Original Article

Role of Efflux Pumps in Reduced Susceptibility to Tigecycline among Clinical Isolates of *Acinetobacter baumannii*

Ashoka Mahapatra, Amresh Pati, Kavita Gupta

Department of Microbiology, All India Institute of Medical Sciences, Bhubaneswar, Odisha, India

Abstract

Background: *Acinetobacter baumannii* (*A. baumannii*) is a very well-known emerging pathogen and has become a major burden on healthcare system especially in intensive care units (ICUs). Tigecycline is the last resort drug for treatment of multidrug-resistant *A. baumannii* infections. However, non-susceptibility to this drug is a rising problem. Resistance to tigecycline is mediated by Resistance-nodulation-cell division (RND) efflux pumps. **Objective:** This study was done to detect efflux pump genes (adeABC) and regulator genes (adeS,adeR) responsible for tigecycline resistance among the clinical isolates of *A. baumannii*. **Materials and Methods:** A total of 150 OXA-51 confirmed clinical isolates were subjected for tigecycline susceptibility test by broth microdilution (BMD) method. All isolates irrespective of their MIC were subjected to conventional PCR for detection of efflux genes (adeABC) and regulator genes (adeRS). **Results:** Prevalence of tigecycline resistance was found to be 14 (9.33%) by the reference broth microdilution method (BMD). Overall prevalence of efflux genes was highest for adeB (69%) and lowest for adeR (29%). Combination of genes especially three, four or five were found more prevalent among resistant isolates with higher minimum inhibitory concentration (MIC). **Conclusion:** Combination of efflux genes confer higher MIC and can be a major contributor for resistance to tigecycline.

Keywords: *Acinetobacter baumannii, efflux pump, tigecycline resistance*

Introduction

Acinetobacter baumannii (*A. baumannii*) is an emerging multidrug-resistant (MDR) pathogen responsible for causing the majority of healthcare-associated infections, especially in intensive care units (ICUs).[1] Earlier, carbapenems were considered the drug of choice for these MDR *A. baumannii* (MDRAB) are ineffective nowadays.[2,3] Tigecycline and polymyxins are the only few drugs left for the treatment of the infections caused by MDRAB. However, resistance to tigecycline has emerged after its wide use in clinical settings, either as a monotherapy or in combination with other antibiotics.[4] Drug removal by efflux pump mechanisms significantly contributes to resistance in *A. baumannii*. One of the major mechanisms of tigecycline resistance is by three resistance-nodulation-cell division (RND) efflux pumps. Three types of RND pumps have been reported in *A. baumannii* such as adeABC, adeFGH, and adeIJK. Over expression of efflux pump adeABC cause resistance to various drugs such as β-lactams, erythromycin, aminoglycoside, chloramphenicol, tetracycline, and tigecycline. The expression of adeABC is regulated by a system with two components which consists of a sensor kinase (adeS) along with a response regulator (adeR).[5,6] Tigecycline resistance is mainly associated with over expression of efflux pump genes adeABC. Mutations or insertions in the regulatory genes result in increased expression of the efflux pump and subsequently leads to resistance to tigecycline.[6] Although tigecycline is an excellent choice for multidrug-resistant *A. baumannii.* infections, nonsusceptibility to this drug is rising due to its overuse without proper antimicrobial susceptibility testing. There is scarcity of data available on the resistance pattern and the efflux genes responsible for tigecycline resistance in *A. baumannii.* The present study was aimed to determine tigecycline susceptibility (by reference BMD method) and presence of efflux genes in the clinical isolates of *A. baumannii* and further to correlate the presence of efflux genes with the mean minimum inhibitory concentration (MIC) of the isolates showing tigecycline resistance.

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Address for correspondence:
Dr. Ashoka Mahapatra, Department of Microbiology, All India Institute of Medical Sciences, Bhubaneswar-751019, Odisha, India.
E-mail: meetasoka@yahoo.co.in

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Materials and Methods

A cross-sectional, observational study was conducted in the Department of Microbiology after approval by Institutional Ethics Committee (Ref Number: IEC/AIIMS BBSR/PG Thesis/2019-20/08) from May 2019 to June 2021. Routine specimens such as blood, bronchoalveolar lavage, sputum, tracheal aspirate, pus, sterile body fluid, and urine from different locations of hospital were transported to the microbiology laboratory within 4 h under aseptic condition. *A. baumannii* isolated from routine specimens were included in this study.

Identification of *A. baumannii*

MacConkey agar and blood agar were used for primary isolation and further identification was carried out using standard procedures such as colony characteristics, Gram-stain, motility, of glucose, growth in citrate, hemolysis, and growth at 37°C and 44°C.[7]. All presumptively identified *Acinetobacter spp.* were further subjected to automated VITEK-2 for species identification and bla-OXA-51 polymerase chain reaction (PCR) for final identification of *A. baumannii*.

Antimicrobial susceptibility was performed by microbroth dilution as per standard protocol. The *A. baumannii* ATCC19606 was used as quality control for this test. Results were interpreted following EUCAST 2020 guidelines for tigecycline break point for *Enterobacteriales*, i.e., isolate showing tigecycline MIC ≤0.5 µg/ml was considered as sensitive and MIC >0.5 µg/ml as resistant.

Detection of efflux pump by polymerase chain reaction

DNA extraction was performed by DNA Spin Column Qiagen miniprep DNA extraction kit. Identification of adeA, B, C, and adeRS genes through PCR was performed to screen for adeABC and adeRS genes. The primer sequences used were OXA51-F-TAATGCTTTGATCGGCCTTG, R-TGG ATTGCACTTCATCTTGG, adeA-F-5'-ATCTTCCTGCACGTGTACAT-3', R-5'-GGCGTTCATACTCACTAACC-3', adeB-F-5'-TTAACGATAGCGTTGTAACC-3', R-5'-TGAGCAGACAATGGAATAGT-3', adeC-F-5'-AGCCTGCAATTAACATCTCAT-3', R-5'-TGGCACTTCACTATCAATAC-3', adeR-F-5'-ACTACGATATTGGCGACATT-3', R-5'-GCGTCAGATTAAGCAAGATT-3', and adeS-F-5'-TTGGTTAGCCACTGTTATCT-3', R-5'-AGTGGACGTTAGGTCAAGTT-3'.

The temperature in the thermal cycler for different PCR was set as per the protocol described by Turton *et al*., in 2006 and Lin *et al*., in 2009.[8,9]. After completion of PCR run, the amplicons were subjected for gel electrophoresis immediately or else stored at −20°C for gel electrophoresis later. Agarose gel 1.5% containing 0.5 µg/ml concentration of ethidium bromide was used for gel electrophoresis and the bands were visualized by Gel doc system (Syngene) Model-Chemi XR 5.

Statistical analysis

Statistical analysis was done using Chi-square test to correlate efflux genes and MIC and to compare the discrete variables and was considered statistically significant at $P < 0.05$.

Results

A total number of 150 nonduplicate *A. baumannii* isolates obtained from various clinical samples were included in the study [Table 1]. Maximum number of *A. baumannii* isolates (48/150, 32%) were recovered from patients in the middle adolescent age group (41–60 years) and were of male gender. Majority of *A. baumannii* isolates were obtained from respiratory samples (81/150, 54%), followed by blood samples (25/150, 16.67%), urine (12/150, 8%), pus (7/150, 4.67%), and others 25/150, 16.67%). Of the 150 isolates of *A. baumannii*, 49.3% (74/150) were obtained from patients admitted to various ICUs, 42% from in patients admitted to various wards (63/150) and 8.7% were from OPDs (13/150). All the isolates were subjected to tigecycline susceptibility test by reference BMD method using tigecycline concentrations ranging from 0.06 µg/ml to 8 µg/ml. Of 150 isolates, 4 isolates had MIC 0.06 µg/ml and majority had MIC in the range 0.125-0.5 µg/ml. Fourteen were tigecycline resistant (14/150, 9.33%) by reference BMD method.

Efflux pump gene adeB was found in 103 isolates (103/150; 69%), followed by adeS in 78 (78/150; 52%), adeA in 74 (74/150; 49%), adeC in 66 (66/150; 44%), and adeR in 43 isolates (43/150; 29%) as shown in Table 2 and Figure 1. Among the 14 tigecycline-resistant isolates, adeB and adeS

Table 1: Distribution of *Acinetobacter baumannii* from different clinical specimens (*n*=150)

Sample type	*n* (%)
Respiratory samples	81 (54)
blood	25 (16.67)
Urine	12 (8)
Pus	7 (4.67)
Others	25 (16.67)
Total	150 (100)

Table 2: Prevalence of various efflux genes (adeABC and adeRS) in *Acinetobacter baumannii*

Efflux gene	Susceptible (*n*=136)	Resistant (*n*=14)	Total (*n*=150)	*P*
adeA	62 (46)	12 (86)	74 (49)	0.00424
adeB	90 (66)	13 (93)	103 (69)	0.04043
adeC	54 (40)	12 (86)	66 (44)	0.00096
adeR	33 (24)	10 (71)	43 (29)	0.0002
adeS	65 (48)	13 (93)	78 (52)	0.00687

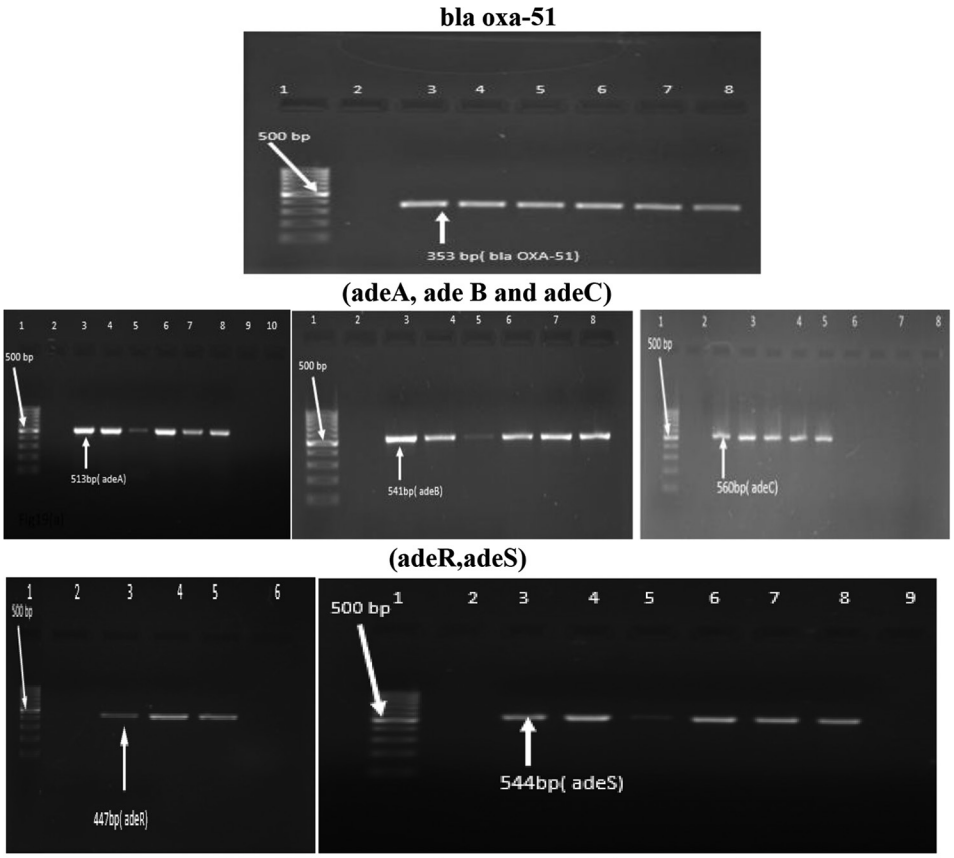

Figure 1: Gel electrophoresis results of bla oxa-51, efflux Pump genes (adeABC) and efflux Pump regulator genes (ade RS)

Table 3: Mean minimum inhibitory concentration of tigecycline in presence of various efflux genes

Genes														
adeA			adeB			adeC			adeR			adeS		
+	−	*P*	+	−	*P*	+	−	*P*	+	−	*P*	+	−	*P*
0.46	0.3	0.0027	0.46	0.19	0.0001	0.51	0.27	0.0001	0.51	0.32	0.0009	0.51	0.23	0.0001

gene were found in 13 (13/14 93%), followed by adeA and adeC gene, each found in 12 isolates (12/14 86%). Efflux pump regulator adeR was found in 10 isolates (10/14 71%). Similarly, among the 136 tigecycline susceptible isolates, adeB and adeS were found in 90 isolates (90/136, 66%) and 65 isolates (65/136, 48%), respectively. adeA and adeC were found in 62 isolates (62/136, 46%) and 54 isolates (54/136, 40%), respectively. adeR was found in 33 susceptible isolates (33/136, 24%).

Mean MICs of tigecycline were compared with the presence and absence of adeABC and adeRS genes as shown in Table 3. The mean MICs of tigecycline were higher in isolates having adeA, adeB, adeC, adeR, and adeS than in the isolates not having those genes. Among the 14 resistant isolates, all efflux genes AdeABC and AdeRS were detected in 6 isolates. A combination of four (adeAB + adeRS, adeABC + adeS, adeABC + adeR) was seen in 5 isolates having MIC 1 µg/ml whereas 1 isolate having MIC 2 µg/ml showed the efflux pump combination of adeBC + adeRS as described in Table 4**.**

No efflux genes (white box) were found in 10 (4 + 6) out of the total 150 isolates. Among the 136 susceptible isolates the presence of single (light blue box) or combination of two (light pink box) or three (brown box) genes was found in 120 within the MIC range of 0.125–0.5 µg/ml and combination of four (yellow box) genes were found in 6 isolates within the MIC range of 0.25–0.5 µg/ml. Among the 14 resistant isolates, combination of three genes (brown box) was seen in 2 (MIC 1 µg/ml). A combination of four (yellow box) and five efflux genes (red box) was found in 12 resistant isolates within the MIC range 1–2 µg/ml [Table 4].

Discussion

Tigecycline has shown satisfactory antimicrobial efficacy against MDRAB. However, reduced susceptibility to

Table 4: Correlation of mean MIC and presence of Efflux genes among *A. baumannii* isolates

Category	MIC in µg/ml (Total no. of isolates with MIC)	No efflux genes	Efflux genes found				
			Single *	Double**	Triple***	Four****	Five*****
Susceptible (136)	0.06 (4)	0	0	0	0	0	0
	0.125 (46)	6	15	25	0	0	0
	0.25 (37)	0	0	17	18	2	0
	0.5 (49)	0	0	7	38	4	0
Resistant (14)	1 (11)	0	0	0	2	5	4
	2 (3)	0	0	0	0	1	2

Single*adeA/adeB/adeC/adeR/adeS. Double**adeAB, adeAC, adeBC, adeA+adeS, adeA+adeR, adeB+adeS, adeB+adeR, adeC+adeR, adeC+adeS, adeRS. Triple***adeABC, adeB+adeR, adeAB+adeS, adeA+adeRS, adeBC+adeR, adeBC+adeS, adeB+adeRS, adeC+adeRS, adeAC+adeS, adeAC+adeR. Four****adeAB+adeRS, adeABC+adeS, adeABC+adeR, adeBC+adeRS. Five*****adeABC+adeRS

tigecycline has been reported in the recent past by the studies conducted in India as well as abroad.[10,11] Efflux pumps such as adeABC play an important role in the resistance mechanisms of tigecycline by throwing drugs away from the target binding site.[12] Most of the clinically relevant efflux systems belong to the RND adeABC superfamily transporters. An increase in the expression of this efflux pump may result in resistance to several antibiotics including tigecycline. The expression of adeABC efflux system is tightly regulated by a two-component system comprising a sensor kinase (adeS) and a response regulator (adeR). Hornsey *et al*. found a correlation between higher MIC values and elevated adeABC expression.[6] Susceptibility of *A. baumannii* to tigecycline vary in different geographical area, moreover, it also depends on the use of guidelines for the interpretation of results and the type of methodologies used. Tigecycline resistance in the present study was observed as 9.33% (14/150) by BMD method. Neither CLSI nor EUCAST or FDA has defined break point for *A. baumannii* against tigecycline. Previous studies have interpreted the breakpoints mentioned for *Enterobacterales* in EUCAST or FDA. However, since 2019, EUCAST have lowered the breakpoint of tigecycline (≤0.5 µg/ml - susceptible and >0.5 µg/ml as resistant) which makes four two-fold difference from the FDA cutoff level (≤2 - Susceptible, 2–8 - Intermediate, and ≥8 - Resistant). Thus, the isolates with MIC 1-8 µg/ml create a gray zone and they might be termed resistant according to EUCAST but susceptible/intermediate as per the FDA criteria. Therefore, in the present study, the current EUCAST 2020 guidelines have been followed (i.e., MIC ≤0.5 µg/ml - susceptible and >0.5 µg/ml - resistant). Majority of the tigecycline resistant isolates were recovered from respiratory samples (6/14, 52%) followed by blood (3/14, 33%). Maximum tigecycline resistant isolates were obtained from the samples of patients admitted to different wards (7/14, 50%) followed by ICUs (6/14, 42.85%). The prevalence of tigecycline resistance in our study (9.33%) was similar to a recent study in China by Yin *et al*. where 9% of isolates were resistant. Kumari *et al*., from India also reported tigecycline resistance to be 8% but the methodology and breakpoint used were different.[13,14] The presence of efflux pumps plays an important role for multidrug resistance in *Acinetobacter spp*.

In the present study, the presence of efflux pump genes adeABC and regulator genes (adeR and adeS) was analyzed in all the 150 clinical isolates of *A. baumannii*. The overall prevalence of adeB was highest (66%) followed by adeA (46%) and adeC (40%). Among the resistant isolates also adeB was the most common (13/14) followed by adeA (12/14) and adeC (12/14). Very few studies have been conducted so far to detect the prevalence of efflux pump genes and regulator genes. Studies conducted earlier have also reported, adeB to be most prevalent efflux genes among adeABC.[15-17] Function of adeA is to act as a membrane fusion protein, adeB captures substrates in the inner membrane of phospholipids bilayer or the cytoplasm, then transports the substrates by adeC (membrane channel protein). The adeRS bicomponent regulation system consists of sensor kinase adeS and responsive regulator adeR.[18] In this study, among regulator genes, adeS (53%) was found more prevalent than adeR (24%). A similar predominance of adeS has been reported from a recent study in South Africa.[19] The mean MICs of tigecycline in the present study was significantly higher for isolates harboring the efflux pump genes than those isolates not having these genes. However, the mean MIC for isolates having adeB (0.46 µg/ml) and adeA (0.46 µg/ml) was found to be same and slightly lower than MIC value observed for isolates having adeC (0.51 µg/ml). Similarly, for isolates having regulator genes adeR and adeS, the mean MIC was 0.51 µg/ml. The mean MICs of tigecycline were higher in isolates having adeA, adeB, adeC, adeR, and adeS than the isolates not having these genes. This shows that each of these efflux genes have individual role for increased MICs in these isolates.

Among the susceptible isolates (n = 136), efflux pump genes were totally absent in 10 of them and those isolates had MIC ≤0.125 µg/ml. Only one efflux pump gene was detected in 15 isolates with MIC 0.125 µg/ml, two efflux pump genes were detected in 49 isolates having MIC ≤0.5 µg/ml. A combination of three/four/five genes was detected in the 14 resistant isolates. Out of 14, 6 had four genes in combination and another six isolates

had five genes in combination. Isolates having four gene combinations had MIC of 1 µg/ml in five and 2 µg/ml in one. Among isolates with five genes in combination, 4 had MIC1 µg/ml and one had MIC 2 µg/ml. This observation depicts that a combination of efflux pump genes is responsible for higher MIC values in isolates and it may confer resistance which agrees to the study by Yang *et al*. where all clinical TGC-resistant XDRAB isolates harbored three genes of the adeABC efflux pumps.[20] A combination of efflux genes other than adeABC has also been detected in *A. baumannii* isolates having higher MICs.[21,22] The presence of efflux genes has also been detected in bacteria other than *A. baumannii*.[23] Previous studies have revealed, AdeABC efflux pump confers resistance to a wide range of antimicrobials, such as aminoglycosides, tetracyclines, fluoroquinolones, chloramphenicol, and tigecycline.[24,25] In the current study, a significant statistical correlation ($P < 0.05$) was observed between resistance to antimicrobials other than tigecycline and presence of efflux pump genes (adeABC and adeRS). Hou *et al*., from China, and Ranjbar *et al*., from Iran, also reported a significant correlation between the presence of efflux pump genes and imipenem resistance in *A. baumannii*.[26,27]

Very few studies pertaining to efflux-mediated drug resistance in *A. baumannii* are available in literature. The presence of efflux pump genes can also confer cross-resistance in MDRAB. Detection of efflux-mediated tigecycline resistance mechanism is equally important for combating antimicrobial resistance. The present study results suggest that, the efflux-based system adeABC is a major contributor for decreased susceptibility to tigecycline.

Conclusion

The prevalence of tigecycline resistance was found to be 9.33% in the current study by BMD method. Both susceptible and resistant isolates harbored the efflux pump genes. However, a combination of genes especially three, four, or five was found more prevalent among resistant isolates. Moreover, combination of efflux genes conferred raised MIC in clinical isolates of *A. baumannii*. The presence of efflux genes in combination can contribute to raised MIC as well as resistance to tigecycline. The current study will help to understand the role of the efflux pump for tigecycline resistance among *A. baumannii* isolates. However, further studies are required for better understanding of efflux mechanism in *A. baumannii* for tigecycline resistance which will help the clinicians for therapeutic purpose.

Ethical clearance

Institutional ethics committee of All India Institute of Medical Sciences, Bhubaneswar (Approval No: IEC/AIIMS BBSR/PG Thesis/2019-20/08).

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Conflicts of interest

There are no conflicts of interest.

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