

Molecular characterization & epidemiology of carbapenem-resistant *Acinetobacter baumannii* collected across India

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Background & objectives: Acinetobacter baumannii is an opportunistic pathogen responsible for causing nosocomial infections. A. baumannii develops resistance to various antimicrobial agents including carbapenems, thereby complicating the treatment. This study was performed to characterize the isolates for the presence of various β -lactamases encoding genes and to type the isolates to compare our clones with the existing international clones across five centres in India.

Methods: A total 75 non-repetitive clinical isolates of *A. baumannii* from five different centres were included in this study. All the isolates were confirmed as *A. baumannii* by $bla_{OXA-51-like}$ PCR. Multiplex PCR was performed to identify the presence of extended spectrum β -lactamases (ESBL) and carbapenemases. Multilocus sequence typing was performed to find the sequence type (ST) of the isolates. e-BURST analysis was done to assign each ST into respective clonal complex.

Results: $bla_{OXA-51-like}$ was present in all the 75 isolates. The predominant Class D carbapenemase was $bla_{OXA-51-like}$ followed by Class B carbapenemase, $bla_{NDM-like}$. Class A carbapenemase was not observed. $bla_{PER-like}$ was the predominant extended spectrum β -lactamase. ST-848, ST-451 and ST-195 were the most common STs. Eight-novel STs were identified. e-BURST analysis showed that the 75 *A. baumannii* isolates were clustered into seven clonal complexes and four singletons, of which, clonal complex 208 was the largest.

Interpretation & conclusions: Most of the isolates were grouped under clonal complex 208 which belongs to the international clonal lineage 2. High occurrence of ST-848 carrying $bla_{OXA-23-like}$ gene suggested that ST-848 could be an emerging lineage spreading carbapenem resistance in India.

Key words $bla_{OXA-23-like}$ - carbapenem-resistant *Acinetobacter baumannii* - CC208 - India - multilocus sequence typing - sequence type

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Acinetobacter baumannii has emerged as a predominant cause of nosocomial infections across the globe^{1,2}. A. baumannii can cause a wide range of infections such as ventilator-associated pneumonia, wound infections, urinary tract infections, bloodstream infections and surgical site infections^{2,3}. Carbapenems are considered to be one of the drugs of choice for treating Acinetobacter infections. However, increased resistance to carbapenem class of antibiotics has been reported worldwide¹. Results from studies have reported carbapenem resistance rate of A. baumannii as 40-75 per cent throughout India⁴.

Carbapenem resistance in *A. baumannii* is mainly due to Class D and Class B carbapenemases belonging to Ambler's classification of β -lactamases. Although various mechanisms contribute to carbapenem resistance, the majority is due to class D carbapenemases such as $bla_{OXA-23-like}$, $bla_{OXA-24-like}$ and $bla_{OXA-58-like}$ ^{5,6}. $bla_{OXA-23-like}$ producing *A. baumannii* responsible for causing outbreaks have been reported from various regions of the world⁷. Class D carbapenemase in transposons, have the ability to rapidly spread in successful clonal lineages of *A. baumannii*⁸.

To control the spread of A. baumannii in the hospital environment, it is necessary to characterize the molecular epidemiology of A. baumannii isolates involved in nosocomial infections9. Multi locus sequence typing (MLST) is highly discriminative and has been successfully applied to various clinically important bacterial pathogens including A. baumannii. MLST offers the possibility of inter-laboratory comparison, thereby providing a powerful tool for epidemiological studies globally¹⁰. Published data on the sequence types (STs) of Indian isolates are limited. The aim of the study was to identify the presence of various types of β -lactamases among the carbapenem-resistant isolates and epidemiological typing of A. baumannii by MLST.

Material & Methods

This study included a total of 75 non-repetitive isolates of carbapenem-resistant *A. baumannii* collected between 2015 and 2017 from five centres across India. These were All India Institute of Medical Sciences (AIIMS, New Delhi), AIIMS trauma centre (New Delhi), Christian Medical College (CMC, Vellore), Jawaharlal Institute of Postgraduate Medical Education & Research (JIPMER, Puducherry) and Postgraduate Institute of Medical Education & Research (PGIMER, Chandigarh). All the study isolates were identified up to the species level as *A. baumannii calcoaceticus* complex (ABCC) using standard biochemical tests. Further confirmation of ABCC as *A. baumannii* was performed using $bla_{OXA-51-like}$ PCR which is intrinsic to this species¹¹.

Antimicrobial susceptibility testing: Susceptibility to carbapenem class of antibiotics was determined in the department of Clinical Microbiology, CMC for all the study isolates by Kirby Bauer disc diffusion method and interpreted according to Clinical Laboratory Standard Institute guidelines¹²⁻¹⁴. The antibiotics tested were imipenem (10 μ g) and meropenem (10 μ g).

Detection of extended spectrum β -lactamase (ESBL) & carbapenemase-encoding genes by multiplex PCR: All the test isolates of *A. baumannii* were grown overnight on blood agar and genomic DNA was extracted using boiling lysis method¹⁵. Conventional multiplex PCR was done for the detection of genes encoding ESBLs such as $bla_{\text{TEM-like}}$, $bla_{\text{SHV-like}}$, $bla_{\text{PER-like}}$ and carbapenemase genes such as $bla_{\text{GES-like}}$, $bla_{\text{SPM-like}}$, $bla_{\text{IMP-like}}$, $bla_{\text{NDM-like}}$, $bla_{\text{KPC-like}}$, $bla_{\text{OXA-48-like}}$ and $bla_{\text{SIM-like}}$, $bla_{\text{NDM-like}}$, $bla_{\text{KPC-like}}$, $bla_{\text{OXA-48-like}}$ and $bla_{\text{OXA-58-like}}$ was also screened by multiplex PCR. The amplicons were visualized in two per cent agarose gel with staining by ethidium bromide. Known positive controls for appropriate genes were used (Courtesy: IHMA, Inc., USA)¹⁶. Targeted sequencing was performed to identify the variant of $bla_{\text{TEM-like}}$ gene.

Insertion sequence mapping PCR: Mapping PCR was performed for a subset of isolates (n=25) to map the position of insertion sequence, ISAba1 with respect to $bla_{OXA-23-like}$ gene¹⁷.

Multilocus sequence typing (MLST): MLST is based on the sequence analysis of seven housekeeping genes and was performed according to Bartual's or oxford scheme¹⁰. The seven housekeeping genes included *gltA* (coding for citrate synthase), *gyrB* (coding for DNA gyrase subunit B), *gdhB* (coding for glucose dehydrogenase B), *recA* (coding for homologous recombination factor), *cpn60* (coding for 60 kDa chaperonin), *gpi* (coding for glucose-6-phosphate isomerase) and *rpoD* (coding for RNA polymerase 70 factor) were checked. All the seven housekeeping genes were amplified and sequenced using previously described primers¹⁰. Each gene sequence was submitted to PubMLST database to find the allelic number and the STs were assigned to each isolate with the seven allelic profiles (*http://pubmlst.org/ abaumannii/*).

e-BURST analysis: e-BURST analysis was performed using the software, e-BURSTv3 (Developed and hosted at The Department of Infectious Disease Epidemiology, Imperial College London) available on the website to assign the STs into respective clonal complexes (*http://eburst.mlst.net/*) and were defined as single locus (SLVs) and double loci variants (DLVs).

Results

Of the 75 *A. baumannii* isolates included in this study, 18 were from AIIMS, 22 from AIIMS trauma centre, 25 isolates from CMC, two isolates from JIPMER and eight isolates were from PGIMER. All isolates were resistant to both imipenem and meropenem.

Molecular characterization of ESBL and carbapenemase genes: bla_{OXA-51-like} gene which is intrinsic to A. baumannii, was present in all the 75 isolates (100%). Among the Class D carbapenemases, bla_{OXA-23-like} gene was the most predominant and present in 73 isolates (97%). None of the isolates harboured $bla_{OXA-24-like}$ and $bla_{OXA-58-like}$ genes. Among the Class B carbapenemases, only $bla_{\text{NDM-like}}$ gene was found in 13 isolates (17%) whereas other genes such as *bla*_{IMP-like}, $bla_{\text{VIM-like}}$, $bla_{\text{SPM-like}}$ and $bla_{\text{SIM-like}}$ were not identified in any of the isolates. None of the isolates had Class A carbapenemase genes like $bla_{\text{KPC-like}}$ and $bla_{\text{GES-like}}$. Among the ESBLs, $bla_{\text{PER-like}}$ gene was found in 43 isolates (57%). The other ESBL genes like $bla_{\text{SHV-like}}$ and $bla_{\text{VEB-like}}$ were not identified in any of the isolates tested. $bla_{\text{TEM-like}}$ gene was found in five isolates (7%). Distribution of carbapenemase and ESBL genes was similar across all the five study centres. Targeted sequencing revealed that five isolates positive for $bla_{\text{TEM-like}}$ belonged to the variant $bla_{\text{TEM-l}}$ gene

Mapping PCR: the ISAba1 element was found upstream of $bla_{OXA-23-like}$ gene in a subset of isolates (n=25) tested.

Multi locus sequence typing: Analysis of multilocus STs of the 75 *A. baumannii* isolates identified 34 different STs. The predominant ST was ST-848, which was

found in 15 isolates (20%), followed by ST-451 in nine isolates (12%), ST-195, in five isolates (7%), ST-218, ST-491 and ST-862 each in three isolates, respectively (4%), ST-208, ST-447, ST-450 and ST-1305 each in two isolates, respectively (3%). Eight novel STs, ST-1500, ST-1501, ST-1502, ST-1503, ST-1504, ST-1505, ST-1506 and ST-1507 were identified in isolates from AIIMS trauma centre. Other less common STs observed in single isolate were ST-229, ST-231, ST-386, ST-391, ST-482, ST-539, ST-620, ST-1051, ST-1114, ST-1223, ST-1289, ST-1306, ST-1307, ST-1308, ST-1335 and ST-1417. ST profiles of the 75 clinical isolates of *A. baumannii* are summarized in the Table.

e-BURST analysis: e-BURST analysis showed that all 75 A. baumannii isolates were clustered into seven clonal complexes (CCs) and four singletons. CC208 was the largest clonal complex and comprised STs 195, 208, 218, 451 and 539 differing in their gpi (SLV), 450, 848, 1114, 1289 and 1305 differing in *gpi* and *gyrB*, 1417 in gpi and recA, 1501 in gpi and cpn60 and 1502 in gpi and rpoD (DLV), respectively. The STs 386, 862, 1306, 1308, 1500, 1506 differing in gpi (SLV) and 1505 differing in *gpi* and *cpn60* (DLV) belonged to CC862, while STs 231, 491 differing in gyrB (SLV) whereas 1223 in gvrB and recA, 1503 and 1504 in gpi and gyrB (DLV) belonged to CC231 and STs 391 and 447 differing in gpi and gyrB (DLV) belonged to CC447. Three STs, ST-229, ST-620 and ST-1507 were shown to be the founder of the CCs 229, 620 and 1507, respectively. Four STs, 1307, 1335, 1051 and 482, were found to be singletons (Figure).

Clonal complex, CC208, was the predominant and observed across all the five centres in this study. CC862 was observed in AIIMS, AIIMS trauma centre and CMC. CC231 was seen among AIIMS, AIIMS trauma centre and PGIMER isolates. CC447 was observed in AIIMS and CMC centres. CC229, CC620 and CC1507 were observed among AIIMS and AIIMS trauma centres, respectively. Four singletons, 482 from PGIMER, 1051 from AIIMS trauma centre, 1307 and 1335 from CMC were observed.

Discussion

The various molecular typing methods for epidemiological characterization include PCR-based DNA fingerprinting methods such as M13, ERIC and DAF4, restriction enzyme-based methods such as pulsed-field gel electrophoresis and amplified fragment length polymorphism and MLST^{18,19}. We followed the

Table. Association of Class D carbapenemases with sequence types of carbapenem-resistant Acinetobacter baumannii								
Number of isolates	carrying intri	intrinsic	Number of isolates carrying acquired OXA gene			Sequence	Clonal	Regional
OXA gene <i>bla</i> _{OXA-51-like}			bla _{OXA-23-like}	bla _{OXA-24-like}	bla _{OXA-58-like}	type	complex	profile
15			15	0	0	848	CC208	Across India
9			9	0	0	451	CC208	
5			5	0	0	195	CC208	
3			3	0	0	862	CC862	
3			3	0	0	218	CC208	
2			2	0	0	208	CC208	
1			1	0	0	391	CC447	South India
1			1	0	0	1114	CC208	
2			2	0	0	1305	CC208	
1			1	0	0	1306	CC862	
1			0	0	0	1307	Singleton	
1			1	0	0	1308	CC862	
1			1	0	0	1335	Singleton	
1			1	0	0	229	CC229	North
1			1	0	0	231	CC231	India
1			1	0	0	386	CC862	
2			2	0	0	447	CC447	
2			2	0	0	450	CC208	
1			1	0	0	482	Singleton	
3			3	0	0	491	CC231	
1			1	0	0	539	CC208	
1			0	0	0	620	CC620	
1			1	0	0	1051	Singleton	
1			1	0	0	1223	CC231	
1			1	0	0	1289	CC208	
1			1	0	0	1417	CC208	
1			1	0	0	1500*	CC862	
1			1	0	0	1501*	CC208	
1			1	0	0	1502*	CC208	
1			1	0	0	1503*	CC231	
1			1	0	0	1504*	CC231	
1			1	0	0	1505*	CC862	
1			1	0 0	0	1506*	CC862	
1			1	0	0	1507*	CC1507	
*Novel sequence types South India-CMC and IIPMER North India-AIIMS AIIMS trauma centre and PGIMER OXA ovacillinase:								
CMC, Christian Medical College: JIPMER, Jawaharlal Institute of Postgraduate Medical Education & Research: AIIMS All India								

Institute of Medical Sciences; PGIMER, Postgraduate Institute of Medical Education & Research

Bartual's scheme¹⁰, as several studies reported high discriminatory power using Bartual's scheme when compared to Pasteur^{20,21}. Carbapenem resistance in *A. baumannii* is mainly due to Class D oxacillinases. In this study, the predominant OXA group associated

with carbapenem resistance across all five centres was $bla_{OXA-23-like}^{16}$. This was in concordance with other studies where 98 and 81 per cent of the isolates were carbapenem resistant due to $bla_{OXA-23-like}$ gene, respectively^{22,23}.



Figure. e-BURST analysis showing clonal complexes and singletons of 34 sequence types of carbapenem-resistant *Acinetobacter baumannii*. Each circle signifies the sequence type. The size of each circle represents to different number of isolates, with larger sizes corresponding to higher frequency of occurrence.

Among the Class B carbapenamases, only $bla_{NDM-like}$ was identified across all the centres except from JIPMER in this study. One study reported $bla_{NDM-like}$ and $bla_{VIM-like}$ as the predominant Class B carbapenemase¹⁶ whereas other studies showed $bla_{IMP-like}$ and $bla_{NDM-like}$ as prevalent Class B carbapenemase²³⁻²⁵. Saranathan *et al*²⁶ reported 31 and 15 per cent of $bla_{IMP-like}$ and $bla_{NDM-like}$, respectively.

Among ESBLs, $bla_{\text{PER-like}}$ was the most common and identified across all the four centres whereas other studies showed 54 and 81 per cent of $bla_{\text{PER-like}}$, respectively^{22,26}. $bla_{\text{TEM-1}}$ was found only among AIIMS, CMC and PGIMER isolates.

In this study, 34 STs were identified from 75 clinical isolates of *A. baumannii*. The most common ST identified was ST-848 which was observed among CMC, AIIMS trauma centre, JIPMER and PGIMER isolates. A study from north India reported ST-146, ST-110, ST-69, ST-103, ST-194, ST-108 and ST-188 as the predominant STs and another study from south India showed ST-538, ST-539, ST-103 and ST-576 as the most common STs in their settings^{23,25}. None of this study isolates had STs similar to the previously reported STs, except ST-539 which was reported earlier from south India suggesting diverse clonal relatedness²³.

The most common clonal complex in this study was CC208, a SLV of the globally disseminated clonal complex CC92 (International clone-II). An earlier study showed CC108 as the predominant clone in India²⁷, while Saranathan *et al*²³ reported CC103 and CC92 as the prevalent clonal complexes in south India. The same investigators also observed the predominance of clonal complexes CC92 followed by CC447²⁶. Although in the current study, none of the isolates belonged to CC92, it was found to be the subgroup founder of CC208.

Molecular epidemiology of clinical isolates of *A. baumannii* showed eight international clonal lineages (ICL) dominating across the globe. Most common clonal lineages ICL1-ICL3 were initially reported in Europe and the United States, later reported from various countries³. Majority of the carbapenem resistant *A. baumannii* outbreaks were associated with the ICL2 isolates harbouring $bla_{OXA-23-like}$ carbapenemase gene³. This was in concurrence with this study where majority of the isolates were clustered under CC208 carrying $bla_{OXA-23-like}$ gene.

The main limitation of this study was insufficient number of isolates from two centres (JIPMER & PGIMER) among the five. Characterizing more number of isolates would help to understand the clonal diversity within this region.

In conclusion, our study showed diverse STs among the clinical isolates of *A. baumannii* across five centres in India. Most of the isolates were grouped under CC208. High occurrence of ST-848 carrying $bla_{OXA-23-like}$ gene suggested that ST-848 might be an emerging lineage spreading carbapenem resistance in India. Further studies with a large number of isolates need to be done to confirm these findings.

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Conflicts of Interest: None.

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