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Research Article

MULTIDRUG RESISTANT GRAM NEGATIVE PATHOGENS ANTIBIOTIC PROFILE AND ITS EFFECTIVE CONTROL USING SECONDARY METABOLITES FROM MARINE ACTINOBACTERIA

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ABSTRACT: Aim: To screen the spread of resistance in ESBLs producer's particularly non lactose fermenting gram negative *Acinetobacter* spp. and *Pseudomonas* spp. and study antimicrobial activity with crude extract from novel marine actinomycetes in India. **Methods:** Fifty clinical isolates in a period of one year were processed and the antibiotic susceptibility was determined by double disk approximation test, the ESBLs production was screened with phenotypic confirmatory methods using disks of amikacin, meropenem, netilimicin, ciprofloxacin, gentamicin, tigecycline and piperacillin along with cephalosporin disks. Antimicrobial activity of the crude extract was determined by agar plug method. **Results:** The isolates collected from different samples were found resistant to third and fourth generation cephalosporins. ESBL production was detected in 56 % to 66 % of the isolates, amikacin and netilmicin showed 50% to 60% resistance they were also found resistant to carbapenems, 86% resistance was observed in *Acinetobacter* spp. Two strains PM21 and PM27 selected from 24 actinobacterial isolates had zone of inhibition >21mm. **Conclusion:** A high level of antibiotic resistance was found in *Acinetobacter* spp. in our study and may reflect the scenario in India. Earlier detection and reporting of ESBL producers will help in treating individual cases and also in controlling the spread of these resistant genes to other sensitive nosocomial isolates. The medical need for new agents is most acute and the future of this work aims to identify one such novel compound from marine actinobacteria.

INTRODUCTION

The leading cause of resistance to β-lactam antibiotics like penicillin, cephalosporin, and cephamycin among gram negative bacteria is due to β- lactamase enzymes produced by these organisms, carbapenem antibiotics are the last resort for their infections. They inactivate antibiotics by hydrolysing the β-lactam ring of penicillins and cephalosporins (Ghysen, 1991).There is an increase in the incidence and prevalence of ESBLs that hydrolyse and cause resistance to oxyimino-cephalosporins and aztreonam by their rapid spread and diversity is a problem worldwide. Most ESBL producing organisms are in the family Enterobacteriaceae commonly in *E.coli* and *Klebsiella pneumonia,* but infections, colonization and nosocomial spread involving other ESBL producing organisms are reported (Thomson *et al*.,2000). These organisms have become a common problem in hospitals and other health care facilities, known risk factors for colonization and infection with organisms harboring ESBL include admission to an ICU, recent surgery, prolonged hospital stay, instrumentation and antibiotic exposure to an extended spectrum cephalosporin (Bush *et al*., 1995). The overuse and misuse of antibiotics have favored the emergence and spread of bacteria that are resistant to multiple antibiotics, threatening the treatment of bacterial infections. Multidrug resistance can occur through many mechanisms, MBLs hydrolyze almost all β-lactam antibiotics including carbapenems and hardly broken by inhibitors. Hence there is a continuous demand for new antibiotics with novel and specific targets to combat evolving life threatening infectious diseases.

Although chemical synthesis and engineered synthetic compounds are accelerated in the production process, nature still has an edge for its versatile and valuable pharmaceuticals especially from actinobacteria. Nature still remains the richest and the most versatile source for new antibiotics and little is known about the microbial diversity of marine sediments (Kpehn *et al*., 2005). India with a long coastal line of over 7,500 km and area of 2.02 million sq km with very rich biodiversity, gives us an opportunity to investigate the untapped reservoir of novel natural products for the benefit of mankind. The marine and estuarine sediments for isolating actinobacteria have not been extensively investigated although its ubiquitous presence and diversity remains inexhaustible than its terrestrial counterparts. Actinobacteria play an important role among the marine bacterial communities, because of its diversity and ability to produce novel chemical compounds of high commercial value (Hopwood, 2007). It has been emphasized that marine sediments contain wide range of unique microorganisms and may be valuable for the isolation of novel strains which could potentially yield a broad spectrum of secondary metabolites (Takizawa *et al*., 1993). The Tamil Nadu coastal region has diverse marine habitats such as seashore, hyper saline lakes, estuaries, saltpans and other varieties of soil habitats. The study was initiated to isolate actinobacteria from the marine sediment sample collected from Tamil Nadu coastal of Parangipettai (*Lat*. 11°.29' N; *Long*. 79°.46'E) sediments and characterize their antibacterial properties against MDR pathogens.

MATERIALS AND METHODS

Multidrug resistant Enterobacteriaceae strains and non lactose fermenting isolates over a period of one year from August 2010 to July 2011 that showed decreased susceptibility to oxyimino-cephalosporins were tested phenotypically for ESBLs production as per CLSI guidelines. The isolates were obtained from different samples urine, blood, sputum, pus, endotracheal aspirates, bronchial secretion, wound and vaginal swabs. About 50 ESBLs positive strains were randomly selected from the total 2,441 isolate collections and stored in tryptic soy broth with 20% glycerol for further analysis. All Gram positive isolates, *Nocardia*, *Proteus* spp. *Providensia, Morgenella morganii, Serratia, Citrobacter diversus* and few other gram negative organisms were excluded since they were not predominantly isolated. Non repetitive ceftazidime or cefotaxime resistant Enterobacteriaceae (*E.coli, Klebsiella* spp. *and Enterobacter* spp.), *Acinetobacter* spp. and *Pseudomonas* spp. 10 strains from each of the microorganisms were selected for the study.

Antibiotic susceptibility testing

All samples were plated right after the collection and were further processed as per standard protocol. Single or mixed growth isolated from all the eligible consecutive samples were cultured and identified by observing the colony characteristics on the blood, Mac-Conkey agar plates and Cystein Lactose Electrolytes Deficient agar (CLED), biochemical reactions were performed using standard microbiological methods. Antimicrobial susceptibility testing was initially determined with cephalosporins, aminoglycosides, penicillins and carbapenems (Hi-Media) as per CLSI guidelines zone diameter was measured and interpreted. For quality control of disc diffusion tests control strains of *E.coli* ATCC 25922, *P.aeruginosa* ATCC 27853 and an in-house carbapenem resistant *Acinetobacter baumannii* were used and they showed the expected zone pattern (NCCLS, 2002).

Test for ESBL enzyme production

Screening for ESBL producers-Double disk approximation method

Test strains were pre incubated in brain heart infusion broth at 37°C to an optical density of 0.5 McFarland turbidity Standard .This suspension was inoculated in Muller Hinton agar plates by swabbing with sterile cotton swabs, the standard disks containing 30 µg of aztreonam, ceftazidime or cefriaxone were placed 15 mm (edge to edge) along with other antibiotic disks and incubated overnight at 37°C for 18 to 24 hours to screen for multidrug resistant strains (Jalier *et al*., 1998). Bacterial susceptibility to all antimicrobials was determined according to CLSI guidelines. When using disk diffusion method *E.coli* and *K.pneumoniae* a zone inhibition diameter lower than the following values ceftazidime (<22mm), cefotaxime and aztreonam (<27mm), ceftriaxone (<25mm), cefpodoxime (<17mm) were investigated with confirmatory tests (Philip *et al*., 1997).

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ESBL phenotypic confirmatory method and MDR screening

ESBL phenotypic confirmatory test was done using standard ceftazidime and cefotaxime disks with and without clavulanic acid (CLSI, 2007). A greater than or equal to 5mm increase in the zone diameter with clavulanate and ceftazidime versus ceftazidime alone confirmed an ESBL production. Multidrug resistant isolates were screened for metallo beta lactamase production using 0.5M EDTA. The presence of an enlarged zone of inhibition towards the EDTA disk was interpreted as positive double disk synergy test for an MBL producer. MIC was determined by agar dilution method for all meropenem resistant isolates, using 64 µg / mL to 0.25 µg / mL concentration.

Isolation of potential actinobacteria

The marine sample was collected from marine sediments by inserting a sterilized polyvinyl corer (10 cm) into the sediments after removing the surface layer. The centre portion of the 2 cm sample was taken out with the help of a sterile spatula. The collected sample was transferred to a sterile polythene bag and taken immediately to the laboratory; the sample was air-dried aseptically for one week. An air-dried sediment sample was incubated at 55°C for 5 min (Balagurunathan, 1992) and plated in Starch Casein Agar media (prepared with 50 % sea water). To minimize bacterial and fungal contamination, all agar plates were supplemented with (100mg/l) of nystatin and nalidixic acid (20mg/l), respectively. All the colonies growing on the Petri plates were separately streaked in Petri plates and maintained in ISP2 agar slants and 30% glycerol broth.

Identification and Screening for antimicrobial activity

The phenotypic characteristics as consistency, aerial mass color, reverse side pigmentation, solubility of pigment, and utilization of carbon sources of the actinobacterial strains were identified (Nonomura, 1974, Ugawa *et al*., 1989). The isolates were tested primarily by cross streak method with test pathogens, incubated at 37°C for 24 hrs and the zone of inhibition was measured (Lemos *et al*., 1985). The potential actinobacteria strains were tested for its biological activity against the test pathogens using 0.5 McFarland's standard broth culture swabbed on MHA plates using sterile cotton swab. To confirm the antimicrobial activity agar plug method was adopted, after scrapping the topical growth from ISP2 media a 5mm agar bored core containing diffused metabolite was impregnated on the surface of the test plate. The plates were incubated at 37°C for 24 hours and the results are tabulated.

RESULTS

The drug resistant isolates from urine, blood, pus, sputum and miscellaneous samples were screened and their resistance percentage was determined (Table-1). Although lactose fermenters like *E.coli* were isolated more, the incidence of ESBLs was 64% compared with 66% of *Enterobacter* spp. isolates. MIC determined by agar dilution method about 28% of organisms had resistance in the concentration of 16 µg / mL, 13% showed resistance for 32 µg/mL followed by 8% resistance for 8 µg/mL for meropenem.

Table-1 Tabulation of all isolates with their resistance percentage

Organisms Isolated	Total No. of Isolates	% of Individual Isolates	% total of ESBL/MDR
E.coli	1447	59.27	63.57
Klebsiella spp	308	12.61	56.1
Enterobacter spp	113	4.62	66.37
Acinetobacter spp	242	9.91	85.95
Pseudomonas spp	331	13.56	56.49

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The number of *E.coli* strains isolated from urine was very high compared to other samples, indicating the complexity of UTI infections. Co-resistance to non-beta lactam antibiotic was observed with higher ($p<0.05$) in non fermentative *Acinetobacter* spp. than *Pseudomonas* spp. and other Enterobacteriaceae results not shown (Table-2).

Table-2 : Tabulation of non lactose fermenting gram negative isolates for individual antibiotics and their P values

	Acinetobacter spp.								
	Misc. Sample			**Blood Sample**			**Pus Sample**		
ANTIBIOTICS	Total Sample	Number Resistant	P value $p < 0.05$	Total Sample	Number Resistant	P value $p < 0.05$	Total Sample	Number Resistant	P value $p < 0.05$
AMIKACIN	124	107	0.86	22	12	0.55	27	17	0.63
AZTREONAM	124	124	1.00	22	-	-	27	20	0.74
CEFTAZIDIME	124	124	1.00	22	-	-	27	-	-
CIPROFLOXACIN	124	124	1.00	22	22	-	27	27	1.00
CEFIPIME	124	124	1.00	22	-	-	27	18	0.67
TIGECYCLIN	124	0	0.00	22	0	-	27	0	0.00
GENTAMICIN	124	124	1.00	22	0	0.00	27	27	1.00
IMIPENEM	124	124	1.00	22	22	1.00	27	-	-
NITILMICIN	124	114	0.92	22	-	-	27	15	0.56
OFLAXOCIN	124	124	1.00	22	-	-	27	-	-
PIPERACILLIN	124	124	1.00	22	-	-	27	27	1.00
TOBRAMYCIN	124	124	1.00	22	-	-	27	21	0.78
MEROPENEM	124	124	1.00	22	21	0.95	27	-	-
				***Pseudomonas* spp.**					
	Misc. Sample			**Blood Sample**			**Pus Sample**		
ANTIBIOTICS	Total Sample	Number Resistant	P value $p < 0.05$	Total Sample	Number Resistant	P value $p < 0.05$	Total Sample	Number Resistant	P value $p < 0.05$
AMIKACIN	52	28	0.54	21	7	0.33	34	31	0.91
AZTREONAM	52	30	0.58	21	-	-	34	27	0.79
CEFTAZIDIME	52	31	0.60	21	-	-	34	-	-
CIPROFLOXACIN	52	32	0.62	21	18	-	34	31	0.91
CEFIPIME	52	32	0.62	21	-	-	34	25	0.74
TIGECYCLIN	52	-	-	21	-	-	34	-	-
GENTAMICIN	52	43	0.83	21	0	0.00	34	33	0.97
IMIPENEM	52	32	0.62	21	21	1.00	34	-	-
NITILMICIN	52	27	0.52	21	-	-	34	28	0.82
OFLAXOCIN	52	32	0.62	21	-	-	34	-	-
PIPERACILLIN	52	30	0.58	21	-	-	34	28	0.82
TOBRAMYCIN	52	30	0.58	21	-	-	34	25	0.74
MEROPENEM	52	-	-	21	18	0.86	34	-	-

Except for amikacin and netilmicin that showed 50% to 60% resistance, most isolates were resistant to carbapenems. ESBL production was detected in 56 to 66 % of isolates, the percentage of resistance observed in *Acinetobacter* spp. was 86%. Resistance percentage for amikacin 56.2%, meropenem 98.5%, netilimicin 61.5%, and other antibiotics like ciprofloxacin, gentamicin and piperacillin showed 99-100% resistance, but all isolates were 100% sensitive for tigecycline, colistin was also found very effective.

From the antibiotic profile it is inferred that non lactose fermenters are more drug resistant than lactose fermenters. In this study few strains of *Burkholderia cepaciae, Citrobacter freundii* and *Enterococci* were also isolated that showed multidrug resistance. The resistance percentage in *Pseudomonas* spp for individual antibiotics was amikacin 56.1%, ciprofloxacin72.1%, gentamicin 77%, piperacillin 57.7%, meropenem 77.5% and netilmicin 67.3%. A total of about 24 different actinobacterial strains were selected based on the different phenotypical features. The following phenotypic characteristics as powdery consistency, pale pink color aerial mass, brown pigment on reverse side with good growth on glucose, sucrose, mannitol, arabinose, rhamnose, cellulose, inositol was recorded. The different growth parameters as pH, temperature and salinity were observed. Antimicrobial activity of the compound tested on the MDR pathogens showed good zones of inhibition 12 to 21 mm (Table-3).

(a). (b).

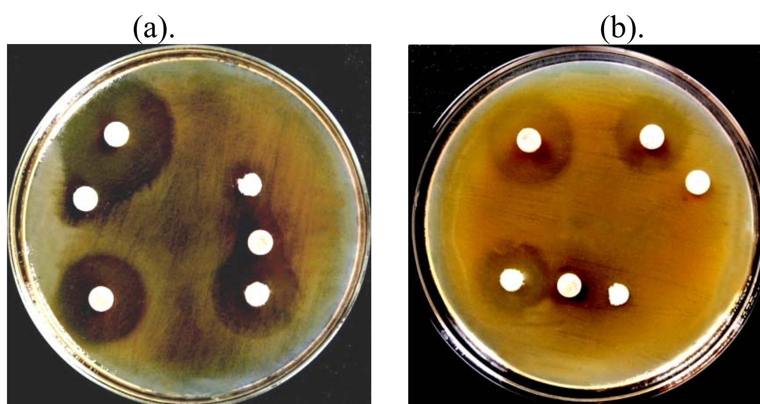

Figure-1 : Plate (a) represents positive and (b) negative ESBL and MBL results, the three disks together are identifying MBL's, meropenem disks are flanked by plain disks with EDTA and Zn (for gp-2 MBL isolates) on comparison with meropenem alone. Amoxy-clav and Ceftazidime disks enhanced zone shows ESBL's.

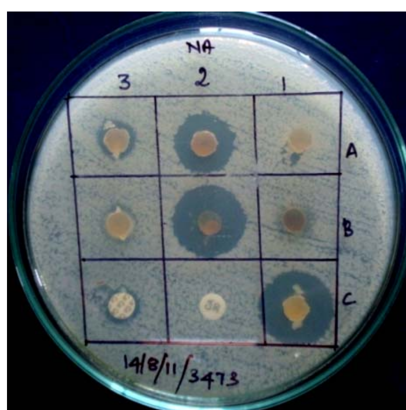

Figure-2: Plate shows actinobacteria bioactive compound's zone of inhibition in comparison with 3rd generation cephalosporin, ceftazidime and cefotaxime antibiotics. The strain A-3473 represents *Acinetobacter baumannii*

Table -3 : Zone of inhibition represented by marine actinobacteria metabolites

Strain Number of Acintobacteria	Serial Number	A-1370 ET secretion	P-2022 sputum	A-481 Pus	A-3473 Sputum	A-674 ET secretion	P-4117 Urine	A-2484 Blood	A-1677 Pus	K-2030 Blood	E-39084 Urine
PM21	A2	13mm	-	20mm	15mm	15mm	12mm	-	19mm	15mm	15mm
PM27	C1	-	-	20mm	20mm	-	-	-	-	-	-
Ceftazidime	C2	16mm	18mm	-		10mm	9mm	-	-	-	10mm
Cefotaxime	C3	15mm	-	10mm	10mm	-	-	-	-	-	-

PM-Parangipetti marine sediment sample.

DISCUSSION

The incidence of ESBL producing strains among clinical isolates have increased over the past few years resulting in limited therapeutic options and their outbreaks all over the world (Ananthakrishanan et al., 2000).With the spread of ESBLs in hospitals it is necessary to know the prevalence so as to formulate a policy of empirical therapy in high risk units where MDR organisms are much higher. The ESBLs isolated were *E.coli* 63.5%, *Enterobacter* spp. 66.3% and *Klebsiella* spp. 56.1%; these resistant strains are treatable with available β-lactam inhibitors. As a result of widespread application of antimicrobial agents the frequency of drug resistance to *Pseudomonas* is raising dramatically 56.4% resistance was observed in this study. *Acinetobacter* spp. acquires resistance to chemotherapeutic agents, 86% of the strains were found resistant and high levels of tigecycline resistance are reported (Nidhi Goel *et al*., 2009, Shiri Navon *et al*., 2007). The *A. baumannii* complex is emerging as a problematic, nosocomial pathogen with the propensity to cause outbreaks in the intensive care unit (ICU) setting (Giamarellou *et al*., 2008). Antimicrobials that were not used frequently in the hospital may show low resistance, such observations have been witnessed by investigators wherein susceptibility is attributed to decreased usage of the antimicrobial, but in this study antibiotics like netilimicin and cefepime are in routine use. MIC of 16 μg/mL or above is only considered as meropenem resistant and most of the ESBLs organisms were found in this range in the study. But strains showing resistance by disk diffusion method were found to have MIC in sensitive range and revealed MICs of 0.5-2 μg/mL which is well below the MIC breakpoint recommended (Weinbren *et al*., 1998).

The p-value ($p < 0.05$) of co resistance transfer for both beta lactam and non beta lactam group of antibiotics is significant in both *Acinetobacter* spp. and *Pseudomonas* spp. If carbapenemases spreads widely many nosocomial Gram-negative infections will be untreatable with the available antibiotics demanding novel antibacterial compounds. There are many reports of marine and terrestrial derived actinobacterial metabolites effect on MRSA, VRSA and ESBLs (Ratnakar *et al*., 2010, Ozgur Ceylan *et al*., 2008). Here we probably report for the first time potential activity of marine actinobacterial bioactive compounds with zone of inhibition <21mm (Remya *et al*., 2008) effective on MBLs producing isolates identified using 0.5 M EDTA, the Zn^+ chelator found in the active site of MBLs enzymes. Based on the phenotypic features inferred from result the strains PM21 and PM27 are tentatively suspected as *Streptomyces* spp. the taxonomic position and molecular characterization will be further elucidated. Marine actinobacteria are potential source of novel antimicrobial compounds yet to be completely explored providing the opportunity for new therapeutic leads in the treatment of MDR pathogens. The demand for novel drugs to the existing antibiotic resistance scenario and the thirst for new leads in pharmaceutical industry can be met with unexplored metabolites from actinobacteria. Work is in progress in our laboratory to characterize the strain for its taxonomic and phylogenetic relatedness and purification of the crude compound for structure elucidation. It is here against Gram- negative opportunists that the medical need for new agents is most acute for the MDR resistant *Acinetobacter* spp. and *Pseudomonas* spp. from marine actinobacteria which can replace the exploding synthetic drugs with natural compounds for their novelty and efficiency in near future.

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