

Molecular Characterization of Carbapenem Resistant Isolates of *Acinetobacter baumannii* in An Intensive Care Unit of A Tertiary Care Centre at Central India

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ABSTRACT

Objective: To detect genes encoding carbapenem resistance in *Acinetobacter baumannii* in an intensive care unit.

Methods: *A. baumannii* isolates were recovered from various clinical specimens of hospitalized patients admitted to the Medical and Surgical intensive care units of a tertiary care centre in Pune. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques. Antibiotic sensitivity test was performed by standard Kirby Bauer disc diffusion technique. PCR amplification and automated sequencing was carried out.

Results: A total of 155 /368 (42.11%) isolates *A. baumannii* were found to have reduced susceptibility to imipenem (diameter of zones of inhibition ≤ 13 mm) by disc diffusion method. Among

these 155 isolates tested 130 (83.87%) isolates showed MIC values for imipenem and meropenem ranging from 16-64 mg/L as per CLSI breakpoints. Among these 155 isolates, Carbapenemase production was confirmed by Modified Hodge test for 93 (60%) isolates. Out of 155 isolates, DDST was positive for 89 (57.41%), CDST was positive for 73(47.09%) and MBL (IP/IP) E-test was positive for 105 (67.74%). bla_{OXA-51} gene was detected in 47/105 (44.76%), bla_{OXA-23} gene in 55/105 (52.38%) and blaOXA-58 like gene in 15/105 (14.28%).

Conclusion: MBL production along with co- production of OXA enzymes are considered to be the important reason for resistance to imipenem in *Acinetobacter* in our health care settings. Hence, early detection of these drug resistant genes by molecular methods is essential in limiting the spread of infection due to these organisms.

Keywords: *A. baumannii*, Carbapenem, Multidrug resistant, Metallo beta-lactamase

INTRODUCTION

Acinetobacter is non fermentative, gram-negative coccobacilli that has emerged as an important opportunistic pathogen, frequently occurring in critically ill intensive care unit (ICU) patients with chronic illness or prolonged hospitalizations [1,2]. *A. baumannii* is ubiquitous in the hospital environment, particularly in the ICU and constitute 80 percent of the total clinical isolates of *Acinetobacter* that has been reported worldwide [3]. Carbapenems are the antibiotics of choice, for the treatment of infections caused by *A. baumannii* when these bacteria are resistant to other β -lactam antibiotics. *A. baumannii* is inherently adaptive for genetic exchange due to the lack of mutS gene which is part of the mismatch repair system that preserves genetic stability and exhibits increased mutation rates [4]. Lately carbapenem resistance is noted amongst *A. baumannii* isolates due to modification of penicillin-binding proteins (PBPs), altered efflux pump activity, carbapenemase production and loss of porins. Carbapenemases production appears to be the most common mechanism involved in drug resistance to carbapenems in these organisms. Infections caused by metallo-beta-lactamase [MBL] producing *A. baumannii* are associated with rising health-care costs and high rates of mortality and morbidity. There has been an increasing trend in mortality in-association with bacteremia (52%) and pneumonia (23-73% [5,6]. Resistance to carbapenems, as mediated by the Ambler class D beta-lactamases (OXA-type) and Ambler class B metallo-beta-lactamases (MBLs), are of greatest concern. Class D carbapenemases are classified into four subgroups; Subgroup 1, the OXA-23 group (including OXA-27 and OXA-49) are the plasmid encoded genes, Subgroup 2 is the OXA-24 group (including OXA-40, OXA-26 and OXA-25), which is chromosomally encoded, Subgroup 3 consists of OXA-51 and its variants, which are chromosomally encoded and Subgroup 4

contains OXA-58, which is a plasmid-encoded gene [7]. The most common metallo- β -lactamases include VIM, IMP, GIM, SIM, SPM and NDM-1 which are located on a variety of plasmids and integron structure that are incorporated as gene cassettes [8]. Insertion of ISAbal, upstream of OXA type class D carbapenemase encoding gene, may provide a promoter to enhance gene expression which ultimately contribute to the increased level of resistance to carbapenems in *Acinetobacter* [9].

This study was undertaken to detect the prevalence of different carbapenemases and molecular detection of resistant genes amongst *A. baumannii* isolates in ICU patients.

MATERIALS AND METHODS

The Bacterial Isolates

A prospective study was conducted in a 1000 bedded tertiary care centre in Pune, India from March 2011 to March 2013. A total of 368 clinically significant, non-duplicate *A. baumannii* isolates were recovered from clinical specimens of hospitalized patients admitted to the Medical and Surgical intensive care units. Collection of sample was done using strict aseptic precautions and was immediately processed. The isolates were obtained from various clinical specimens such as cerebrospinal fluid, bone marrow, blood, pus, urine, lower respiratory secretions (endotracheal secretions, bronchoalveolar lavage and bronchial wash) and other sterile body fluids. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques [10]. The organisms were identified up to the species level using VITEK-GNI cards (bioMérieux, Marcy l'Etoile, France). The significance of the isolates was based on presence of intracellular bacteria, presence

of the organism in the Gram stain, pure growth in culture with a significant colony count and clinical history. All the strains were preserved in 15% glycerol-supplemented Luria-Bertani medium at -80°C for molecular analysis.

Antimicrobial Susceptibility Testing and Determination of MIC

Antibiotic sensitivity test was performed by standard Kirby Bauer disc diffusion technique as per the guidelines of the Clinical Laboratory Standards Institute (CLSI) with commercially available discs (Hi Media, Mumbai, India) on Mueller Hinton agar plates [11]. The antibiotics tested were as follows (potency in $\mu\text{g}/\text{disc}$): Piperacillin (100), Ticarcillin (75), Piperacillin-Tazobactam (100/10), Ticarcillin-Clavulanic acid (75/10), ceftazidime (30), cefotaxime (30), cefepime (30), ceftriaxone (30), Imipenem (10), Meropenem (10), Gentamicin (10), Tobramycin (10), Amikacin (30), Netilmicin (30), Ciprofloxacin (5), Levofloxacin (5), Lomefloxacin (10) and Ofloxacin (5). *E. coli* ATCC 25922, *E. coli* ATCC 35218 and *P. aeruginosa* ATCC 27853 strains were used for quality control. Minimum inhibitory concentrations (MIC) of antibiotics were determined by VITEK-2 as per CLSI break points in isolates showing resistance to carbapenems [11]. Also MICs were further determined by the E-test method against imipenem, meropenem, tigecycline and colistin (bioMérieux, Marcy l'Etoile, France).

Screening for the Carbapenemase Production

A. baumannii isolates, with a reduced susceptibility to meropenem and imipenem (diameter of zones of inhibition $\leq 13\text{mm}$) by disc diffusion method, were screened for the production of carbapenemase. The phenotypic detection of the carbapenemase production was performed by the modified Hodge test by using a meropenem disc (10 μg) as per CLSI guidelines [11]. The screening of metallo-beta-lactamase production was performed by the double-disc synergy tests (DDST) and combined-disc synergy test (CDST) as described previously by using an imipenem discs [12]. Also MBL (IP/IP) E-test was carried out to detect MBL as per manufacturer's instructions.

Molecular detection of the Beta-lactamase genes

DNA from the isolates were extracted using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions. PCR-based detection of Ambler class B MBLs (bla_{IMP} , bla_{VIM} , bla_{SPM} , bla_{GIM} , bla_{SIM} and $\text{bla}_{\text{NDM-1}}$) and class D ($\text{bla}_{\text{OXA-23}}$, $\text{bla}_{\text{OXA-24}}$, $\text{bla}_{\text{OXA-51}}$ and $\text{bla}_{\text{OXA-58}}$) were carried out on the isolates by using Gene Amp 9700 PCR System (Applied Biosystems, Singapore). PCR primers and reaction conditions for PCR as described previously [12-14]. PCR amplification for the detection of class-1 integron cassettes (integron PCR), *Int1* and *Int2* integrase genes (integrase gene PCR) were performed with primers as described previously [15]. Amplified PCR products were resolved by electrophoresis at 80 V for 2 h on 2% agarose gels with 0.5 Tris-borate-EDTA buffer containing ethidium bromide and were visualized under UV light and photographed. The amplicons were purified using QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany). Automated sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (<http://blast.ncbi.nlm.nih.gov>).

RESULT

A total of 155 (42.11%) isolates *A. baumannii* were found to exhibit reduced susceptibility to imipenem (diameter of zones of inhibition $\leq 13\text{mm}$) by disc diffusion method. Among 155 isolates tested, 130 (83.87%) isolates showed MIC values for imipenem and meropenem ranged from 16-64 mg/L as per CLSI breakpoints. All of these 155 isolates were found to be susceptible to tigecycline and Colistin in vitro as per MIC breakpoints (for tigecycline was

$<2\text{mg}/\text{L}$ and for colistin $<0.5\text{ mg}/\text{L}$). Among these 155 isolates, Carbapenemase production was confirmed by Modified Hodge test for 93 (60%) isolates. Out of these 155 isolates, DDST was positive for 89 (57.41%), CDST was positive for 73 (47.09%) and MBL (IP/IP) E-test was positive for 105 (67.74%). Among these isolates 79 were from surgical and 26 were from medical ICUs. Distribution of these isolates based upon body site depicted in [Table/Fig-1]. These 105 isolates were further studied. PCR detection of the *int1* and *int2* genes determined the presence of Class 1 integrons in 95.23% (100 of 105) of the *A. baumannii* isolates, whereas only five *A. baumannii* strain (4.76%) contained a class 2 integron. Among 105 MBL (IP/IP) E-test positive *A. baumannii*, bla_{VIM} was positive for 52 (49.5%). Based on sequencing two variants of bla_{VIM} as $\text{bla}_{\text{VIM-2}}$ and $\text{bla}_{\text{VIM-6}}$ were found in given study. $\text{bla}_{\text{OXA-23}}$ like was detected in 55 isolates, $\text{bla}_{\text{OXA-51}}$ like was detected in 47 isolates, $\text{bla}_{\text{OXA-58}}$ like was detected in 15 isolates and $\text{bla}_{\text{OXA-24}}$ like was detected in 10 isolates. $\text{bla}_{\text{OXA-51}}$ alone was detected in 12 isolates while $\text{bla}_{\text{OXA-23}}$ alone was detected in 16. Coexistence of $\text{bla}_{\text{OXA-23}}$ with $\text{bla}_{\text{OXA-51}}$ was detected in 35. ISAb1 was positive for all the *A. baumannii* isolates. Among ISAb1 positive isolates of *A. baumannii*, twelve isolates showed co-presence of bla_{VIM} with $\text{bla}_{\text{OXA-51}}$ and another twelve showed co-presence of bla_{VIM} with $\text{bla}_{\text{OXA-58}}$ respectively. Ten isolates showed bla_{VIM} with $\text{bla}_{\text{OXA-24}}$ while two isolates showed co-presence of bla_{VIM} , $\text{bla}_{\text{OXA-23}}$ and $\text{bla}_{\text{OXA-51}}$ [Table/Fig-2].

Body site	Number of Isolate
Tracheal aspirate	45
Blood culture	24
BAL	08
PUS culture	18
Sputum culture	10

[Table/Fig-1]: Distribution of carbapenem resistant *Acinetobacter baumannii* (n=105) among different body site

Class I Integron (n=100)		Class II Integron (n=5)	
Name of Gene	No. (%)	Name of Gene	No. (%)
$\text{bla}_{\text{OXA-23}}$ alone	16	$\text{bla}_{\text{OXA-24}}$ alone	05
$\text{bla}_{\text{OXA-51}}$ alone	02	Total	05
$\text{bla}_{\text{OXA-58}}$ alone	01	-	-
bla_{VIM} alone	15	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-23}}$	02	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-51}}$	10	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-58}}$	12	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-24}}$	05	-	-
$\text{bla}_{\text{OXA-23}}+\text{bla}_{\text{OXA-51}}$	33	-	-
$\text{bla}_{\text{OXA-23}}+\text{bla}_{\text{OXA-58}}$	01	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-23}}+\text{bla}_{\text{OXA-51}}$	02	-	-
$\text{bla}_{\text{VIM}}+\text{bla}_{\text{OXA-23}}+\text{bla}_{\text{OXA-58}}$	01	-	-
Total	100	-	-

[Table/Fig-2]: Distribution of carbapenem resistant genes in *Acinetobacter baumannii* (n=105)

DISCUSSION

There is a worldwide emergence of *Acinetobacter baumannii* as one of the most prominent nosocomial pathogen. Multidrug resistant phenotypes emerged as a result of misuse of broad-spectrum antibiotics against this bacterium. Natural resistance mechanisms of *Acinetobacter spp.* to several β -lactam antibiotics also aids in its resistance profile. Long term survival in the health care setting is also a contributing factor that acts synergistically with emerging resistance profiles. Resistance nodulation cell-division (RND), Multidrug and toxic compound extrusion (MATE) and

Major facilitator super family (MFS) are the efflux systems usually associated along with carbapenemase production in *A. baumannii* for antimicrobial resistance. Intensive care units provide an ideal environment for the dissemination of resistant determinant genes within the organisms. There are multiple risk factors associated with both environment and the patient that allow the development and spread of such pathogens. ICU patients often have frequent hospital admissions with their respective underlying medical conditions and there has been increase in the risk of colonization by multi-drug-resistant pathogens. Carbapenem resistant isolates are generally resistant to most other classes of antibiotics, while usually retaining susceptibility to tigecycline and colistin. Resistance against carbapenem in and on itself is considered sufficient to define an *A. baumannii* isolate as highly resistant. Carbapenem resistance because of the OXA-type (class D) carbapenemases is growing problem [16-18]. OXA enzymes are the most important reason for resistance to imipenem and meropenem in *Acinetobacter* worldwide. OXA-type carbapenemases can be more effective against imipenem and meropenem and ISAb_a sequence can induce high level of resistance in this strains [8,14]. bla_{OXA-51} gene was detected in 47/105 (44.76%), bla_{OXA-23} gene in 55/105 (52.38%) and bla_{OXA-58} like gene in 15/105 (14.28%). Previous study from Korea reported co-presence of ISAb_a1 with bla_{OXA-23} (38.9%) and ISAb_a1 with bla_{OXA-51} (69.4%) respectively [19] while findings from other studies from Italy reported presence bla_{OXA-23} with bla_{OXA-58} [20]. ISAb_a1 was positive for all *A. baumannii* isolates tested while Only 10 isolates carried bla_{OXA-24} like gene.

CONCLUSION

A. baumannii is a notorious pathogen in ICU settings. Carbapenem resistance because of OXA type carbapenemases is a growing problem. MBL production along with co- production of OXA enzymes are considered to be the important reason for resistance to IPM in *Acinetobacter* in our health care settings. Hence, early detection of these drug resistant genes by molecular methods is essential in limiting the spread of infection due to these organisms.

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