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Research Article

DETECTION AND PREVALENCE OF EFFLUX PUMP-MEDIATED DRUG RESISTANCE IN CLINICAL ISOLATES OF MULTIDRUG-RESISTANT GRAM-NEGATIVE BACTERIA FROM NORTH KERALA, INDIA

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ABSTRACT

Objectives: The present study was carried out to detect the prevalence of efflux pump-mediated drug resistance in clinical isolates of multidrug-resistant (MDR) Gram-negative bacteria isolated from North Kerala.

Methods: Clinical isolates (*n* = 123) of MDR Gram-negative bacteria were collected from various clinical laboratories in North Kerala, and their efflux-mediated drug resistance was detected by two simple phenotypic assays - ethidium bromide (EB)-agar cartwheel method and efflux pump inhibitor (EPI)-based microplate assay, employing phenylalanine-arginine β-naphthylamide as inhibitor.

Results: The 123 Gram-negative MDR strains tested comprised *Escherichia coli*, *Pseudomonas aeruginosa, Acinetobacter* spp., and *Klebsiella* spp. The EB-agar cartwheel method of screening revealed efflux activity in 20% (n=25) of the strains with representatives from all 4 genera. The efflux activity was revealed at a minimum concentration of EB at 1 mg/l. *P. aeruginosa* strains showed the highest activity, many folds higher up to a concentration of 2.5 mg/l. The confirmatory EPI-based microplate assay showed efflux activity only in 15% (n=18) strains with 6% (n=7) active against more than one antibiotic. Efflux pump-mediated drug resistance was found to be most prevalent in *P. aeruginosa* (34.8%, n=8 out of 23), followed by that in *E. coli* (18.6%, n=8 out of 43), *Acinetobacter* spp. (9%, n=1out of 11), and *Klebsiella* spp. (2%, n=1 out of 46).

Conclusion: This study reports on the emergence of efflux pump-based multidrug-resistance in North Kerala. Our results showed that 15% of drug resistance in Gram-negative MDR strains is attributable to efflux-related mechanisms, thereby emphasizing the need for inclusion of efflux-related tests in the diagnostic regimen for MDR clinical bacteria.

Keywords: Gram-negative bacteria, Multidrug-resistance, Efflux pumps, Ethidium bromide, Efflux pump-inhibitor.

INTRODUCTION

Development of antibiotic resistance is one of the major causes of treatment failure of bacterial infections which is a worldwide health-care problem. Bacteria resist antibiotic action through several mechanisms, including target alteration, drug inactivation, decreased permeability, and increased efflux [1]. Of these, bacterial efflux pumps are a major concern since they confer bacteria the ability to drive away a variety of structurally unrelated antibiotics before their effect is realized [2,3]. Based on their composition, energy source, the number of membrane-spanning regions, and the types of substrate exported, these pumps are classified into five: major facilitator super family, the adenosine triphosphate-binding cassette super family, the small multidrug resistance family, the resistance-nodulation-cell division (RND) super family, and the multidrug and toxic compound extrusion family [4-6]. While in most cases, genes encoding multidrug efflux transporters are located on bacterial chromosome [7], such genes have also been found in both Gram-positive and Gram-negative bacteria on transmissible elements [8].

In Gram-negative bacteria, efflux-mediated drug resistance is more complex due to the molecular architecture of the cell envelope [7]. Efflux pumps of the RND family are prominent in clinically significant MDR Gram-negative bacteria. Mex in *Pseudomonas aeruginosa* and Acr in *Escherichia coli* can be cited as examples which are organized as tripartite systems comprising a cytoplasmic membrane transporter, a periplasmic membrane adaptor protein, and an outer-membrane channel protein [5]. The present study was undertaken to detect the prevalence of efflux pump-mediated drug resistance in Northern parts of Kerala.

METHODS

Clinical isolates

A total of 123 clinical isolates of MDR Gram-negative bacteria, collected from various clinical laboratories in North Kerala from December 2012 to January 2014, were included in our study. These isolates included four genera, *Klebsiella* spp., *E. coli, P. aeruginosa,* and *Acinetobacter* spp. The isolates were identified based on colony morphology and standard biochemical tests [9].

Antimicrobial susceptibility testing

Antibiotic sensitivity test was done by standard disc diffusion method (Kirby-Bauer Method) on Mueller–Hinton agar (MHA) plates. Commercially available antibiotic discs (HiMedia Mumbai, Maharashtra, India) used were: amikacin - 30 mcg, ampicillin - 10 mcg, aztreonam - 30 mcg, cefotaxime - 30 mcg, ceftazidime - 30 mcg, cefepime - 30 mcg, chloramphenicol - 30 mcg, ciprofloxacin - 5 mcg, gentamicin - 10 mcg, meropenem - 10 mcg, nalidixic acid - 30 mcg, ofloxacin - 5 mcg, piperacillin/tazobactam - 100/10 mcg, and tetracycline - 30 mcg. The choice of antibiotics and interpretation of bacterial sensitivity were determined according to the Clinical and Laboratory Standards Institute recommendations [10].

Ethidium bromide (EB)-agar cartwheel method (screening method)

Bacterial strains were grown in 5 ml of Luria–Bertani (LB) medium at 37°C with agitation (220 rpm) until they reached an optical density (OD) of 0.6 at 600 nm. Tryptic soy agar (HiMedia Mumbai, India) plates containing EB concentrations ranging from 0 to 2.5 mg/l were prepared on the same day of the experiment and protected from light.

Table 1: Antibiotic sensitivity profile

Bacteria	Antibiotic resistance (%)													
	AK	AMP	AT	CTX	CAZ	CPM	C	CIP	GEN	MRP	NA	OF	PIT	TE
Klebsiella spp.	76	100	87	91	91	91	54	96	78	59	91	85	85	83
E. coli	42	100	77	100	98	98	23	100	37	51	98	98	79	81
P. aeruginosa	78	100	61	96	83	78	87	74	83	74	100	83	83	91
Acinetobacter spp.	91	100	100	100	91	100	82	100	91	82	91	91	100	100

AK: Amikacin, AMP: Ampicillin, AT: Aztreonam, CTX: Cefotaxime, CAZ: Ceftazidime, CPM: Cefepime, C: Chloramphenicol, CIP: Ciprofloxacin, GEN: Gentamicin, MRP: Meropenem, NA: Nalidixic acid, OF: Ofloxacin, PIT: Piperacillin/tazobactam, TE: Tetracycline, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa*

The plates were then divided into sectors by radial lines. Cultures were then swabbed on EB agar plates starting from the center of the plate toward the edges and incubated at 37°C for 16 hrs in dark. The cultures were placed on a ultraviolet transilluminator and photographed using a gel documentation system (AlphaImager2200, USA). The minimum concentration of EB that produced fluorescence of the bacterial mass was recorded [11], taking corresponding MTCC strains as negative controls.

Efflux pump inhibitor (EPI)-based microplate assay (confirmatory method)

MDR strains were grown in LB medium until they reached an OD of 0.6 at 545 nm. 1 ml of Mueller–Hinton broth was added into 24-well microtiter plate which also included control wells. Antibiotic discs to be tested were distributed into the wells of the plate and incubated at 37°C for 1 hr. After the incubation, the efflux inhibitor, phenylalanine-arginine β-naphthylamide (PAN) (sigma Aldrich Chemicals. Pvt. Ltd) at a concentration 20 mg/l was dispensed to the corresponding wells of the microplate. Bacterial suspension (0.1 ml) was inoculated into all the wells and the plates were incubated at 37°C for 16-18 hrs. The determination of the effect of PAN was made by comparing the growth of the bacterium in the well containing a given antibiotic disc with that of the corresponding well containing the antibiotic disc plus PAN. The contents of the wells with no growth or poorer growth along with the controls were then plated on MHA plates to determine the number of colony forming units (CFU) [11].

RESULTS

A total of 123 clinical isolates of MDR Gram-negative bacteria were collected from various clinical laboratories in North Kerala, and screened for the presence of efflux-pump by phenotypic methods. The 123 Gram-negative MDR strains tested belonged to 4 genera comprising 37% (n=46) *Klebsiella* spp., followed by 35% (n=43) *E. coli*, 19% (n=23) *P. aeruginosa,* and 9% (n=11) *Acinetobacter* spp. The antibiotic sensitivity profile of all isolates are given in Table 1. All strains were found to be ampicillin-resistant.

The EB-agar cartwheel method used for the identification of presumptive overexpressed efflux systems showed efflux activity in 25 strains (Fig. 1 and Table 2). Clinical isolates without efflux pump activity were represented in all the 4 genera which were found to fluoresce at 0.5 mg/l concentration of EB as observed in the MTCC strains taken as negative controls. The minimum concentration of EB at which strains with efflux activity showed fluorescence was 1mg/l. At this concentration, strains of all 3 genera - *E. coli*, *Acinetobacter* spp., and *Klebsiella* spp. were found to fluoresce. Interestingly, *P. aeruginosa* strains showed much higher efflux activity in comparison to strains from other genera. Out of 23 *P. aeruginosa* strains, as many as 11 strains effectively effluxed the fluorochrome dye at concentrations many folds higher up to 2.5 mg/l (Table 2).

The 25 strains identified to possess efflux activity by the cartwheel method were subjected further to an EPI-based microplate assay employing selected antibiotics which showed zero inhibition zone. In the presence of the efflux inhibitor, PAN, some isolates displaying efflux activity completely reverted to a phenotype sensitive to the antibiotic(s) concerned (denoted as "reversal" in Table 3). In other words, resistance to specific antibiotics in these strains was solely due to efflux-pumping activity. Isolates with reduced growth compared to controls were indicative of only a partial contribution of efflux activity toward antibiotic resistance (denoted as "reduction" in Table 3). Based on the above-mentioned criteria, only 18 strains tested positive for efflux activity. Of these, 7 isolates displayed efflux activity against more than one antibiotic (Table 3). Further, efflux pump-mediated drug

Table 2: Determination of efflux activity at varying concentrations of ethidium bromide as fluorochrome

Number of bacterial species in each genus	Concentration of ethidium bromide at which bacteria started to fluoresce (mg/l)	Efflux activity
Klebsiella spp.		
45	<0.5	-
1	1	+
E. coli		
31	<0.5	-
12	1	+
P. aeruginosa		
12	<0.5	-
5	1	+
5	1.5	+
1	2.5	+
Acinetobacter spp.		
10	<0.5	-
1	1	+

E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa

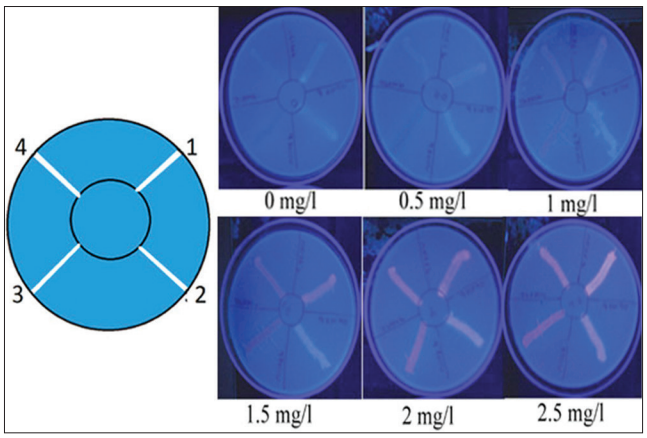

Fig. 1: Accumulation of fluorescent chromophore - Tryptic soy agar Petri plates containing varying concentrations of ethidium bromide, swabbed with *Pseudomonas aeruginosa* strains. Schematic representation of a Petri plate on the left of the figure denotes the position of bacterial strains - (1) *P. aeruginosa* MTCC 2453 (negative control), (2) *P. aeruginosa* (positive clinical isolate produced fluorescence at 1 mg/l concentration of ethidium bromide), (3 and 4) *P. aeruginosa* (negative clinical isolates)

Table 3: Effect of EPI (PAN) on antibiotic resistance

Bacterial strains	Antibiotics	CFU (×10^5)		EPI activity
		With antibiotic alone	With antibiotic+ EPI	
Klebsiella spp.				
K1	C	204000	3200	Reduction
E. coli				
E1	CTX	53500	1100	Reduction
E2	AK	7700	0.001	Reduction
	TE	20	0.001	Reduction
E3	AK	13400	0	Reversal
	PIT	148	0	Reversal
	TE	36	0	Reversal
E4	NA	4500	73	Reduction
E5	C	85	9.1	Reduction
	TE	6000	0	Reversal
E6	TE	1600	0	Reversal
E7	TE	2.33	0	Reversal
E8	NA	40000	12	Reduction
P. aeruginosa				
P1	CTX	30800000	23800	Reduction
	C	642000	0	Reversal
	NA	1382000	2	Reduction
	TE	5820000	0	Reversal
P2	C	3400	0	Reversal
P3	MRP	4100000	0.002	Reduction
	OF	387000000	74000	Reduction
	TE	37200	0.006	Reduction
P4	C	450000	0	Reversal
P5	C	37300	0.002	Reduction
P6	CTX	1970000	0	Reversal
	C	15300	0	Reversal
P7	TE	151000000	6.5	Reduction
P8	CTX	24000000	92	Reduction
	MRP	11500	0	Reversal
Acinetobacter spp.				
A1	TE	23500	1.09	Reduction

AK: Amikacin, CTX: Cefotaxime, C: Chloramphenicol, MRP: Meropenem, NA: Nalidixic acid, OF: Ofloxacin, PIT: Piperacillin/tazobactam, TE: Tetracycline, CFU: Colony forming units, EPI: Efflux pump inhibitor, PAN: Phenylalanine-arginine β-naphthylamide, *E. coli: Escherichia coli, P. aeruginosa: Pseudomonas aeruginosa. E. coli and P. aeruginosa* strains were found to efflux more than one antibiotic

resistance was found to be most prevalent in *P. aeruginosa* (34.8%, n=8), followed by that in *E. coli* (18.6%, n=8), *Acinetobacter* spp., (9%, n=1) and *Klebsiella* spp. (2%, n=1).

DISCUSSION

Efflux systems play a key mechanistic role in the development of drug resistance in Gram-negative bacteria. These pump solutes out of the cell, thereby allowing the microorganisms to regulate their internal environment by removing toxic substances such as antimicrobial agents, metabolites, and quorum-sensing signal molecules [12]. RND pumps known to be present in Gram-negative bacteria subsequently allow for acquisition of additional resistance mechanisms resulting in high bacterial pathogenicity - invasion, adherence, colonization, and survival of bacteria in the host [13].Efflux blockers are increasingly being investigated as a tool for effective deployment of antimicrobial drugs [14]. In this study, we have employed PAN, reported to be one of the first inhibitors of RND pumps [15,16]. Our study reveals the emergence of efflux pump-mediated drug resistance in MDR Gram-negative bacteria in North Kerala. The EB-agar cartwheel screening method showed efflux activity in 25 strains. The likelihood of false positives in such a screening cannot be ruled out as it has been reported that bacterial permeability to EB may also be highly decreased due to the down-regulation of porins [11]. Hence, the 25 strains mentioned above were subjected to EPI-based confirmatory method which tested positive for 18 strains with 7 of them exhibiting efflux activity against more than one antibiotic. The effect of EPI on the resistance against a given antibiotic was classified essentially as described by Martin *et al*., 2010, as (i) reversal - corresponding to no growth, due to bacteria being fully susceptible to the antibiotic; (ii) reduction - poorer growth in comparison to control, indicating that efflux contributed partially to the resistance; and (iii) no effect - no change in the growth in the presence or absence of the EPI, revealing the existence of resistance mechanisms other than efflux pumping [11]. It may be relevant to mention here that PAN is reported to have differential effects which are concentration-dependent acting only as an EPI alone at low concentrations with additional membrane-destabilizing effects at high concentrations resulting in increased membrane permeabilities [16-18]. This aspect assumes critical importance clinically as this mechanism can potentially revert bacterial resistance to antibiotics.

CONCLUSION

Routine antimicrobial sensitivity tests fail to detect efflux pump-mediated drug resistance. The current study showed that as much as 15% of drug-resistance in Gram-negative MDR strains is attributable to efflux-related mechanisms and that efflux activity-based antibiotic resistance is more prevalent among *P. aeruginosa* in comparison to that in *E. coli*, *Acinetobacter* spp. and *Klebsiella* spp. Hence, it is suggested that detection of efflux pump overexpression should also be included in the diagnostic regimen to facilitate implementation of appropriate therapy to the ailing patients.

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