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PREVALENCE OF VIM- AND GIM-PRODUCING *ACINETOBACTER BAUMANNII* FROM PATIENTS WITH SEVERE URINARY TRACT INFECTION

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Carbapenems are administered as the final drug of choice for treating complicated nosocomial infections caused by multidrug-resistant *Acinetobacter baumannii* strains. It is currently a worldwide issue that metallo-β-lactamases (MBLs) as carbapenem-hydrolyzing enzymes are one of the major drug resistance mechanisms. This investigation is thus aimed to assess the prevalence and characterize the MBL-producing strains of *A. baumannii* both by phenotypic assays and by genotypic characterization. A total of 73 isolates of *A. baumannii* were phenotypically and genotypically characterized from patients ($N = 1{,}000$) with severe urinary tract infection. Tested strains were subjected to double disc synergy testing (DDST) by Kirby–Bauer disc diffusion method with imipenem (IMP) and IMP/EDTA combination discs. Plasmid DNA was molecularly screened for MBL-encoding bla_{IMP}, bla_{VIM}, bla_{GIM}, and bla_{NDM} genes by PCR for the genetic relatedness of the MBL genes with carbapenem resistance. Carbapenem resistance profile showed 100%, 45%, and 49% non-susceptibility against imipenem, doripenem, and meropenem, respectively. Altogether 42.46% ($n = 31$) of the isolates showed MBL production upon double disc phenotypic test with IMP and IMP/EDTA discs. The bla_{VIM} and bla_{GIM} were detected in 34.24% ($n = 25$) and 16.43% ($n = 12$) of the isolates, respectively, while the co-occurrence of bla_{VIM} and bla_{GIM} was 2.73% among the isolates. DDST-positive isolates showed 21.19% and 9.58% strains positive for bla_{VIM} and bla_{GIM}, respectively, whereas 1.36% of the strains for both genes. None of the strains yielded bla_{IMP} and bla_{NDM} genes. The findings of this study showed prevalence of carbapenem resistance among *A. baumannii* from urine samples and the frequency of bla_{VIM} *and* bla_{GIM}.

Keywords: *A. baumannii*, carbapenems, bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{NDM}

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Introduction

Acinetobacter baumannii, a pleomorphic, aerobic Gram-negative bacterium, has emerged as one of the critical multidrug-resistant nosocomial pathogens worldwide [1]. *A. baumannii* is considered as one among the top seven pathogens threatening the patient's healthcare and as an unmet medical need [2]. *A. baumannii* infections are considered with great concern due to their resistance pattern exhibited to several classes of antibiotics, especially carbapenems [3] and are considered as sentinels of drug resistance with the designation as carbapenem-resistant *A. baumannii* [4]. Among several mechanisms related to carbapenem resistance, the resistance exhibited due to carbapenem-hydrolyzing enzymes is frequently considered worldwide [5]. Based on the Ambler classification, these enzymes belong to class B metallo-β-lactamase (MBL) and class D OXA-type carbapenemases and most of them are mediated by plasmids [6].

MBLs are further classified into several families with bla_{VIM}, bla_{IMP}, bla_{GIM}, and more recently bla_{NDM} and are located in the gene cassettes of class 1 integrons with bla_{IMP} in class 3 integrons [7, 8]. For the optimal MBL activity, divalent cations are required as cofactors with further action of one or two zinc ions for their catalytic activity with chelators as inhibitory agents [9]. These MBLs are highly potent in hydrolyzing all the β-lactam antibiotics except the monobactams such as aztreonam [10] and there are no known MBL inhibitors [11]. Dissemination of MBL-encoding genes is highly popular among the plasmids or by integron-borne mobile gene cassettes through horizontal gene transfer mechanisms [12]. As a dominant MBL variant, bla_{NDM} [13] and $bla_{\text{VIM-2}}$ [14] have recently emerged with worldwide reports.

Earlier studies have reported the emergence of bla_{VIM}-, bla_{IMP}-, and bla_{GIM}-based carbapenem resistance among *A. baumannii* [15, 16] along with the increased incidences of bla_{NDM}-based resistance [17, 18]. Detection of these MBLs is often based on the inhibitor-based test using metal ion chelators, such as ethylene diamine tetra acetic acid (EDTA) or thio-based compounds [19]. Among several phenotypic detections, Clinical Laboratory Standards Institute (CLSI) [21] advocates the application of modified Hodge's test, CarbaNP test, and/or a molecular-based assay for the confirmation of the MBL producers among *Enterobacteriaceae* and *A. baumannii* strains. Genotypic characterization of the MBL-based genetic determinants bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{NDM} is detected by polymerase chain reactions (PCRs) and clonal relatedness is analyzed by various molecular methods [20].

With this background, the present investigation aimed to phenotypically and genotypically characterize the MBL producers among *A. baumannii* strains with the phylogenetic assessment of the MBL-based genetic determinants such as

bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{NDM} screened from the patients with severe urinary tract infections from South India.

Materials and Methods

Study design

A total of 73 consecutive and non-repetitive *A. baumannii* isolates, which were isolated and identified for a period of 12 months (2014–2015) from urine samples of patients with severe urinary tract infections ($N = 1{,}000$), were phenotypically and genotypically confirmed by conventional microbiological analytical tests and PCR, respectively, at the Department of Microbiology. These characterized strains were subjected to antibiotic susceptibility test by standard Kirby–Bauer disc diffusion method using imipenem (IMP; 10 μg), doripenem (10 μg), and meropenem (10 μg) for the carbapenem resistance profile of the selected strains under study.

Phenotypic confirmatory test

With the observation and record of the carbapenem resistance, all the strains were further subjected for phenotypic double disc synergy testing (DDST) as per the CLSI (2012) [21]. All the test strains were prepared as fresh broth cultures and lawn was made onto sterile Mueller–Hinton agar (MHA; Hi-Media, Mumbai). For the MBL detection, two discs were used for the profile such as IMP (10 μg) (Hi-Media) and IMP/EDTA. An amount of 0.5 M EDTA was prepared by dissolving 186.1 g of disodium EDTA-2H_2O in 1,000 ml of distilled water (pH 8.0) and was sterilized by autoclaving. An amount of 10 μl was added onto one of the IMP discs to obtain the desired concentration of IMP/EDTA (10/750 μg). The discs were placed onto the surface of the MHA at a distance of 20 mm from each. The plates were then incubated for a period of 24 h at 37 °C. The increase in zone size of ≥7 mm around the IMP/EDTA disc than IMP was interpreted as MBL producers.

Molecular detection of bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{NDM} genetic determinants in MBL producers

Extraction of plasmid DNA and PCR amplification. All the strains were stored at −80 °C in 80%/20% (v/v) glycerol in Luria–Bertani media for genetic stability of

resistance upon storage [22]. Plasmid DNA was extracted from fresh cultures of *A. baumannii* using Qiagen extraction kit in accordance with the manufacturer's instructions and was stored in −20 °C until further use. An amount of 15 µl of amplification reaction mixtures was prepared by mixing 7.8 µl of 2× Master Mix (Takara, Japan) in 5.6 µl of double distilled water. Specific forward primer and reverse primer (Eurofins Genomic India Pvt Ltd., Bangalore, India) of bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{NDM} were added using the standard PCR conditions (Table I). PCR amplification was carried out and the resulting PCR amplicons were examined in 1% agarose gel electrophoresis containing ethidium bromide, which was visualized in a gel documentation system. The 100 bp DNA ladder was used to confirm the amplicon size.

Results

Preliminary screening for the carbapenem resistance tests showed 100%, 45%, and 49% non-susceptibility against imipenem, doripenem, and meropenem, respectively, as per CLSI zone interpretative criteria (Figure 1). DDST with IMP and IMP/EDTA discs yielded 42.46% ($n = 31$) of the isolates as MBL producers (Table II). A total of 37 isolates (50.68%) showed resistance to all the carbapenems tested with 31.50% ($n = 23$) DDST-positive (Figure 2).

Genotypic characterization of the MBL genetic determinants showed the presence of bla_{VIM} and bla_{GIM} in 34.24% ($n = 25$) and 16.43% ($n = 12$) of the isolates. Co-occurrence of bla_{VIM} and bla_{GIM} was observed in 2.73% ($n = 2$) of the isolates. DDST-positive isolates showed 21.19% ($n = 16$) positive for bla_{VIM} and 9.58% ($n = 7$) positive for bla_{GIM} determinants (Table III and Figure 3). Among the two isolates with both bla_{VIM} and bla_{GIM} genes, only one strain was DDST-positive. However, none of the strains yielded bla_{IMP} and bla_{NDM} genes.

Discussion

A wide range of nosocomial infections encompassing meningitis, septicemia, pneumonia, and skin and wound infections are caused by *A. baumannii* and are considered as a major challenge in the patient healthcare [23]. Carbapenems such as imipenem, doripenem, meropenem, and ertapenem are considered as the last resort antibiotics in the treatment of severe and complicated infections established by multidrug-resistant *A. baumannii* [24]. In recent decades, resistance to carbapenems is highly reported [25]. The present investigation has also recorded a total of 37 isolates ($N = 73$) as carbapenem-resistant strains. All the strains fall under the

Table I. Primer sequence and PCR conditions to detect bla_{IMP}, bla_{VIM}, bla_{GIM}, and bla_{NDM-1} among ESBL producers of *A. baumannii*

Target gene	Primers	Sequence (5′–3′)	Annealing temperature (°C)	Amplicon size (bp)	Reference
bla_{IMP}	IMP – F′	GAATAGAATGGTTAACTCTC	53	188	[26]
	IMP – R′	CCAAACCACTAGGTTATC			
bla_{VIM}	VIM – F′	GTTTGGTCGCATATCGCAAC	53	382	
	VIM – R′	AATGCGCAGCACCAGGATAG			
bla_{GIM}	GIM – F′	TCAATTAGCTCTTGGGCTGAC	53	726	
	GIM – R′	CGGAACGACCATTTGAATGG			
bla_{NDM-1}	NDM – F′	GGTTTGGCGATCTGGTTTTC	52	621	
	NDM – R′	CGGAATGGCTCATCACGATC			

Note: ESBL: extended-spectrum β-lactamase; IMP: imipenem.

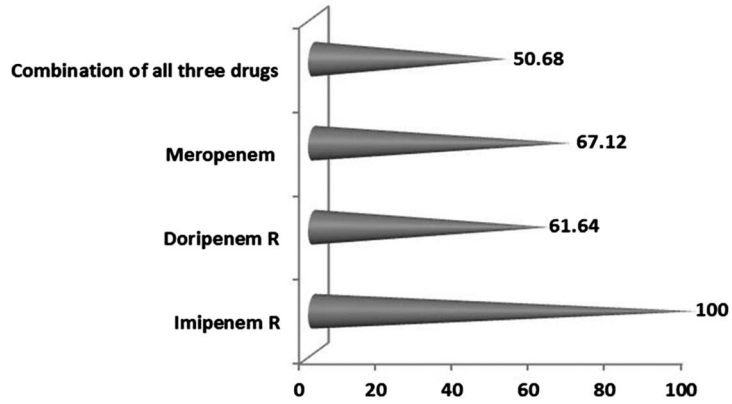

Figure 1. Frequency of resistance exhibited *by A. baumannii* toward the antibiotics tested

Table II. Preliminary screening and phenotypic confirmation of ESBL producers among the *A. baumannii* isolates as per CLSI (2012) [21]

Isolate	Preliminary screening	DDST	
A. baumannii ($N = 73$)	100% ($n = 73$)	Antibiotics	Positive (%)
		Imipenem and imipenem + EDTA	41.46 ($n = 31$)

Note: ESBL: extended-spectrum β-lactamase; DDST: double disc synergy test; EDTA: ethylene diamine tetra acetic acid; CLSI: Clinical Laboratory Standards Institute.

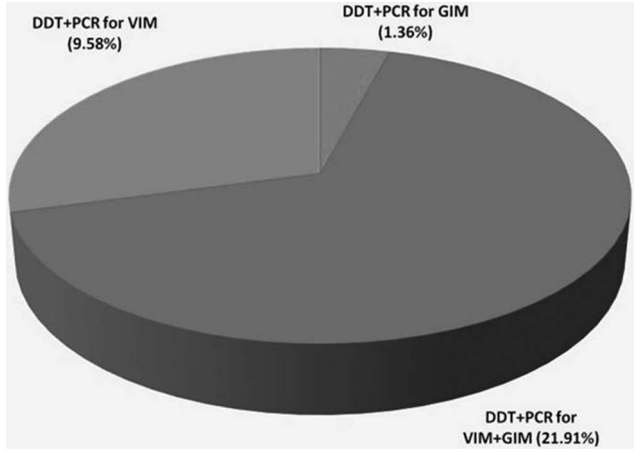

Figure 2. The specificity of detection methods employed for identifying drug-resistant strains

Table III. Distribution of bla_{IMP}, bla_{NDM}, bla_{VIM}, and bla_{GIM} genes among the MBL producers of *A. baumannii*

MBL-positive isolates	Target genes studied (%)				
A. baumannii ($N = 73$)	bla_{IMP}	bla_{NDM}	bla_{VIM}	bla_{GIM}	$bla_{VIM} + bla_{GIM}$
	0	0	34.24 ($n = 25$)	16.43 ($n = 12$)	2.73 ($n = 2$)

Note: MBL: metallo-β-lactamase.

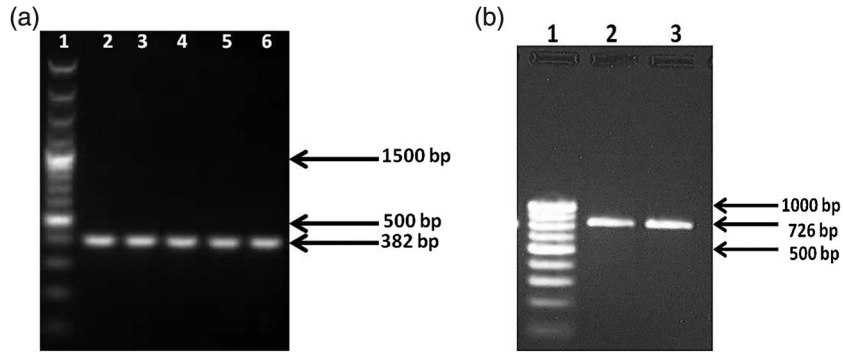

Figure 3. Electrophoretogram of (a) bla_{VIM} gene and (b) bla_{GIM} amplicons run along with 100 bp DNA ladder (Lane 1)

imipenem-resistant strains of *A. baumannii*. One hundred percent of the strains showing imipenem resistance are also reported in an earlier study from South India [27]. Imipenem showing intrinsic resistance in *A. baumannii* is reported [28] and in many earlier studies the isolates of *A. baumannii* for carbapenemase and MBL production were categorized based on imipenem susceptibility and resistance patterns [29]. Several studies have recorded the higher incidences of imipenem non-susceptibility/resistance [30, 31]. This study has recorded nearly 60%–65% of non-susceptibility against doripenem and meropenem with only 15.06% and 13.69% susceptibility against the same, respectively. Similar observations were recorded from Turkey with 66.6% resistance against meropenem and 49.9% against doripenem [32]. Another study from USA showed 68% and 80% non-susceptibility to meropenem and doripenem, respectively [33]. On the contrary, an earlier study from Punjab recorded only 6% of the isolates to exhibit non-susceptibility against doripenem and meropenem [34]. Along with these routinely administered carbapenems, administration of ertapenem induced no impact on the susceptibility pattern of imipenem and was directly associated with the reduced use of imipenem and ciprofloxacin among *A. baumannii* [35]. However, this study

limits *per se* the omission of ertapenem under carbapenem-resistant profile for the test organisms under the study.

Phenotypic detection of MBL production was observed using IMP and IMP/EDTA potentiation disc test. Among the tested isolates, with 100% resistance against imipenem and nearly 63% resistance against doripenem and meropenem, phenotypic confirmation was achieved only in 42.46% of the isolates. In addition, DDST positivity co-related only with 31.50% ($n = 23$) of the isolates confirmed by Kirby–Bauer diffusion assay. Among the 73 imipenem-resistant isolates, only 31 were DDST-positive, which might be due to the bla_{IMP} gene cassettes associated with integrons that do not phenotypically express the bla_{IMP} type of genetic determinants [36]. About 21.91% and 9.58% of the isolates among 31 DDST-positive *A. baumannii*, expressing bla_{VIM} and bla_{GIM}, suggest the role of VIM- and GIM-type β-lactamases in inducing carbapenem resistance. Although variants of IMP and VIM are frequently reported, MBL genetic determinants can be restricted to certain geographical regions with members of SPM, GIM, and SIM variants [37]. Genotypic characterization of MBL determinants with phenotypic-positive DDST showing 6 and 19 negative isolates may be again related to the variants exhibited among class I integron structures, which are frequently detected among *A. baumannii* [38, 39]. Comparative analysis between phenotypic and genotypic data observed in the present investigation suggests DDST to be highly reliable and easy to perform for the preliminary screening of MBL production with the reports varying from 7.5% [40] to 70.9% [27].

Molecular detection of the genetic determinants of MBL production such as bla_{VIM}, bla_{IMP}, bla_{GIM}, and bla_{NDM} was observed using PCR. All the imipenem-resistant isolates ($n = 73$) of *A. baumannii* showed bla_{IMP} and bla_{NDM} negative. In comparison with the carbapenem-resistant profile (imipenem – 100%, doripenem – 61.64%, and meropenem – 67.12%) and DDST-positive isolates ($n = 31$), only 25 (34.24%) and 12 (16.43%) showed the presence of bla_{VIM} and bla_{GIM}. This variation might be due to the other non-enzymatic mechanisms, such as presence of efflux pumps, role of outer membrane proteins etc., exhibiting the carbapenem resistance property among *A. baumannii* [41]. A widespread distribution of MBL producers among *A. baumannii* is observed worldwide such as 70%–90% in India, 27.1% in Pakistan, and considerable numbers in Europe, Australia, and Africa [42, 43].

Among the MBL genetic determinant, co-occurrences of the genes are also not uncommon. The studies record the different patterns of co-occurring MBL genes from different countries [44]. In view with this, the present study also records the co-occurrence of bla_{VIM} and bla_{GIM} in two isolates (2.73%). Comparative analysis between phenotypic and genotypic detection also shows a significant report. The study records 21.91% ($n = 16$) and 9.58% ($n = 7$) with DDST + bla_{VIM} and DDST + bla_{GIM} positivity, respectively, with one 1.36% of

the isolate showing DDST + bla_{VIM} + bla_{GIM} positivity. In an earlier study from Nepal, co-existence of bla_{OXA-23} and bla_{NDM-1} was detected [45] with the presence of other class B MBLs, such as bla_{VIM} and bla_{GIM}. One of the recent studies showed the presence of bla_{VIM} only in imipenem-resistant genomic species of 13TU – *A. baumannii* and not in other isolates [46]. These results when compared with the present investigation vividly portray the differences in the phenotypic and genotypic traits against the carbapenems among the *A. baumannii* species existing in different geographic locale.

A. baumannii traits acquire different kinds of antimicrobial resistance and are emerging as a dreadful nosocomial pathogen leading to complications in the treatment and control. Frequency of MBLs and the distribution of their genetic determinants restrict the administration of carbapenems against *A. baumannii*. Thus, this study concludes by stating the need for the proper and periodical antimicrobial surveillance programs for using carbapenems against *A. baumannii* isolates, as there exists a variation in the resistance pattern and the associated genes in inducing the carbapenemase resistance.

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Conflict of Interest

None.

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