ORIGINAL RESEARCH

Molecular Characterization of Metallo Beta Lactamase Producing Clinical Isolates of Acinetobacter

Shyamasree Nandy[1], Ashish K. Asthana[2], Molly Madan[3]

ABSTRACT

Introduction: Metallo-beta-lactamase (MBL)-producing Acinetobacter is a therapeutic challenege all over the world. The aims of this study were to determine the prevalence of MBL genes and evaluate the antimicrobial susceptibility profile of carbapenem non-susceptible isolates of Acinetobacter.
Material and Method: During a period of 12 months (November 2012 to October 2013) 94 isolates of carbapenem non susceptible Acinetobacter were collected from different clinical specimens. All isolates were tested for antimicrobial susceptibility by Kirby-Bauer disk diffusion method. Carbapenem non-susceptible isolates were further screened for production of MBL by DDST, CDT with Imipenem, Meropenem and EDTA,Etest and were then subjected to PCR for detection of MBL genes.
Result: Among 94 Carbapenem (resistant to either or both Imipenem and Meropenem) non-susceptible isolates of Acinetobacter 25 (26.59%) were found to be MBL producers. Of 25 MBL-producing isolates, 24% carried the bla(IMP) gene and 76% carried the bla(VIM) gene. All MBL-producing isolates were multidrug resistant.
Conclusion: Our study was intended to find out the prevalence of MBL in Acinetobacter (26.59% of total carbapenem resistant isolates). All the MBL producing isolates were resistant to almost all the group of drugs except Tigecycline(100% sensitivity).This indicate that generally MBL producing isolates carry multidrug resistant integrons. Hence,identification and AST of MBL producing Acinetobacter is essential for proper prophylaxis.

Keywords: Metallo beta lactamases, Acinetobacter, bla VIM, bla IMP.

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[1]phD Student, [2]Assistant Professor, [3]Professor and HOD, Department of Post Graduate Studies, Department of Microbiology, Subharthi Medical College, Subharthi University

Corresponding author: Shyamasree Nandy, Department of Post Graduate Studies of Microbiology, Subharthi Medical College, Subharthi University

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INTRODUCTION

One of the most important hospital pathogen which has been added recently to the Infectious Diseases Society of America's dangerous pathogens hit-list is A.baumannii.[1] The combination of its environmental resilience and its wide range of resistance determinants renders it a successful nosocomial pathogen.[2]
Among various species of Acinetobacter, A. baumannii (AB) accounts for about 80% of infections.It acts as an opportunistic pathogen in humans, affecting immunocompromised patients.Thus it is becoming increasingly important cause of nosocomial infection.A.baumanni infections typically occur in critically ill, hospitalized patients.Community-acquired infections are more common in tropical climates.[3]
Acinetobacter can survive on dry surfaces for up to a month and are commonly carried on the skin of health care workers. As a result this increases the chances of patients being colonized and medical equipment being contaminated. Infact *A. baumannii* first received huge attention when severe wound infections, burn wound infections and osteomyelitis were reported in soldiers who had major injuries during military operations in Iraq or Afghanista. They were then send back to the USA or the UK. These isolates were often MDR. It was assumed that the organism might have been inoculated at the time of injury. The source might be skin or contaminated soil. However, it is now considered that the soldiers acquired their infecting organism during emergency care at field hospitals or following cross-transmission during their hospitalization in military hospitals.[4]
Acinetobacter can acquire new mechanism of resistance via plasmids, integrons, and transposons. Many

outbreak strains of A. baumannii have a class 1 integron. Acinetobacter produces various enzymes for degrading antibiotics. Genes coding for these enzymes passes from cell to cell via plasmids, integrons, and transposons. Mutated genes can also be acquired from other bacteria.[5]

Way back in 1993 there was only one reported case of transferable MBL. Over last 2 decades the cases of MBL production in Acinetobacter has increased significantly. MBL open the β-lactam ring with the help of a metal cofactor. It can degrade all classes of β-lactams except monobactams. The MBLs were discovered in the mid-1960s.They were initially regarded as resistance determinants of low clinical importance, compared to serine-β-lactamases. For, they were detected in only a few species of minor pathogenic potential (e.g., *Bacillus cereus, Stenotrophomonas maltophilia*, some *Aeromonas* species, a cluster of strains of *Bacteroides fragilis*, and some flavobacteria). That view changed completely with the appearance of acquired MBLs in late eighties. IMP-1 was the first acquired MBL to be identified. It was discovered in the early 1990s (late eighties) in Japanese hospitals in carbapenem-resistant isolates of *Serratia marcescens, P. aeruginosa*, and other gram-negative pathogens. Initially it was thought that acquired MBLs encoded by genes carried on mobile DNA elements, among major gram-negative pathogens, including members of the family Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Acinetobacter* species is an isolated problem being reported only from Japan in a negligible number of cases. But during the past decade the MBLs has spreaded all over the world rapidly and the cases are rising.[6] The present study aims to determine the prevalence of MBL producing Acinetobacter in a tertiary care teaching hospital.

MATERIAL AND METHOD

The strains of Acinetobacter were collected from clinical samples including pus,urine,blood,endotracheal aspirate ,other body fluid (i.e. sputum,peritoneal fluid etc), swab (i.e swab from pus,wound,throat swab etc). The MIC of Imipenem & Meropenem were determined by broth dilution method.[7] The isolates that had an MIC >8µg/ml were considered as carbapenem resistant.[8] Any isolate of Acinetobacter that were either or both resistant to Imipenem or Meropenem were screened for MBL production.All 94 isolates were non duplicate. The strains were stocked in 16% glycerol broth at -20 ^{0}C.

Control strain used: P. aeruginosa ATCC 27853

Preparation of EDTA

A 0.5 M EDTA solution was prepared by dissolving 93.05 gm of disodium EDTA. $2H_2O$(HiMedia Labs), in 500ml of distilled water.[9] Ph was adjusted to 8 by adding NaOH. The mixture was sterilized by autoclaving.10 µl of 0.5M EDTA was added to Imipenem and Meropenem disc and Blank disc, dried and stored at -20^{0}C for further use. It was seen that EDTA discs retained its efficacy till 16 weeks. EDTA solution can also be stored at -20^{0}C. But adding EDTA during every test is a cumbersome process. Hence,we had prepared the discs ,stored and had used them within 16 weeks.

Imipenem (IMP)-EDTA combined disc test:

The IMP-EDTA combined disk test was performed as described by Yong *et al*, 2002.[9] Test organisms was inoculated on to Mueller Hinton agar as recommended by the CLSI (M100-S22).[8] One 10 µg Imipenem disks (Himedia) and one Imipenem EDTA disc was placed. Opacity was adjusted to 0.5 McFarland opacity standards. The inhibition zones of the Imipenem and Imipenem-EDTA disks was compared after 16 to 18 hours of incubation in air at 35°C. In the combined disc test, if the increase in inhibition zone with the Imipenem and EDTA disc was ≥ 7 mm than the Imipenem disc alone, it was considered as MBL positive.

Meropenem EDTA combined disc test

The MRP-EDTA combined disk test was performed as described by Walsh et al, 2005[10] & Varaiya et al, 2008.[11] Test organisms was inoculated on to plates with Mueller Hinton agar as recommended by the CLSI (M100-S22).[8] One 10 µg Meropenem disks (Himedia) and one Meropenem EDTA disc was placed. Opacity was adjusted to 0.5 McFarland opacity standards. The inhibition zones of the Imipenem and Meropenem-EDTA disks was compared after 16 to 18 hours of incubation in air at 35°C. In the combined disc test, if the increase in inhibition zone with the Meropenem and EDTA disc was ≥ 7 mm than the Meropenem disc alone, it was considered as MBL positive.

Imipenem-EDTA double disc synergy test (DDST):

This test was performed as described by Lee et al, 2003.[12] Organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI (M100-S22).[8] An Imipenem (10 µg) (Himedia) disc was placed 20 mm centre to centre from another EDTA(750 ug) disc. Opacity was adjusted to 0.5 McFarland opacity standards. Enhancement of the zone of inhibition in the area between Imipenem and the EDTA disc in comparison with the zone of inhibition on the far side of the drug was interpreted as a positive result.

MBL Epsilometer test

The isolates found to be producing MBL was further tested by MBL E Test strip (Himedia).The strip is coated with mixture of Imipenem+ EDTA on upper half with highest concentration tapering downwards and the lower half is coated with Imipenem in a concentration gradient in reverse direction. When the ratio of the value obtained for Imipenem (IPM): the value of Imipenem + EDTA (IPM+EDTA) was more than to 8 or if zone was observed on the side coated with Imipenem+EDTA and no zone is observed on the opposite the side coated with Imipenem the isolate was considered as MBL positive.

Polymerase Chain Reaction for detection of MBL genes

Amplification

The presence of bla IMP,bla VIM and bla NDM gene was tested in all the 25 test isolates. The primer sequence used was as given in below Tabular.

Polymerase chain reaction was carried out in 50 µl reaction mixture containing 5 µl template,1 µl forward primer, 1 µl reverse primer, 25 µl PCR buffer (containing dNTPs, Taq polymerase). Samples were then subjected to initial denaturation at 95^0 C for 3 minutes, followed by 30 cycles of 95^0C for 30 seconds, 45^0C for 1 minute for VIM and 66^0C for IMP gene, 72^0C for 1 minute and a final extension at 72^0C for 10 minutes to complete the elongation of the PCR intermediate products. For detection of NDM gene amplification was carried out under the following thermal cycling conditions: 10 min at 94°C; 36 cycles of amplification consisting of 30 s at 94°C, 40 s at 52°C, and 50 s at 72°C; and 5 min at 72°C for the final extension. Isolates positive for metallo beta lactamase production was found to be carrying either VIM or IMP.NDM was not detected in any of the isolates.

Gel Electrophoresis

The PCR products were analyzed by electrophoresis in 1.0% agarose gel to detect specific amplified product of 432 bp , 500 bp and 660bp by comparing with standard molecular weight marker of 100 base pair (DNA ladder). The amplified products of the study samples were visualized by trans-illuminator, photographed by a digital camera and transferred to computer data for labeling and storage.

Antibiotic susceptibility Testing

Antimicrobial sensitivity was performed on Mueller Hinton agar plates by Kirby-Bauer disk diffusion method according to CLSI full guidelines (Clinical and Lab Standards Institute (CLSI, 2012).[8] Following antibiotic disks(Hi media, Mumbai, India) were used Ciprofloxacin-5 µg,Levofloxacin 5 µg ,Gentamicin-10 µg, Amikacin-30 µg, Tobramycin-10 µg, Ceftazidime-30 µg,Ceftazidime Clavulanic acid 30/10 µg, Piperacillin100 µg,Piperacillin tazobactam 100/10 µg,Cefepime 30 µg, Imipenem-10 µg, Meropenem-10 µg, Tigecyclin 15 µg. P. aeruginosa ATCC 27853 was used as control.

RESULT

MBL producers were found in lesser number in females. But the percentage of MBL production among the carbapenem resistant isolates is more in female patients having Acinetobacter baumanni infection (42.85%). No cases of MBL production in female patient is recorded in Acinetobacter lwofii and Acinetobacter sp. infection. Highest percentage of MBL production in male patient is seen in case of Acinetobacter sp. infection(37.25%). Overall percentage of MBL production seen in A.baumanni is 31.14%,in A.lwofii 15% and in A.sp. 23.07%.

In the study 26 pus, 34 Endotracheal aspirate, 09 sputum, 05 urine,16 blood and 04 other sample including wound swab,necrotic tissue and body fluid were included.Acienetobacter baumanni was mostly found in ET aspirate followed by blood. Acienatobacter lwofii and Acinetobacter sp. was mostly isolated from pus sample.

Among the MBL producer Acinetobacter the highest sensitivity was found towards Tigecyclin(Resistance =0%) followed by Gentamicin and Tobramycin(Resistance 68%).100% resistance was shown against Meropenem, Ceftazidime and Piperacilin.

Discussion: Carbapenems have a broad spectrum of antibacterial activity.Hence,they are often used as a

Target gene	Primer sequence		Amplicon size	Reference
	Forward	Reverse		
IMP	GTTTATGTTCATACWTCG	GGTTTAAYAAAACAACCAC	432	13
VIM	TTTGGTCGCATATCGCAACG	CCATTCAGCCAGATCGGCAT	500	13
NDM	GGTGCATGCCCGGTGAAATC	ATGCTGGCCTTGGGGAACG	660	14

	Total number of Male patient included	No of MBL p roduced	Percentage	Total number of Female patient included	No of MBL produced	Percentage
Acinetobacter baumanni	40	10	25%	21	09	42.85%
Acinetobacterlwofii	17	03	17.64%	03	00	00%
Acinetobacter sp.	08	03	37.5%	05	00	00%
Total	65	16	24.61%	29	09	31.03%

Table-1: Sex wise distribution of MBL producers

	Abaumanni		A.lwofii		A.sp.		Total
	MBL	Non MBL	MBL	Non MBL	MBL	Non MBL	
PUS	03	11	03	05	00	04	26
Endotracheal aspirate	09	17	00	03	3	02	34
Sputum	02	03	00	03	00	01	09
Urine	01	03	00	00	00	01	05
Blood	04	08	00	04	00	00	16
Others	00	00	00	02	00	02	04
Total	19	42	03	17	03	10	94

Table-2: Distribution of specimen

Antibiotics	MBL positive(25)	MBL Negative(69)
Imipenem	20(80%)	40(57.97%)
Meropenem	25(100%)	58(84.05%)
Ceftazidime	25(100%)	69(100%)
Ceftazidime clavulanic acid	24(96%)	54(78.26%)
Piperacillin	25(100%)	69(100%)
PiperacillinTazobactam	24(96%)	54(78.26%)
Cefepime	21(84%)	55(79.71%)
Tobramycin	17(68%)	45(65.21%)
Gentamicin	17(68%)	45(65.21%)
Amikacin	20(80%)	42(60.87%)
Ciprofloxacin	24(96%)	54(78.26%)
Levofloxacin	20(80%)	49(71.01%)
Tigecylin	0(00%)	00(00%)

Table-3: Antibiotic Susceptibility Pattern of both MBL positive and Negative Acinetobacter (n=94)

Organisms	*bla*IMP	*bla*VIM
Acinetobacterbaumanni	6	13
Acinetobacterlwofii	0	3
Acinetobacter sp.	0	3
Total	06(24%)	19(76%)

Table-4: Distribution of various genes in the MBL producers(n=25).

last resort in treatment.These are resistant to hydrolysis by most β-lactamases including extended spectrum β-lactamases (ESBL) and AmpC β- lactamases.There has been an increase in reports of carbapenem resistance in Acinetobacter species and P. aeruginosa worldwide.

In India the studies done on metallo beta lactamase producing nonfermentors are numerous.The results vary all over the country. The prevalence of metallo beta lactamase producers among carbapenem resistant isolates(Resistant to either or both Imipenem and Meropenem) in the present study was found to be 26.59%. In 2010 a study from Vellore Christian Medical college reported 42.6% MBL production which is much higher compared to the present study.[15] Some other studies reported 33.33%[16], 50%[17], 20%[18], 41.2%[19], 55%[20] and 72.72%.[21] The difference in prevalence can be attributed to conditions under which they were tested and heterogenous nature of MBLs.

In the present study Endotracheal aspirate comprised for the majority of specimen followed by pus,blood,urine,sputum and other sample.This study is different from the study by Ranjan et al, 2014 where the majority of specimen included was pus (48.28%)[22] and the study done by Wankhede et al,2011 where the majority

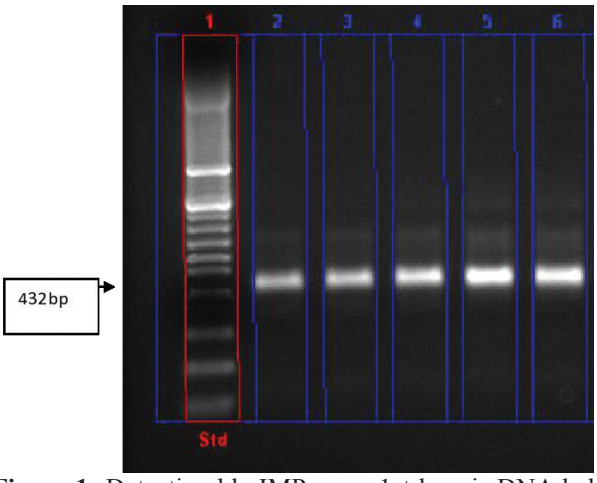

Figure-1: Detection bla IMP gene. 1st lane is DNA ladder, 2nd lane is positive control and 3rd to 6th lane is positive sample

of specimen was wound swab(44.11%).[23]

The isolates included in this study were taken from 65 male patient and 29 female patient. The male to female ratio is 2.24:1. This is comparable with studies done by Javaiya et al, 2008 who showed the male female ratio to be 2:1.[24] The percentage of MBL production in female is 31.03% % which is slightly higher than in male patients (24.61%).

The genes detected in the present study are bla IMP and blaVIM. No other gene were detected in the present study. Among 25 MBL producer majority of the isolates had VIM gene.[19] Only 06 IMP gene were detected. A study from Mahatma Gandhi Institute of Medical Sciences, Sevagram, Wardha, Maharashtra reported bla-VIM MBL gene only in 7 (16.28%) of the 43 screen test positive.[25] Another study from Pondicherry University reported only bla-IMP-1 in 42% (23 isolates)of A. baumannii.[26]

In Acinetobacter Tigecyclin was found to be 100 % effective and next most effective drug were Tobramycin and Gentamycin(Sensitivity=32%).Among the Quinolones Levofloxacin was more effective compared to Ciprofloxacin.100% resistance was seen against Ceftazidime,Meropenem and Piperacillin. In the study by John et al, 2011 the sensitivity towards Tigecyclin was reported to be 100% and the resistance towards Netillimycin and Pieperacillin was 61.5% & 100% respectively.[27] The present study is in accordance to this findings.However it differed in case of Gentamycin (John et al, 2011[27] reported 100% resistance) and Amikacin(John et al, 2011[27] reported 56.7% resistance).

In this study it was seen that all MBL producing Acinetobacter showed aminoglycoside resistance. In Acinetobacter the most effective aminoglycoside was Tobramycin and Gentamycin. Amikacin was found to be the least effective drug in case of Acinetobacter MBL positive isolates. None of the MBL producers were sensitive to all the aminoglycosides. All the MBL producers showed some kind of resistance mechanism towards aminoglycosides. This hints that these organisms carry multiple resistance genes which makes the therapeutic options very limited.

CONCLUSION

Our study was intended to find out the prevalence of MBL in Acinetobacter which we found to be 26.59% of total carbapenem resistant isolates. Our study and all other studies also indicate that generally MBL producing isolates carry multidrug resistant integrons.Infact the only drug that showed 100% susceptibility towards MBL producing isolates was Tigecyclin.Hence,identification and AST of MBL producing Acinetobacteris is essential for proper prophylaxis.

REFERENCES

1. Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant Acinetobacter baumannii. Nature Reviews Microbiology 2007;5:939-951.
2. Nordmann P. Acinetobacter baumannii, le pathogène nosocomial par excellence. Pathologie Biologie 2004;52:301-303.
3. Bergogne-Berezin E, Towner KJ. Acinetobacter spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin Microbiol Rev 1996;9:148-165.
4. Davis KA, Moran KA, McAllister CK, Gray PJ. Multidrug-resistant Acinetobacter extremity infections in soldiers. Emerging Infect Dis 2005;11:1218-1224.
5. Camp C, Tatum OL. A review of Acinetobacter baumannii as a highly successful pathogen in times of war. Lab Medicine 2010;41:649-657.
6. Rossolini GM. Acquired metallo-beta-lactamases: an increasing clinical threat. Clin Infect Dis 2005 1;41:1557-1558.
7. Bailey W. E. and Scott E. G. (1962). Diagnostic microbiology.Diagnostic microbiology.
8. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. 19th informational supplement; Wayne, PA:CLSI; 2012. CLSI document M100-S22.
9. Yong D, Lee K, Yum JH, Shin HB, Rossolini GM, Chong Y. Imipenem-EDTA disk method for differentiation of metallo-beta-lactamase-producing clinical isolates of Pseudomonas spp. and Acineto-

bacter spp. J Clin Microbiol 2002;40:3798-3801.
10. Walsh TR, Toleman MA, Poirel L, Nordmann P. Metallo-beta-lactamases: the quiet before the storm? Clin Microbiol Rev 2005;18:306-325.
11. Varaiya A, Kulkarni M, Bhalekar P, Dogra J. Incidence of metallo-beta-lactamase-producing Pseudomonas aeruginosa in diabetes and cancer patients. Indian J Pathol Microbiol 2008;51:200-203.
12. Lee K, Lim YS, Yong D, Yum JH, Chong Y. Evaluation of the Hodge test and the imipenem-EDTA double-disk synergy test for differentiating metallo-beta-lactamase-producing isolates of Pseudomonas spp. and Acinetobacter spp. J Clin Microbiol 2003;41:4623-4629.
13. Srinivasan VB, Rajamohan G, Pancholi P, Stevenson K, Tadesse D, Patchanee P, et al. Genetic relatedness and molecular characterization of multidrug resistant Acinetobacter baumannii isolated in central Ohio, USA. Annals of clinical microbiology and antimicrobials 2009;8:21-29.
14. Bonnin RA, Naas T, Poirel L, Nordmann P. Phenotypic, biochemical, and molecular techniques for detection of metallo-beta-lactamase NDM in Acinetobacter baumannii. J Clin Microbiol 2012;50:1419-1421.
15. Manoharan A, Chatterjee S, Mathai D, SARI Study Group. Detection and characterization of metallo beta lactamases producing Pseudomonas aeruginosa. Indian J Med Microbiol 2010 Jul-;28:241-244.
16. De AS, Kumar SH, Baveja SM. Prevalence of metallo-beta-lactamase producing Pseudomonas aeruginosa and Acinetobacter species in intensive care areas in a tertiary care hospital. Indian J Crit Care Med 2010;14:217-219.
17. Azim A, Dwivedi M, Rao PB, Baronia AK, Singh RK, Prasad KN, et al. Epidemiology of bacterial colonization at intensive care unit admission with emphasis on extended-spectrum beta-lactamase- and metallo-beta-lactamase-producing Gram-negative bacteria--an Indian experience. J Med Microbiol 2010;59:955-960.
18. Joseph NM, Sistla S, Dutta TK, Badhe AS, Rasitha D, Parija SC. Ventilator-associated pneumonia in a tertiary care hospital in India: role of multi-drug resistant pathogens. The Journal of Infection in Developing Countries 2010;4:218-225.
19. Chakraborty D, Basu S, Das S. A study on infections caused by metallo beta lactamase producing gram negative bacteria in intensive care unit patients. Am J Infect Dis 2010;6:34-39.
20. Varaiya A, Kulkarni N, Kulkarni M, Bhalekar P, Dogra J. Incidence of metallo beta lactamase producing Pseudomonas aeruginosa in ICU patients. Indian J Med Res 2008;127:398-400.
21. Chacko B, Varaiya A, Dedhia B. Imipenem resistant metallo β lactamse producing Pseudomonas aeruginosa. Indian journal of medical microbiology 2008;26:398-398.
22. Ranjan S, Banashankari G, Babu PS. Comparison of epidemiological and antibiotic susceptibility pattern of metallo-beta-lactamase-positive and metallo-beta-lactamase-negative strains of pseudomonas aeruginosa. Journal of laboratory physicians 2014;6:109-110.
23. Wankhede S, Iyer V, Bharadwaj R. THE STUDY OF MBL PRODUCERS IN GRAM NEGATIVE ISOLETS FROM icus AND WARDS. 2011.
24. Javiya VA, Ghatak SB, Patel KR, Patel JA. Antibiotic susceptibility patterns of Pseudomonas aeruginosa at a tertiary care hospital in Gujarat, India. Indian J Pharmacol 2008;40:230-234.
25. Purohit M, Mendiratta DK, Deotale VS, Madhan M, Manoharan A, Narang P. Detection of metallo-beta-lactamases producing Acinetobacter baumannii using microbiological assay, disc synergy test and PCR. Indian J Med Microbiol 2012;30:456-461.
26. Uma Karthika R, Srinivasa Rao R, Sahoo S, Shashikala P, Kanungo R, Jayachandran S, et al. 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 2009;58:430-435.
27. John S, Balagurunathan R. Metallo beta lactamase producing Pseudomonas aeruginosa and Acinetobacter baumannii. Indian J Med Microbiol 2011;29:302-304.