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Original Article

Microbiology Section

Impact of Efflux Pump Inhibitor Carbonyl-Cyanide M-Chlorophenylhydrazone in Multidrug Resistant *Acinetobacter* Species Isolates from Sterile Body Fluids

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ABSTRACT

Introduction: Antimicrobial resistance of *Acinetobacter baumannii* (*A. baumannii*) are rapidly emerging, becoming non-responsive to most of the commonly prescribed antibiotics and leaving us with few treatment options and galloping treatment costs.

Aim: To study the effect of Efflux Pump Inhibitor (EPI) Carbonyl Cyanide 3-Chlorophenylhydrazone (CCCP) on Multidrug Resistance (MDR) *A. baumannii* isolates from different sterile body fluids.

Materials and Methods: A total of 40 *Acinetobacter* species isolates from different sterile body fluids i.e., Cerebrospinal Fluid (CSF), ascitic fluid, pleural fluid, and peritoneal fluid were collected and identified by Matrix Assisted Laser Desorption/ Ionisation-Time Of Flight (MALDI-TOF), Biomerieux, France. Minimum Inhibitory Concentration (MIC) of *A. baumannii* was determined by automated VITEK-2 Antimicrobial Susceptibility Testing (AST) system (Biomerieux, France). In addition, MIC of the isolates, grown on Mueller-Hinton Agar (MHA) plate with 15 µg/mL with EPI CCCP (Sigma Aldrich, US) was determined. For Tigecycline, MIC was determined by Broth Microdilution (BMD) method.

Results: Out of 40 isolates, 34 (85%) were *A. baumannii* and 6 (15%) were *Acinetobacter junii*. Most of the *Acinetobacter* spps were MDR and only susceptible to few antibiotics. Most effective antibiotic was Tigecycline 25 (73.52%) followed by Co-trimoxazole 10 (29.41%). Similarly, Out of 40 isolates, 2 to 64 folds reductions in MIC was observed due to CCCP in 10 (25%) isolates for various antibiotics. Likewise, for Tigecycline, 2 to 4 folds reductions in MIC value (One strain changed from intermediate to sensitive) was observed by VITEK-2 AST which corroborated with reduction in MIC by BMD after addition of CCCP.

Conclusion: MDR *A. baumannii* are spreading rapidly. There is the need to overcome the antimicrobial resistance by investigating resistance inhibiting substance that will help to restore antimicrobial susceptibility and bringing back the existing antibiotics in prescription.

Keywords: *Acinetobacter baumannii*, Broth micro-dilution, Efflux pump inhibitors, Minimum inhibitory concentration, Multidrug resistance

INTRODUCTION

A. baumannii is a major cause of various human infections such as bacteremia, meningitis, pneumonia, urinary tract infection, skin and soft tissue infection [1] throughout the world, and it is gaining due public health concern because of emerging MDR strains during the last few decades [2]. We are heading towards post-antibiotics era where alarming number of previously curable infections are changing into non-curable and are threatening to life [3]. However, antimicrobial resistance is the natural phenomenon, irrational use of antibiotics boosts-up the emergence of drug-resistance strains [4]. *A. baumannii* was susceptible to most of the antibiotics till 1970s. Drug resistance in *A. baumannii* can be intrinsic or acquired mostly through the acquisition of plasmids, transposomes or integrons which harbors clusters of genes encoding resistance to myriad families of antibiotics [5-8]. Transition of extra chromosomal material renders over expression of efflux pump that causes MDR [9] due to reduction in drug accumulation inside bacteria, and resulting in the increased MIC. Currently, efflux pumps are the newest and one of the most complicated bacterial resistance mechanisms that played a crucial role on drug resistance in *A. baumannii* [10]. Till date, it has been proven that five different families of efflux pump are present in *A. baumannii* i.e., ATP Binding Cassette (ABC) transporters, Resistance Nodulation-Cell Division (RND), Multidrug Toxic Composite Extrusion (MATE) transporters, Small Multidrug Resistance (SMR) and Major Facilitation Super family (MFS). Most common efflux pump families in *A. baumannii* responsible for MDR are ABC and RND [11,12].

Some molecules i.e., synthetic or natural have the potential to act specifically on efflux pump to restore the action of antimicrobial agents, known as EPIs [13] i.e., CCCP. Bacterial cell envelope has a crucial role in arbitrating resistance to antibiotics through its physiological properties, efflux pump and porine channels. More attention has been drawn to protonophores i.e., CCCP that reduces ATP production and increase membrane permeability in bacteria [14-16] by interfering with the transmembrane electrochemical gradient and proton motive force. In addition CCCP offers good effect on reversal effect of drug resistance on *A. baumannii* [17]. This study was designed and conducted to observe the effect of CCCP on MIC of various antibiotics used for the treatment of *A. baumannii* from different sterile body fluids.

MATERIALS AND METHODS

A cross-sectional study was designed and conducted at the Department of Microbiology, All India Institute of Medical Sciences, New Delhi, India from June 2018 to September 2019. Total 40 representative MDR isolates of *Acinetobacter* species from various sterile body fluids i.e., CSF, pleural fluid, ascitic fluid and peritoneal fluid were collected and identified by MALDI-TOF. Inclusion criteria were collection of isolates only from sterile body fluids. Isolates were further processed for the determination of MIC for various antibiotics

and was determined by automated VITEK-2 AST system as per manufacturer instruction (Biomerieux, France).

Bacterial Identification by MALDI-TOF

Sterile body fluids (ascitic, CSF, peritoneal fluid and pleural fluid) were inoculated on Blood, chocolate and Mac conkey agar plate and were incubated at 37°C for 24-48 hours. Smear of isolates grown on culture plate were made on MALDI-TOF slide. A 0.5 µL matrix (α-cyano-4 hydroxycinnamic acid) was added on the smear and kept it for one minute at room temperature. Slide was kept in MALDI-TOF (VITEK-MS, Biomerieux, France) machine for acquisition and identification [18].

Determination of Minimum Inhibitory Concentration (MIC)

MIC of *A. baumannii* was determined by automated VITEK-2 AST system (Biomerieux, France). An isolated colony of *A. baumannii* was picked up from the culture plate and mixed in normal saline. Turbidity was measured in Turbidometer (Biomerieux, France) and was adjusted to 0.5-0.6 CFU/mL as per manufacturer instruction. 145 µL bacterial suspension was transferred to the tube containing 3 mL normal saline and was mixed properly. VITEK Card N281 was used for AST and MIC determination. Result of the MIC was taken after 18-24 hours of incubation in VITEK-2 AST system. For the determination of MIC of Tigecycline (Sigma Aldrich, USA), along with VITEK-2 AST, BMD was also performed and taken as standard.

Addition of CCCP in MHA plate

To check and confirm the mechanism of efflux pump, CCCP was added to MHA plate. Final concentration of CCCP (Sigma Aldrich, USA) in MHA was maintained 15 µg/mL [19]. All the isolates were subcultured on MHA plate with CCCP. Then MIC was measured again by VITEK-2 AST system for all the *A. baumannii* isolates grown on MHA plate. In addition, for Tigecycline (Sigma Aldrich, USA), MIC was also determined by BMD (which is considered as reference standard for determination of MIC) in the presence of efflux pumps inhibitor, CCCP (CCCP, Sigma Aldrich, USA). Result of the MIC performed in VITEK-2 AST system in the presence of CCCP and without CCCP was compared. Similarly, MIC result of Tigecycline with CCCP by VITEK-2 AST system and BMD were also compared.

STATISTICAL ANALYSIS

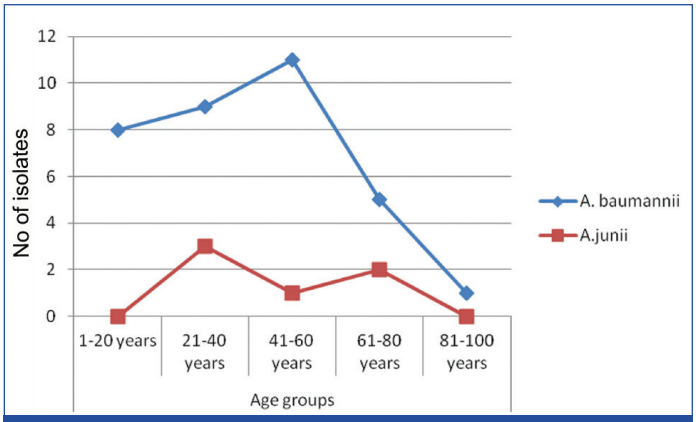
Data were entered with coding and analysed in Microsoft Excel Version 10. Percentage of *Acinetobacter* species and their antibiotic susceptibility result was calculated.

RESULTS

The experiment result was interpreted taking *A. baumannii* isolates from all age groups. A total of 40 isolates of *Acinetobacter* species were collected from different sterile body fluids. Of the total, 31 (77.5%) were male and 9 (22.5%) were female. The mean age of the patient was 40.27±20.50 years (Min 3-Max 93 years). Most of the *A.baumannii* (12, 35.29%) were present in 41-60 years of age group as shown in [Table/Fig-1]. Out of 40 isolates, 34 (85%) were *A. baumannii* and 6 (15%) were *A. junii*. Distribution of *Acinetobacter* species in different samples is shown in [Table/Fig-2].

A. baumannii and *A. junii* showed different resistance pattern. Most of the *A. baumannii* were MDR and only susceptible to few antibiotics. Most effective antibiotic against *A. baumannii* was Tigecycline 25 (73.52%) followed by Co-trimoxazole 10 (29.41%) shown in [Table/Fig-3].

Effect of EPI (CCCP) on various antibiotics were observed, *Acinetobacter* species were cultured on MHA with 15 µg/mL CCCP and MIC was determined by VITEK-2 AST system. Out of 40 isolates, 2 to 64 folds reductions in MIC value were observed in 10 (25%) isolates for various antibiotics. Max^{n} reduction was observed in



[Table/Fig-1]: Showing the distribution of *Acinetobacter* species in different age group.

Isolates	Samples				Total
	CSF	Pleural fluid	Ascitic fluid	Peritoneal fluid	
Acinetobacter baumannii	12 (35.29%)	11 (32.35%)	07 (20.59%)	04 (11.76%)	34
Acinetobacter junii	02 (33.33%)	03 (50.0%)	01 (16.67%)	-	06
Total	14 (35.0%)	14 (35.0%)	08 (20.0%)	04 (10.0%)	40

[Table/Fig-2]: Showing distribution of *Acinetobacter* species in different samples.

Antibiotics	Isolates					
	Acinetobacter baumannii (34)			*Acinetobacter junii* (6)		
	S	I	R	S	I	R
Piperacillin/ Tazobactam	1 (2.94%)	1 (2.94%)	32 (94.11%)	6 (100%)	-	-
Ceftriaxone	1 (2.94%)	2 (5.88%)	31 (91.17%)	6 (100%)	-	-
Cefaperazone/ Sulbactam	3 (8.82%)	1 (2.94%)	30 (88.23%)	4 (66.66%)	-	2 (33.33%)
Cefepime	4 (11.76%)	-	30 (88.23%)	3 (50.0%)	-	3 (50.0%)
Imipenem	3 (8.82%)	2 (5.88%)	29 (85.29%)	3 (50.0%)	-	3 (50.0%)
Meropenem	3 (8.82%)	2 (5.88%)	29 (85.29%)	6 (100%)	-	-
Amikacin	5 (14.70%)	-	29 (85.29%)	6 (100%)	-	-
Gentamicin	5 (14.70%)	-	29 (85.29%)	6 (100%)	-	-
Ciprofloxacin	4 (11.76%)	-	30 (88.23%)	6 (100%)	-	-
Tigecycline*	25 (73.52%)	9 (26.47%)		6 (100%)	-	-
Trimethoprim+ Sulphamethoxazole	10 (29.41%)	-	24 (70.58%)	4 (66.66%)	-	2 (33.33%)

[Table/Fig-3]: Showing the antibiotic resistance pattern of *Acinetobacter baumannii* and *A. junii*.
S=Sensitive, I=Intermediate and R=Resistance; *FDA interpretive criteria for enterobacteriaceae was used

Meropenem (64 fold) followed by Trimethoprim-sulphomethoxazole (32 fold) [Table/Fig-4].

In addition, significant reduction (8-fold) in MIC value was also observed in 2 (5%) isolates against Ciprofloxacin. Of the 2 isolates, one was *A. baumannii* and one was *A. junii*. Likewise, for Tigecycline, 2 to 4 fold reductions in MIC value (One strain changed from intermediate to sensitive) was observed by VITEK-2 AST which corroborated with reduction in MIC by BMD after addition of CCCP.

DISCUSSION

Acinetobacter species are most important bacterial agent causing different infections in human. Globally, more attention is given on *A. baumannii* due to the emergence of MDR leaving only limited option for the treatment. In the present study, most of the antibiotics tested were resistance, ranging from 70-100%. However, most effective antibiotic was Tigecycline which was 100% susceptible in *A. junii*. However, in *A. baumannii*, 25 (73.52%) isolates were sensitive and 9 isolates (26.47%) were intermediate in sensitivity.

Isolates	Antibiotics											
	Tigecycline		Trimethoprim+Sulphamethoxazole		Gentamicin		Imipenem		Meropenem		Cefepime	
	MIC 1	MIC2	MIC1	MIC2	MIC1	MIC2	MIC1	MIC2	MIC1	MIC2	MIC1	MIC2
A.baumannii Strain no. 50	4 (I)	2	160®	80®	-	-	-	-	-	-	-	-
A.baumannii Strain no. 14	-	-	-	-	16®	4	-	-	-	-	-	-
A.baumannii Strain no. 15	-	-	160®	20	4	1	8®	4	16®	0.25	-	-
A.baumannii Strain no. 53	2	0.5	320®	20	16®	2	16®	0.25	-	-	64®	4
A.baumannii Strain no. 42	-	-	160®	20	-	-	-	-	-	-	64®	32®
A.baumannii Strain no. 43	-	-	160®	20	-	-	8®	0.25	16®	0.25	-	-
A.baumannii Strain no. 13	4 (I)	2	-	-	-	-	-	-	-	-	-	-
A.junii Strain no. 51	-	-	-	-	2	1	8®	4	-	-	4	1
A.junii Strain no. 37	-	-	160®	80®	-	-	8®	0.5	16®	0.25	32®	1
A.baumannii Strain no. 39	-	-	-	-	8®	4	-	-	-	-	-	-

[Table/Fig-4]: Showing the change in MIC (µg/ml) value of the isolates grown on MHA with CCCP.
MIC1: Isolates cultured on MHA, MIC2: Isolates cultured on MHA with CCCP, ®: Resistant

It was followed by co-trimoxazole in which 10 (29.41%) and 4 (66.66%) isolates were susceptible in *A. baumannii* and *A. junii*, respectively. Finding of this study is in concordance with the various studies that has shown increasing resistant in *A. baumannii* against different antibiotics worldwide [20]. Likewise, 88.23% isolates were resistance to ciprofloxacin which was less than the finding of Asadollahi P et al., who showed 100% resistance of *A. baumannii* to ciprofloxacin [21] though Shi WF et al., and Leseva M et al., has shown 52% and 62% are resistant to ciprofloxacin [22,23]. *A. baumannii* exhibits intrinsic MDR to a gamut of antibiotics due to an innate expression of efflux pumps, chromosomally encoded enzymes, and low membrane permeability. Plethora of chromosomally encoded efflux systems and Outer Membrane Porins (OMPs) has been identified that are responsible for MDR in *A. baumannii* [24]. Increased efflux as a result of over expression of efflux pumps is a common mechanism of MDR in *A. baumannii*, and resistance to a wide range of antibiotics.

In addition, MIC of different antibiotics was also measured after culture of *A. baumannii* on MHA plate with CCCP and without CCCP. Significant finding in the reduction of MIC was observed in 25% of the isolates against various antibiotics. Maximum reduction in MIC was observed in meropenem (64 folds) followed by trimethoprim (32 folds). As CCCP acts a protonophore that binds reversibly to protons (H^+) and transport them across the cell membrane that causes membrane depolarisation, eradication of electrochemical concentration gradient and less production of ATP [14,25]. It may be the reason of MIC reversal as well as conversion of resistance strain to sensitive strain after inhibiting efflux pump activity which indicates the role of efflux pump as the main mechanism behind emergence of resistance strain of *A. baumannii*. In this study, BMD for Tigecycline in addition to VITEK-2 AST was also performed for the determination of MIC. There were 4-fold reductions in MIC (determined by BMD) of Tigecycline after addition of CCCP. Similar reduction was observed in MIC of Tigecyclin by Osei Sekyere J et al., [26]. Concurrent result was observed for the MIC of Tigecyline determined by VITEK-2 AST after addition of CCCP. We infer that CCCP reverses drug resistance in *A. baumannii*. Though, CCCP is an experimental agent with no therapeutical value clinically, further studies are essential to decode the mechanism underlying the reversal effect of CCCP to bring it to the therapeutic use.

Limitation(s)

A limitation of this study was small sample size.

CONCLUSION(S)

MDR *A. baumannii* possess great challenge to the health system. As efflux pump is the main mechanism for MDR that could spread rapidly among the *A. baumannii* isolates, there is an urgency to search for an EPI to counteract the alarming situation of MDR.

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