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ORIGINAL RESEARCH

Molecular Characterization of Invasive Carbapenem-Resistant *Acinetobacter baumannii* from a Tertiary Care Hospital in South India

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ABSTRACT

Introduction: *Acinetobacter baumannii* is an important opportunistic pathogen responsible for causing nosocomial infections. Carbapenems are considered to be the drug of choice to treat infections caused by multidrug-resistant *A. baumannii*. The prevalent mechanism of carbapenem resistance in *A. baumannii* is enzymatic degradation by β-lactamases. Therefore, the aim of the study is to determine the prevalence and distribution of molecular determinants among the clinical isolates of carbapenem-resistant *A. baumannii*.

Methods: A total of 103 consecutive, non-duplicate carbapenem-resistant *A. baumannii* isolated from blood and endotracheal aspirates (ETAs) were included in the study. The CarbAcineto NP test was performed for the screening of carbapenemase production. Polymerase chain reaction (PCR) was performed to detect extended spectrum β-lactamases (ESBLs), metallo-β-lactamases (MBLs) and oxacillinases (OXAs). PCR was done for the detection of IS*Aba1* elements, and mapping PCR was performed to identify the position of *ISA*ba1 with respect to the OXA-23-like gene.

Results: Among the 103 *A. baumannii* isolates, 94 were phenotypically identified as carbapenemase producers. *bla*PER was the most common among the ESBLs. Among MBLs, *bla*NDM was predominant followed by the *bla*VIM gene. *bla*OXA-51 and *bla*OXA-23 were the most common and present in all 103 isolates. Almost 80% of the isolates had *ISA*ba1 upstream *bla*OXA-23 gene.

Conclusion: The *bla*OXA-23 and *bla*NDM genes are the most common type of oxacillinases and metallo β-lactamases, respectively, and contribute to carbapenem resistance in clinical

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isolates of *A. baumannii*. The presence of *ISA*ba1 upstream of the *bla*OXA-23 gene suggests that the insertion element acts as a promoter for its increased expression.

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Keywords: *Acinetobacter baumannii*; Carbapenem resistance; Insertion sequence; Metallo-beta lactamases; Oxacillinases

INTRODUCTION

Acinetobacter baumannii is a leading cause of nosocomial infections with increased morbidity and mortality [1]. *A. baumannii* is intrinsically resistant to most antibiotics and has the ability to acquire resistance genetic determinants from the environment as well [2]. Antimicrobial resistance to β-lactams in *A. baumannii* includes: (1) enzymatic mechanisms or production of β-lactamases and (2) non-enzymatic mechanisms such as modification of membrane permeability by either loss of or decrease in expression of outer membrane porins or an increased expression of efflux pumps [2, 3].

Carbapenems are the drug of choice to treat infections caused by multidrug-resistant *A. baumannii*. However, resistance to carbapenems is increasing and has been reported worldwide. Carbapenem resistance in *A. baumannii* can be either carbapenemase mediated or non-carbapenemase mediated. Carbapenemase-mediated resistance is mainly due to the production of class A (serine proteases), class B (metallo-beta-lactamases) and class D (oxacillinases) carbapenemases, whereas non-carbapenemase-mediated resistance involves upregulation of the efflux pumps and/or loss of outer membrane porins [4].

Resistance to carbapenem in *A. baumannii* is most frequently due to oxacillinases, which can be intrinsic or acquired. The intrinsic *bla*OXA-51 gene is considered to be specific for *A. baumannii*, since the said gene is carried in its chromosome. Acquired OXA enzymes, which are encoded by the *bla*OXA-23, *bla*OXA-40 and *bla*OXA-58 genes, are more predominant in *A. baumannii*, whereas MBLs encoded by the *bla*IMP, *bla*VIM, *bla*NDM and *bla*SIM genes are more predominant in non-baumannii Acinetobacter isolates [5].

The insertion sequence has a major role in the overproduction and dissemination of OXA genes in *A. baumannii*. Overexpression of OXA genes is driven by insertion sequences, which provide promoter sequences, thereby contributing to high levels of carbapenem resistance. The insertion sequence *ISA*ba1 belongs to the IS4 family and has been associated with several resistance genes in *A. baumannii*, such as *bla*OXA-23, *bla*OXA-51 and *bla*OXA-58 [6].

Limited data are available on the resistance pattern and molecular determinants responsible for carbapenem resistance in *A. baumannii* from India. The objectives of this study were (1) to determine the trend in the antibiotic resistance profile for invasive *Acinetobacter species* and (2) to detect the prevalence of the resistance genes responsible for beta lactamases such as extended spectrum and carbapenamases in *Acinetobacter* species.

METHODS

Bacterial Isolates

The study was conducted at a tertiary care hospital, South India, from January 2014 to July 2015. A total of 103 non-duplicate, consecutive,

carbapenem-resistant isolates recovered from invasive infections of *Acinetobacter* species were included in this study. The isolates were obtained from invasive clinical specimens including blood and endotracheal aspirates (ETAs). The isolates were identified up to the species level as *Acinetobacter baumannii calcoaceticus* complex (ABCC) by standard biochemical tests and confirmed as *Acinetobacter baumannii* using *bla*OXA-51 PCR, which is intrinsic to this species [7].

Antimicrobial Susceptibility Testing

Susceptibility to different classes of antibiotics was determined by the Kirby Bauer disc diffusion method and interpreted according to the Clinical Laboratory Standard Institute guidelines [8, 9]. Antibiotics tested were ceftazidime (30 μg), cefepime (30 μg), piperacillin/tazobactam (100/10 μg), cefoperazone/Sulbactam (75/30 μg), amikacin (30 μg), netilmycin (30 μg), tobramycin (10 μg), aztreonam (30 μg), levofloxacin (5 μg), tetracycline (30 μg), co-trimoxazole (1.25/23.75 μg), imipenem (10 μg), meropenem (10 μg) and polymyxin B (10 μg).

Phenotypic Characterization

Phenotypic detection of carbapenemase was done by the CarbAcineto NP test. In brief, all the study isolates to be tested were grown on a Mueller-Hinton agar plate for 24 h, and the isolated colonies were re-suspended in two 1.5-ml centrifuge tubes (A and B) containing 100 μl NaCl (5 M). In tube A, 100 μl of solution A (phenol red solution with zinc sulfate) was added, and in tube B, 100 μl of solution A with imipenem (6 mg/ml) was added. The tubes were incubated at 37 °C for a maximum of 2 h. The carbapenemase activity was detected by color change in tube B resulting from hydrolysis of imipenem leading to a decrease in pH value [10]. Concurrently, BAA-1705 and BAA-1706 were included as positive and negative controls, respectively.

Molecular Characterization

PCR for the Detection of Carbapenemase Genes

Uniplex PCR was performed to detect the presence of the intrinsic *bla*OXA-51 gene. Multiplex PCR was performed to detect ESBL genes such as *bla*TEM, *bla*SHV, *bla*PER, *bla*VEB and *bla*GES. A second multiplex PCR was performed for MBL genes such as *bla*KPC, *bla*IMP, *bla*VIM, *bla*SIM, *bla*NDM and *bla*SPM. A third multiplex PCR was performed to detect the presence of acquired OXA genes, namely *bla*OXA-23, *bla*OXA-24 and *bla*OXA-58. Uniplex PCR was performed to detect the presence of the insertion sequence (IS) element, *ISA*ba1 and PCR mapping was performed to map the position of the IS element relative to the *bla*OXA-23 gene.

Primers used in this study were described earlier [11–16]. Briefly, for PCR amplification, 2 μl of template DNA was added to 18 μl reaction containing 10 μl of Qiagen master mix, 2 μl of primer mix (1 μl each of the respective forward and reverse primers) and 6 μl of molecular-grade water. The cyclic conditions for the intrinsic OXA-51 gene were: initial denaturation at 94 °C for 3 min, 35 cycles of 94 °C for 45 s, 57 °C for 45 s and 72 °C for 1 min, followed by extension of 72 °C for 5 min. For ESBL and MBL genes, initial denaturation at 95 °C for 15 min, 30 cycles of 94 °C for 30 s, 59 °C for 1 min 30 s and 72 °C for 1 min 30 s were followed by extension of 72 °C for 10 min.

For acquired OXA genes, the initial denaturation was at 95 °C for 15 min, 30

cycles of 94 °C for 30 s, 52 °C for 1 min 30 s and 72 °C for 1 min 30 s, followed by extension of 72 °C for 1 min 30 s.

For the IS element PCR, briefly, 5 μl of template DNA was added to 20 μl reaction containing 12.5 μl of Hot Start Master Mix, 1 μl each of forward and reverse primer and 5.5 μl of molecular-grade water. For mapping PCR, 5 μl of template DNA was added to a 20-μl reaction containing 12.5 μl of Hot Start Master Mix, 1 μl each of IS forward and OXA-23 reverse primer, and 5.5 μl of molecular-grade water. The cyclic conditions for IS element PCR and mapping PCR were as follows: initial denaturation at 95 °C for 15 min, 35 cycles of 95 °C for 45 s, 56 °C for 45 s and 72 °C for 2 min, followed by extension of 72 °C for 10 min.

This article does not contain any new studies with human or animal subjects performed by any of the authors.

RESULTS

A total of 103 invasive clinical isolates of *Acinetobacter* species were included in this study. Thirty isolates were from blood specimens, and 73 isolates were from ETA.

All 103 isolates (100%) were confirmed as *Acinetobacter baumannii* by the presence of the *bla*OXA-51 gene.

Antimicrobial Susceptibility

Antimicrobial susceptibility testing revealed that all the isolates were resistant to ceftazidime, cefepime, piperacillin/tazobactam, cefoperazone/sulbactam, aztreonam, imipenem, meropenem, amikacin, netilmycin, tetracycline, tobramycin, levofloxacin and co-trimoxazole. Almost 102 (98%) of the study isolates were susceptible to polymyxin B.

Phenotypic Detection

Among the 103 clinical isolates, the CarbAcineto NP test was positive in 94 (91.2%) isolates and negative in 9 (8.7%) (Table 1).

PCR for ESBL, MBL and OXA Genes

Among the 103 isolates tested for ESBLs, the PER gene was present in 42 isolates (41.5%) followed by the TEM gene in 8 (7.9%), SHV gene in 1 (0.9%) and VEB gene in 1 (0.9%) isolate. PER and TEM genes coexisted in seven (6.9%) isolates followed by PER and SHV coexisting in one isolate (2%). None of the isolates had the GES gene (Table 1).

Twenty isolates were positive for the NDM gene (19.2%), and six isolates were positive for the VIM gene (5.7%). One isolate (0.9%) had both VIM and NDM genes. None of the isolates had KPC, IMP, SPM and SIM genes (Table 1).

The acquired OXA carbapenemase, *bla*OXA-23 gene was present in 101 isolates (98%). Two isolates (2%) had both *bla*OXA-23 and *bla*OXA-24 genes. None of the isolates had the *bla*OXA-58 gene (Table 1).

*ISA*ba1 and Mapping PCR

*ISA*ba1 and mapping PCR was performed for all the clinical isolates ($n = 103$). The *ISA*ba1 element was found upstream of the corresponding *bla*OXA-23 gene in 82 isolates (79.6%). Seven isolates (6.7%) were positive for the *ISA*ba1 element but not found upstream of the *bla*OXA-23 gene. Fourteen isolates (14%) were negative for the *ISA*ba1 element (Table 1).

Table 1 CarbAcineto NP test and distribution of different types of β-lactamase genes among invasive isolates of *Acinetobacter baumannii*

Samples	Phenotypic characterization		Molecular characterization										
	CarbAcinetoNP test		ESBL type				Carbapenemases					*ISA*ba1 (%)	*ISA*ba1-*bla*OXA-23 like (%)
							MBL type		OXA type				
	Positive (%)	Negative (%)	PER (%)	TEM (%)	SHV (%)	VEB (%)	VIM (%)	NDM (%)	51-like (%)	23-like (%)	24- like (%)		
Blood ($n = 30$)	30 (32)	0 (0)	6 (14)	2 (25)	0 (0)	1 (100)	0 (0)	6 (30)	30 (30)	30 (30)	0 (0)	29 (33)	24 (29)
ETA ($n = 73$)	64 (68)	9 (100)	36 (86)	6 (75)	1 (100)	0 (0)	6 (100)	14 (70)	73 (70)	73 (70)	2 (100)	60 (67)	58 (70)
Total ($n = 103$)	94	9	42	8	1	1	6	20	103	103	2	89	82

CarbAcineto: *NP* Nordmann and Poirel, *ESBL* extended spectrum beta-lactamases, *MBL* metallo-beta-lactamases, *OXA* oxacillinases, *ISAba1* insertion sequence, *ETA* endotracheal aspirate, *PER Pseudomonas aeruginosa*. Carbapenemase: *VIM* Verona integron metalloenzyme, *NDM* New Delhi metallo-beta-lactamase

DISCUSSION

Multidrug-resistant *A. baumannii* is increasingly reported in healthcare-associated infections worldwide. This scenario has left carbapenems as the drug of choice to treat severe infections caused by MDR *A. baumannii*. However, increased resistance to carbapenems due to diverse intrinsic and acquired resistance mechanisms is emerging. Production of class D OXA carbapenemases and class B metallo-β-lactamases plays a predominant role in contributing to carbapenem resistance to *A. baumannii* worldwide [17].

The SENTRY study reports that the susceptibility rate of imipenem to *A. baumannii* has declined from 73.7% in 2001–2004 to 37.4 in 2009 in the Asia-Pacific region [18]. The Tigecycline Evaluation and Surveillance Trial (TEST) study between 2005 and 2009 reported the overall susceptibility rate for imipenem and meropenem was 54.1% and 51.8%, respectively [1]. A recent study from South India has reported 34–93.6% carbapenem susceptibility in *A. baumannii* isolates [19].

Due to the intrinsic low permeability of carbapenems, phenotypic detection of carbapenemase is difficult in *Acinetobacter* sp. A number of phenotypic tests such as the modified Hodge test and inhibition-based tests have been proposed for the rapid detection of carbapenemases in *Acinetobacter* sp. These tests were efficient in detecting IMP and VIM producers, leaving the most predominant carbapenemases such as OXA and NDM producers undetected [10]. Although CLSI 2015 recommends the use of the carba NP test, it has not been well validated for the detection of carbapenemase-producing *Acinetobacter* sp. The rate of imipenem hydrolysis achieved with OXA enzymes is too low to be detected with the carba NP test [10]. Therefore, a modified version of the Carba NP known as the CarbAcineto NP was proposed for the phenotypic detection of carbapenemase production in *Acinetobacter* sp. The sensitivity and specificity of the CarbAcineto NP test are reported to be 100% and 94.7%, respectively, in comparison to the carba NP test where the sensitivity is 11.9% only [10]. However, the CLSI is non-committal about this modification.

In this study, 94 out of 103 clinical isolates were positive for the CarbAcineto NP test, and all were positive for the *bla*OXA-23 gene. Nine isolates were negative for the CarbAcineto NP.

Among the study isolates, OXA carbapenemases were detected in 101 (98%) isolates of carbapenem-resistant *A. baumannii*. The *bla*OXA-23-like oxacillinases were the most common type. A study from East India also showed the OXA-23 genes as the prevalent type of oxacillinase contributing to carbapenem resistance [17]. Another study from South India showed the prevalence of the OXA-23 gene to be 56.8% [19]. Among the metallo β-lactamases (MBLs), 20 isolates were positive for the NDM gene while 6 were positive for the VIM gene. This study showed NDM as the predominant MBL gene. However, studies by Saranathan et al. and Amudhan et al. showed IMP-like and VIM-like as the prevalent MBL genes [19, 20]. Of the 103 isolates tested, 52 (50%) were positive for 1 of the ESBL genes such as PER, TEM and SHV. In addition, we found coexistence of PER, TEM and OXA-23 in six isolates, PER, TEM, VIM and OXA-23 in one isolate, and PER, SHV and OXA-23 in one isolate.

Class D OXA enzymes possess weak carbapenemase activity. However, the presence of an insertion sequence upstream of the OXA gene promotes their expression and can modulate the mobility of OXA genes. Evans et al. reported that a promoter for the

*bla*OXA-23-like gene is provided by *ISA*ba1 when it is inserted 25-bp upstream of the gene [21]. Also studies have shown that transposons such as Tn2006 can be formed by two *ISA*ba1 elements bracketing the *bla*OXA-23 gene, which in turn involves in the dissemination of resistance determinants [22]. In this study, we found that 79.6% of the isolates had *ISA*ba1 upstream of the OXA-23-like gene, which could provide a promoter for OXA-23 gene overproduction.

Even though the *bla*OXA-51-like gene is chromosomally encoded and has weak carbapenemase activity, studies have reported that the presence of *ISA*ba1 upstream to this gene can provide a promoter that allows the hyper-production of carbapenemase, resulting in carbapenem resistance [6]. In this study, 6% of the carbapenem-resistant *A. baumannii* isolates had an *ISA*ba1 element, which was not found upstream but seen elsewhere than the *bla*OXA-23 gene. Carbapenem resistance in these isolates might be due to overexpression of the *bla*OXA-51 gene. Future studies are required to confirm the role of insertion sequence-induced overexpression of the *bla*OXA-51 gene. Studies have reported that carbapenem resistance in *A. baumannii* is mainly due to carbapenemase mediated [4]. However, non-carbapenemase-mediated resistance mechanisms such as reduced membrane permeability due to porin changes and overexpression of efflux pumps make a trivial contribution toward carbapenem resistance in *A. baumannii* [23]. Heritier et al. reported that the synergism between acquired oxacillinases and the RND efflux pump, AdeABC, was implicated in the increased levels of resistance toward carbapenems [24]. Therefore, future studies on the role of non-carbapenemase-mediated mechanisms in *A. baumannii* are required.

CONCLUSION

In conclusion, this study reports the emergence of *bla*OXA-23-like and *bla*NDM-like genes as the predominant cause of carbapenem resistance in *Acinetobacter baumannii*. Additionally, in this study we found the *ISA*ba1 insertion sequence, which plays an important role in the overexpression of *bla*OXA-23.

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Compliance with Ethical Standards. This article does not contain any new studies with human or animal subjects performed by any of the authors.

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Anandan and Balaji Veeraraghavan declare no conflict of interest.

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