.47%) followed by wound/pus swab culture 42/590 (7.1%) and least for culture was throat swab 26/590 (4.40%). Out of 223/522(42.7%) growth in urine samples, Gram negative bacilli were seen in 165/223 (74%)

cases, out of which MBL were seen in 39/48(81.2%) cases. On the other hand 42 cases of wound swab showed growth in 39/42(92.85%) cases out of which 22/39(56.41%) showed Gram negative bacilli with 9/48(1.87%) MBL. Among 26 throat swab, growth were seen in 9/26(34.61%) cases out of which 4/9(44.4%) cases were seen to grow Gram negative bacilli with no cases of MBL. (Figure 1) Therefore growth of Gram negative bacilli and MBL production among the urine pathogens were maximum (81.2%) followed by wound swab (1.87%) with no MBL strains among throat swab which was unlike a study done by Franklin et al where maximum MBL isolate were found to be from respiratory tract specimens 34(40%) while wound swab showed 8(10%) and urine only 5(6%)(14). This was probably because urine samples in our study were more, and also our specimens were collected from all patients admitted in the tertiary care hospital unlike Franklin et al where sample were exclusively from intensive care unit. Out of a total of 191 Gram negative cases most common isolate was Escherichia coli 84/191(44%) followed by *Klebsiella pneumoniae* 35/191(18%), *Pseudomonas aeruginosa* 31/191(16%), *Acinetobacter baumannii* 21/191(16%), *Proteus mirabilis* 11/191(6%) and *Proteus vulgaris* 9/191(5%) which was similar to Shamim M et al(15). (Figure 2)Screening for MBL was done by CMDT. Among the four tests done i.e., Double disk synergy test, Combination Disk Method, Modified Hodge method and E-test, most sensitive was found to be the Combination disk synergy test which was 48/191(25.13%) which was similar to Ghazzawy I et al(16). DSST showed almost similar prevalence i.e., 45/191(23.56%). Least was found with MHT 26/191(13.61%) and E- test 23/191(12.04%) which was similar to Khanda et al(13). (Figure 4:A,B,C) (Table 1) (p<0.05)Highest percentage of cases of MBL were found from *Klebsiella pneumoniae* 15/45(42.85%) followed by *Acinetobacter baumannii* 12/84(14.28%), *Pseudomonas aeruginosa* 11/31(39.09%) and *Proteus vulgaris* 2/9(22.22%) Least was seen among *Escherichia coli* 12/84(14.28%). *Proteus mirabilis* showed no cases of MBL which was similar to a study done by Rasheed J K et al (17)and Struelens M J et al(18). (Figure 3)

V. Conclusion

Thus to conclude, metallo-β-lactamase containing pathogenic strains from various clinical samples are a constant niggle among the clinicians. The present perspective observational study shows a preponderance of Gram negative bacilli among various clinical samples collected from the patients admitted in a tertiary care hospital. Also among them a high rate of MBL producers were observed. Most common isolate producing MBL was Klebsiella pneumoniae which is a proven fact from other research articles. Others being *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Escherichia coli.* Treatment of the patients infected with these MBL producer is formidable and demands challenge with the current limited antibiotic options with only Colistin, Polymyxin B and Tigecycline depending on the type of clinical infection, where these antibiotics are toxic with a lot of side effects. In order to avoid this situation a meticulous epidemiological survey of various clinical samples should be done to isolate and identify the MBL strains from patients admitted to the hospital and to validate it by both phenotypic and genotypic methods and also to specify proper antibiotic usage to the clinicians. Also on the basis of our study, we concluded Combination Disc Synergy test to be the most sensitive among all phenotypic tests for the detection of MBL which is also easy to perform method. Therefore, an introduction of this test can be standardised to be done routinely along with the Kirby Bauer's disc diffusion method in microbiological laboratories for identification of these treacherous MBL strains.

5.1 Further Scope

Confirmation of the isolated Gram negative bacilli by MALDI-TOF and genotypic confirmation of the MBL strains by PCR for identification of particular genes IMP, VIM, SPM, GIM, SIM types and also, a new strain which has recently emerged i.e., NDM (New Delhi MBL).

Acknowledgements

References

[1].   Bonomo RA. New Delhi metallo-??-lactamase and multidrug resistance: A global SOS? Clinical Infectious Diseases. 2011;52(4):485–7.
[2].   Forozesh Fard M, Irajian G, Moslehi Takantape Z, Fazeli H, Salehi M, Rezania S. Drug resistance pattern of Pseudomonas aeruginosa strains isolated from cystic fibrosis patients at Isfahan AL Zahra hospital, Iran (2009-2010). Iran J Microbiol. 2012;4(2):94–7.
[3].   Butt T, Usman M, Ahmad RN, Saif I. Emergence of metallo-beta-lactamase producing Pseudomonas aeruginosa in Pakistan. J Pak Med Assoc. 2005;55(7):302–4.
[4].   Walsh TR, Toleman MA, Sarma JB, Irfan S, Woodford N, Livermore DM. New Delhi metallo-??-lactamase 1 - Authors' reply. Vol. 10, The Lancet Infectious Diseases. 2010. p. 752–4.

[5].   Yan JJ, Wu JJ, Tsai SH, Chuang CL. Comparison of the double-disk, combined disk, and Etest methods for detecting metallo-β-lactamases in gram-negative bacilli. Diagn Microbiol Infect Dis. 2004;49(1):5–11.
[6].   Rolain JM, Parola P, Cornaglia G. New Delhi metallo-beta-lactamase (NDM-1): Towards a new pandemia? Clinical Microbiology and Infection. 2010;16(12):1699–701.
[7].   CLSI . Performance standards for antimicrobial susceptibility testing. CLSI supplement M100S. CLSI supplement M100S. Wayne, PA :Clinical and Laboratory Standards Institute. 2017.
[8].   Tam VH, Chang KT, LaRocco MT, Schilling AN, McCauley SK, Poole K, et al. Prevalence, mechanisms, and risk factors of carbapenem resistance in bloodstream isolates of Pseudomonas aeruginosa. Diagn Microbiol Infect Dis. 2007;58(3):309–14.
[9].   Hirakata Y, Yamaguchi T, Nakano M, Izumikawa K, Mine M, Aoki S, et al. Clinical and bacteriological characteristics of IMP-type metallo-beta-lactamase-producing Pseudomonas aeruginosa. Clin Infect Dis. 2003;37(1):26–32.
[10].   Laupland KB, Parkins MD, Church DL, Gregson DB, Louie TJ, Conly JM, et al. Population-based epidemiological study of infections caused by carbapenem-resistant Pseudomonas aeruginosa in the Calgary Health Region: importance of metallo-β-lactamase (MBL)-producing strains. J Infect Dis [Internet]. 2005;192(9):1606–12.
[11].   Peleg AY, Franklin C, Bell JM, Spelman DW. Dissemination of the metallo-beta-lactamase gene blaIMP-4 among gram-negative pathogens in a clinical setting in Australia. Clin Infect Dis. 2005;41(11):1549–56.
[12].   Miriagou V, Cornaglia G, Edelstein M, Galani I, Giske CG, Gniadkowski M, et al. Acquired carbapenemases in Gram-negative bacterial pathogens: Detection and surveillance issues. Vol. 16, Clinical Microbiology and Infection. 2010. p. 112–22.
[13].   Khanda Abdulateef Anoar, Omer Kaafaasa. Detection of metallo β -lactamase enzyme in some gram negative bacteria isolated from burn patients in sulaimani city , Iraq. Eur Sci J. 2014;10(3):485–96.
[14].   Franklin C, Liolios L, Peleg AY. Phenotypic detection of carbapenem-susceptible metallo-??-lactamase- producing gram-negative bacilli in the clinical laboratory. J Clin Microbiol. 2006;44(9):3139–44.
[15].   Mumtaz S, Zafar S, Ahmad M. Original Article Frequency of Enteric Gram Negative Rods Isolated from Various Clinical Samples. 2012;2012(2):93–6.
[16].   Iman FEG, Marwa AM, Doaa AY. Phenotypic and genotypic methods for detection of metallo beta lactamases among carbapenem resistant Enterobacteriaceae clinical isolates in Alexandria Main University Hospital. African J Microbiol Res [Internet]. 2016;10(1):32–40.
[17].   Rasheed JK, Kitchel B, Zhu W, Anderson KF, Clark NC, Ferraro MJ, et al. New Delhi metallo-??-lactamase-producing enterobacteriaceae, United States. Emerg Infect Dis. 2013;19(6):870–8.
[18].   Struelens MJ, Monnet DL, Magiorakos a P, Santos O'Connor F, Giesecke J. New Delhi metallo-β-lactamase 1-producing Enterobacteriaceae: emergence and response in Europe. Euro Surveill [Internet]. 2010;15(46):1–10.