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Genotyping of MDR Acinetobacter baumannii to detect the Co-existence of MBL and Carbapenemase Genes Including NDM-1 and the Sequencing of the Most Prevalent Resistance Genes

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ABSTRACT Multidrug-resistance is quite common among non-fermenting gram negative rods including Acinetobacter baumannii (A.baumannii). The rapid spread of multidrug-resistant A.baumannii in clinical settings has made the treatment options difficult for clinicians. Molecular characterization of suspected pathogens might help to provide an idea about the acquisition/presence of resistance genes among them and to provide appropriate therapy.

Materials and Methods:

251 isolates of A.baumannii were collected from various samples and screened by phenotypic methods, were subjected to PCR to detect the presence of drug resistance genes [Metallo-β-lactamase (MBL) and Oxacillinase genes (OXA)] - blaIMP, blaVIM, blaNDM-1, blaOXA-23, blaOXA40, blaOXA51 and blaOXA58. Five isolates were selected and sequencing was done by Sanger's sequencing method for each gene.

Results and Discussion:

The 251 isolates showed the co-existence of various MBL and Oxacillinase [Carbapenemase] genes that contributed to their drug resistance. The analysis of sequencing done showed mutations in blaOXA-51, blaOXA-58 and blaNDM-1 genes. There was no difference in blaOXA-23gene sequence.

Conclusion:

The co-existence of multiple Oxacillinases [Carbapenemases] and MBLs along with other resistance mechanisms might result in treatment failure. Genotypic screening should be performed for the detection of resistance genes in nosocomial MDR and XDR A.baumannii isolates.

KEYWORDS : MDR-AB, MBL, Carbapenemase, Genotyping, Sequencing

Introduction:

The emergence and dissemination of resistance genes among nosocomial gram negative bacteria has led to clinical and epidemiological problem in patient care and public health. Global surveillance programs conducted over the last decade has shown an unparalleled increase in resistance rates among clinical *Acinetobacter* isolates. Genus *Acinetobacter* is a diverse group of gram negative bacteria and has 33 species that are identified by molecular methods and some of them such as *A.baumannii* are of clinical importance. *A.baumannii* has gained much interest in recent decades owing to its increasing prevalence in hospital setting. Currently it is considered to be one of the most difficult bacterial species to treat and prevent in hospital setting. Once introduced into the hospital, it may become difficult to get rid of it. The ability of this organism to survive on artificial surfaces for an extended period of time in spite of disinfection has made it a tough bacterium to eradicate from hospital environment. Numerous reports worldwide have identified this organism as a major threat to hospitalized patients leading to high mortality rates, particularly immunocompromised patients in ICU. It is necessary to understand the repertoire of resistance determinants and their organization and origins to explain their acquisition and dissemination.[1]

Inappropriate use of broad-spectrum antibiotics and the developing resistance mechanisms of bacteria are the key factors in the emergence and spread of resistant infections. According to Infection control specialists and hospital epidemiologists, several interventions are necessary to stop the horizontal transmission of multidrug-resistant gram negative bacilli. A dramatic increase of *Acinetobacter* strains with decreased susceptibility to many antibiotics including carbapenems have been observed in recent years throughout the world. Since 2000, carbapenem-resistant and multidrug-resistant *A.baumannii* strains [MDR and XDR strains] have emerged due to the presence of multiple carbapenemases. Extended spectrum β-lactamases [ESBLs] conferring resistance to broad spectrum cephalosporins, carbapenemases conferring resistance to carbapenems and 16S rRNA methylase conferring resistance to all clinically relevant aminoglycosides are the most important causes of concern. There are reports of resistance to fluoroquinolones, polymyxins [colistin] and tigecycline that has led to pan drug-resistance.

The strains belonging to international clone II (IC2) or sequence type 2 (ST2) is the most prevalent epidemic lineage associated with multidrug-resistance and nosocomial outbreaks.[2] Besides their intrinsic resistance to antibiotics due to presence of native β-lactamase genes, they have acquired a wide array of antibiotic resistance mutations and genes, located either on the chromosome or plasmids. Furthermore, clinical strains of CRAB possess *bla* genes that are transferred via mobile genetic elements such as insertion sequences, transposons or plasmids. According to recent studies, *A.baumanniii* is known to possess **New Delhi Metallo β-lactamase [NDM].** It is a broad-spectrum β-lactamase [an MBL] which has been renamed now as **Plasmid encoded carbapenemase resistant metallo β-lactamase [PCM]**. It can inactivate all β-lactams except aztreonam and was reported for the first time in *Klebsiella pneumoniae* isolated from urine of a 59 year old male patient of Indian descent.[3]The same gene was identified in *E.coli* that was resistant to all antibiotics including carpapenems from the stool sample of the same patient. The occurrence of same novel resistance gene in two different genera suggested that it was transferable. Molecular studies coupled with conjugation experiments confirmed that bla_{NDM-1} gene was located on transferable plasmids. Since then, high incidence of NDM/PCM producers belonging to different genera of gram negative bacteria including *Acinetobacter* have been reported globally. Series of further variants of NDM-1, ie., NDM-2 to 7 have been identified. NDM/PCM producers show resistance to all aminoglycosides, macrolides, sulfamethoxazole and carbapenems. Recent studies have investigated the presence of multiple carbapenemase encoding genes in clinical isolates of *A.baumannii* & detected their presence along with bla_{NDM-1} gene.

Molecular characterization of this pathogen might help to provide appropriate antimicrobial therapy and good clinical outcome. Not many extensive reports are available from India especially from Karnataka, regarding the genes prevalent in MBL and Carbapenemase producing *A.baumannii.* Hence this study was carried out to identify the presence of multiple drug resistance genes such as carbapenemase and MBLs including NDM-1 gene among the XDR-AB clinical isolates, from a tertiary care hospital, Mangaluru, coastal Karnataka. An attempt was also done to sequence the most prevalent genes present in these isolates.

Materials & methods:

A total of 251 isolates of *Acinetobacter* were collected from various clinical samples from patients of ICU who were on ventilation, postoperative critical care and organ support following multiple trauma. Chronically ill patients suffering from various forms of malignancies were included in the study as well. The isolates were identified, confirmed and antimicrobial susceptibility testing was performed to detect MDR and XDR isolates.The isolates were then subjected to various phenotypic tests - Double Disc Synergy Test (DDST), Combined Disc Test (CDT), Modified Hodge Test (MHT) and MBL E-test to detect the MBL production, AmpC disk test for AmpC production and Phenotypic Confirmatory Disc Diffusion Test (PCDDT) for ESBL production according to the standard procedures as cited in our previous report.[14] *A.baumannii* MTCC 1425 was used as control strain for all the tests. The isolates were then subjected to PCR to detect the presence of drug resistance genes [Metallo-β-Lactamase (MBL) and Oxacillinases (OXA) genes] - ***bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{NDM-1}$, *bla*$_{OXA-23}$, *bla*$_{OXA-40}$, *bla*$_{OXA-51}$ and *bla*$_{OXA-58}$.**

From the freshly cultured bacterial isolates, the DNA was isolated by HotShot Plus Thermal SHOCK method (Truett et al, 2000) using an alkaline lysis reagent and a neutralizing reagent. The isolates were subjected to thermal shock to extract the DNA from the cell [heated at 95°C for 10 minutes and cooled to 4°C]. The isolated DNA was mixed with the PCR master mix and subjected to polymerase chain reaction with precise annealing temperature according to the primers used (table-1).

After amplification, 15µl of each mixture were loaded into the wells made on 1.5% agarose gel for gel electrophoresis. The gel was ran until the size of the insert was clearly observed. Then the gel was visualized under UV illuminator, the reactions were compared with known DNA ladder and the correct product size was identified [Figs-1A &B].

Sequencing of *Acinetobacter baumannii* genes:

Among the seven genes detected in MDR AB, the most prevalent ones were chosen for sequencing, namely *bla*$_{OXA-51}$,*bla*$_{OXA-58}$,*bla*$_{OXA-23}$and*bla*$_{NDM-1}$.Five isolates for each gene were selected and sequencing was done by Sanger's sequencing method. The DNA was isolated from the above isolates and confirmed by PCR and gel electrophoresis. Elution of the PCR product was done by Gel elution. The quality of the PCR product was checked and was subjected to sequencing. The sequencing report was further analysed using Bioedit software. The sequences of *bla*$_{OXA-51}$, *bla*$_{OXA-58}$, *bla*$_{OXA-23}$and*bla*$_{NDM-1}$were compared with the original gene sequences [GEN Bank sequences] using Clustal W tool in the Bioedit software.

Results:

The 251 isolates belonged to *A.baumannii* complex and were extremely drug resistant isolates [XDR AB].

Among the 251 isolates subjected for PCR, 83(33.1%) were positive for *bla*$_{IMP}$gene, 60(23.9%) for *bla*$_{VIM}$, 103 (41%) for *bla*$_{NDM-1}$, 144(57.4%) for *bla*$_{OXA-23}$, 100(39.8%) for *bla*$_{OXA-40}$, 146(58.2%) for *bla*$_{OXA-58}$ and 227(90.4%) for *bla*$_{OXA-51}$ genes respectively [table-2]. Table-3 shows the distribution of these resistance genes in relation to Imipenem susceptibility.

Among the 251 isolates, 227(90.4%) were *A.baumannii* as they had *bla*$_{OXA-51}$ gene [intrinsic carbapenemase gene present in chromosome]. The remaining 24(9.6%) were comprised of *A.pittii* [genomospecies 3] and *A.nosocomialis* [genomospecies 13TU].

Out of the 251 clinical isolates of *A.baumanii*, 21(8.4%) isolates carried only one resistance gene, 45(17.9%) had two genes, 59(23.5%) had three genes, 72(28.7%) had four genes, 41(16.3%) had five genes, 10(4%) had six genes and three isolates had all the seven resistance genes.

Figures-2a & b shows the gene sequencing graph of *bla*$_{NDM-1}$ and *bla*$_{OXA-58}$ genes. The analysis of sequencing done by Bioedit software showed mutations in *bla*$_{OXA-51}$, *bla*$_{OXA-58}$ and *bla*$_{NDM-1}$ genes. They showed variations in the gene sequence when compared to the original Gen bank sequence. There was no difference in *bla*$_{OXA-23}$gene sequence.

Discussion:

Multi-drug resistant *A.baumannii* (MDR AB) is a significant pathogen in health care settings. The infection tend to occur especially in immunosuppressed patients, in patients with serious underlying diseases and in those subjected to invasive procedures and treated with broad spectrum antibiotics especially in ICUs. Nosocomially acquired MDR AB pose a real challenge to the clinician. Carbapenems are generally the last resort in the treatment of life threatening infections caused by MDR AB. However, emergence of Carbapenem hydrolysing β-lactamases of Ambler class B (MBLs) and class D (Oxacillinases/CHDLs), which is the most important mechanism of carbapenem resistance, have caused serious problem in treatment of MDR AB. While *bla*$_{OXA-51}$ is involved in intrinsic resistance with chromosomal origin, the other genes including *bla*$_{NDM-1}$ cause resistance via plasmids. These organisms are resistant to carbapenems, fluoroquinolones and aminoglycosides and have been reported from various hospital settings in India as well as from several other countries. Colistin resistant isolates are rare but occurrence has been reported. By acquiring various kinds of resistance mechanisms, *A.baumannii* has evolved as one of the most difficult nosocomial pathogens to control and treat.

In the present study, out of 251 isolates chosen for PCR, 202 (80.5%) were imipenem resistant along with other antibiotics but were sensitive to either tigecycline or colistin indicating that they were XDR-AB. Pan drug resistant isolates were not seen in our tertiary care hospital.

There are many studies worldwide, reporting the co-existence of various resistance genes in MDR and XDR *A.baumannii* isolates. They have stated that the co-production of OXA and metallo-β-lactamase enzymes was not an uncommon phenomenon in *A.baumannii*. [1,3-8,10-12] This increases the resistance spectrum by horizontal transfer of resistance factors intra- and inter-species.[19] In the present study the various MBL and Oxacillinase (Carbapenemase) genes, contributed to the drug resistance, was observed as given in the table-2. Phenotypic detection of MBLs and Carbapenemases in these isolates was reported in an earlier study.[14]

There has been a recent report from South India in the occurrence of oxacillinases production which also demonstrated predominant presence of *bla*$_{VIM}$ along with *bla*$_{OXA-23}$[13] and *bla*$_{OXA-23}$ with *bla*$_{NDM-1}$[8] among clinical isolates of CRAB. It has been illustrated that among different types of OXA carbapenemases, *bla*$_{OXA-23}$ like and *bla*$_{OXA-51}$ like genes were the major genetic factors and most common types involved in resistance development. The result of molecular analysis of the present study was in agreement with reports of all those published studies about co-existence of multiple resistance genes except that it differed in the prevalence of each gene (table -2).

Since their discovery in 2007, NDM-1 positive bacteria have started to spread globally through health care facilities. Among the genes encoding for MBLs, this study documented highest prevalence of the novel *bla*$_{NDM-1}$ (41%) gene in accordance with reports from other Indian studies.[4,5] The prevalence of other two MBLs, i.e., *bla*$_{IMP}$ and *bla*$_{VIM}$ were low, 33.1% and 23.9% respectively, though many studies have reported that they were the most common MBLs seen in *A.baumannii*, their prevalence ranging from 51 to 100%.[4-7]

Only few studies have demonstrated low prevalence of *bla*$_{OXA-58}$.[10-12] But in comparison, this study documented higher prevalence of OXA-58 i.e., 58.2% and was the second most prevalent oxacillinase after OXA-51. The prevalence of *bla*$_{OXA-24/40}$was 39.8% which was similar to few other published studies.[13] Lowest prevalence of OXA-24/40 (0.4%) was seen with Wu, et al.[12]

Investigations of the present study revealed that, out of 251 clinical isolates of XDR *Acinetobacter baumannii*, 28.7% carried four genes and three isolates (1.2%) had all the seven genes. These findings indicated the increased prevalence of resistance genes in XDR AB. Prevalence of these genes were found to be higher in the present report compared to other studies.

This is the first comprehensive study which has probed for higher number of multiple genes, i.e., seven resistance genes – three MBLs including the novel NDM/PCM and four oxacillinases in clinical isolates of *A.baumannii* from coastal Karnataka and confirms multiple genetic mechanisms of carbapenemases production among clinical isolates of *A.baumannii* in our setting. However, the present work did not investigate the source of carbapenemases, i.e., class-I integron and ISAba1.

An interesting observation was made in this study that among the 49 imipenem sensitive isolates, 20 isolates showed the presence of two or more resistance genes but were negative by all the four phenotypic tests and the isolates were sensitive to carbapenems. This finding indicated that the genes might be present but phenotypically not expressed and could be a cause of concern as these isolates might help in dissemination of these resistance genes. It also implies that the scenario might change in due course. Hence both phenotypic screening and genotyping should be performed for the detection of resistance genes in nosocomial MDR and XDR *A.baumanni* isolates.

Whole genome sequencing (WGS) is now poised to make an impact on hospital infection prevention and control, delivering cost-effective identification of routes of infection within a clinically relevant time frame and allowing infection control teams to track and even prevent the spread of drug resistant nosocomial pathogens. WGS provides a promising new method for investigating the epidemiology of outbreaks, particularly when coupled to clinical, locational and temporal data.

In the present study, Whole Genome Sequencing could not be done as it was far from our scope but sequencing of most prevalent resistance genes such as $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-23}}$ and $bla_{\text{NDM-1}}$ was done to check for any mutations or variations. Bioinformatics of sequencing of the $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$, and $bla_{\text{NDM-1}}$ showed variations in the gene sequence when compared to the original Gen bank sequence indicating that our isolates were different and had undergone mutation. There was not much difference in $bla_{\text{OXA-23}}$ gene sequence.

A.baumanni isolates harbouring multiple resistance genes should be seriously considered and addressed with alternative and newer therapeutic strategies, strict infection control measures and continuous surveillance. Initial screening of the putative carbapenemase producers would help to organize intervention and early directed therapy.

Conclusion:

The *Acinetobacter* strains (80.5%) isolated from various clinical samples in the present study, were all XDR-AB isolates showing resistance to multiple classes of antibiotics. All the carbapenem resistant isolates carried multiple resistance genes that contributed for their resistance.

In the present study, $bla_{\text{OXA-51}}$ (90.4%) was the most prevalent carbapenemase producing gene among *A.baumannii* clinical isolates followed by $bla_{\text{OXA-58}}$ (58.2%), $bla_{\text{OXA-23}}$ (57.4%), $bla_{\text{NDM-1}}$ (41%) and $bla_{\text{OXA-40}}$ (39.8%) with low prevalence of bla_{IMP} (33.1%) and bla_{VIM} (23.9%). About 41% of our isolates were NDM-1 producers and for the first time reported such high prevalence of NDM-1 from coastal Karnataka, south India.

Bioinformatics showed variations in the gene sequence of $bla_{\text{OXA-51}}$, $bla_{\text{OXA-58}}$ and $bla_{\text{NDM-1}}$ indicating that our isolates had undergone mutation. Co-existence of multiple MBL and carbapenemase genes in clinical isolates of *A.baumannii* is a significant threat in hospitals and is a problem to reckon with.

Fig-1: Gel documentation photos of PCR results – A.$bla_{\text{NDM-1}}$, B. $bla_{\text{OXA-58}}$

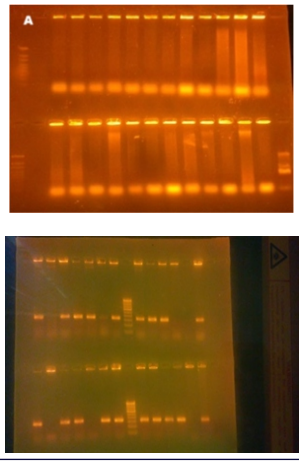

Fig-2a: Chromatogram plot of $bla_{\text{NDM-1}}$ gene of XDR *A.baumannii*

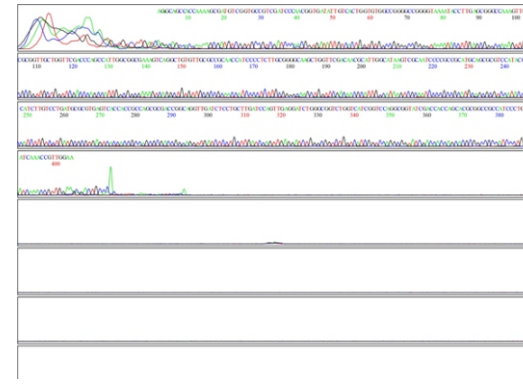

Fig-2b:- Chromatogram plot of $bla_{\text{OXA-58}}$ gene of XDR *A.baumannii*

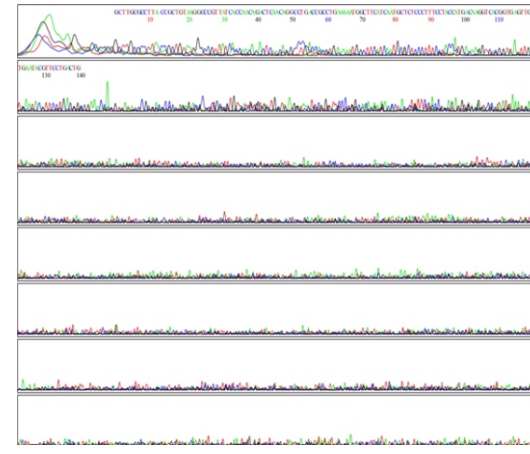

Table-1: PCR conditions and Primer details

Step	Temperature	Time
Initial Denaturation	95°C	30 seconds
30 cycles -Final Denaturation -Annealing -Initial Elongation	95°C 53°C-59°C [Depended on Primer] 72°C	30 seconds 30 seconds 1 min, 30 seconds
Final Extension	72°C	8 minutes
Hold	8°C	

Primer	Sequence	Annealing temp(°C)	Product size (bp)
$bla_{\text{OXA-23}}$	F(5'- ATG TTA TGG AGC AGC AAC G -3') R(5'- TTA GTT GCT TGG TTT TG -3')	59°C	500
$bla_{\text{OXA-40}}$	F(5'- ATG TTC AAA CTT TTG AGT AAG -3') R(5'- CTA CTC AAC GAC TGA GCG -3')	53°C	250
$bla_{\text{OXA-58}}$	F(5'- ATG AAT AAA TAT TTT ACT TGC -3') R(5'- CTT AAA TAA TAT TCA GCT G -3')	53°C	600
$bla_{\text{OXA-51}}$	F(5'- TAA TGC TTT GAT CGG CCT TG -3') R(5'- TGG ATT GCA CTT CAT CTT GG -3')	53°C	350
bla_{IMP}	F(5'- ATG AAA TTA TTA AAA ATA TTG -3') R(5'- CTT ATA AAT AAT GAA AAA C -3')	58°C	500
bla_{VIM}	F(5'- ATG AAA AAA TTT ATA CTT C -3') R(5'- TTA AAT GAT TCC AAG ATT TTC -3')	57°C	645
$bla_{\text{NDM-1}}$	F(5'-ATTAGCCGCTGCATTGAT-3') R(5'-GGCATGTCGAGATAGGAAGT-3')	59°C	156

Table-2: Distribution of MBL and Oxacillinases in 251 A.baumannii isolates

Acinetobacter isolates	MBLs	Carbapenemases					
	blaIMP n(%)	blaVIM n(%)	blaNDM-1 n(%)	blaOXA-23 n(%)	blaOXA-40 n(%)	blaOXA-58 n(%)	blaOXA-51 n(%)
Positive	83 (33.1)	60 (23.9)	103 (41.0)	144 (57.4)	100 (39.8)	146 (58.2)	227 (90.4)
Negative	168 (66.9)	191 (76.1)	148 (59.0)	10 7(42.6)	151 (60.2)	105 (41.8)	24 (9.6)

Table-3:- Distribution of resistance genes in relation to Imipenem susceptibility in 251 A.baumannii isolates

Imipenem	Total isolates n(%)	PCR result	MBLs	Carbapenemases					
			IMP n(%)	VIM n(%)	NDM-1 n(%)	OXA-23 n(%)	OXA-40 n(%)	OXA-58 n(%)	OXA-51 n(%)
Resistant	202(80.5)	Positive	77 (38.1)	59 (29.2)	92 (45.5)	138 (68.3)	88 (43.6)	129 (63.9)	188 (93.1)
		Negative	125 (61.9)	143 (70.8)	110 (54.5)	64 (31.7)	114 (56.4)	73 (36.1)	14 (6.9)
Sensitive	49(19.5)	Positive	06 (12.2)	01 (2.0)	11 (22.5)	06 (12.2)	12 (24.5)	17 (34.7)	39 (79.6)
		Negative	43 (87.8)	48 (98.0)	38 (77.5)	43 (87.8)	37 (75.5)	32 (65.3)	10 (20.4)

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