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CO EXISTENCE OF AMPC AND EXTENDED SPECTRUM BETA LACTAMASES RESISTANCE IN METALLO BETA LACTAMASES PRODUCING STRAINS OF PSEUDOMONAS AERUGINOSA

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ABSTRACT

Pseudomonas aeruginosa is one of the commonest multi drug resistant bacteria causing nosocomial infection and Metallo-Beta-Lactamase production is one of the important reasons. The following study was conducted to determine incidence of MBL producing *P.aeruginosa.* and co existence of AmpC and ESBL production. Screening for MBL was done using imipenem disk and all the isolates found resistant were further subjected to phenotypic confirmation of MBL production using Disc Potentiation test, Modified Hodge test and E- test. Out of 220 isolates screened, 14 were detected to be imipenem resistant; all 14 were positive by Disc Potentiation test and E-test (6,36%) and only 11 isolates were positive by Modified Hodge test. Out of 14 MBL producing isolates, 8 were AmpC producers (57.14%) and all were ESBL producers. Imipenem (carbapenems) use should be restricted and used only in life threatening conditions or in Multi Drug Resistant isolates like *P.aeruginosa.*

KEY WORDS: **Metallo beta lactamases, ESBL, AmpC, P.aeruginosa**

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INTRODUCTION

Pseudomonads are heterogenous group of gram negative bacteria and *Pseudomonas aeruginosa* (*P.aeruginosa*) the major pathogen of the group is mainly associated with hospitalized patients and in patients with cystic fibrosis (1). *P.aeruginosa* unfortunately has become resistant to a variety of antimicrobial agents such as beta lactams, aminoglycosides, chloramphenicol, quinolones, and sulfonamides (2). Due to the broad spectrum activity and stability to hydrolysis by most beta lactamases, the carbapenems have been a drug of choice for treatment of infections caused by penicillin or cephalosporin-resistant gram negative bacilli especially, Extended Spectrum Beta-Lactamase (ESBL) producing gram negative infections (3).Carbapenemase resistance has been observed in non fermenting bacilli especially *P.aeruginosa.* Resistant to carbapenems is due to decreased outer membrane permeability, efflux systems, alteration of penicillin binding proteins and carbapenem hydrolyzing enzymes – carbapenemase (4). Metallo beta lactamase(MBL) belongs to group of beta lactamases which requires divalent cations of zinc as cofactors for their enzyme activity and are capable of inactivating beta lactam drugs including carbapenems MBL-producing *P.aeruginosa* isolates have been reported to be important cause of nosocomial infections associated with clonal spread. These constitute 20%-40% of all nosocomial isolates. The appearance of MBL genes their spread among bacterial pathogen is a matter of concern with regard to the future of anti microbial chemotherapy . The present study has been planned for the detection of MBL which would be an aid in the judicious use of carbapenems and prevent further dissemination in hospital settings

MATERIALS AND METHODS

This prospective study was conducted in the Department of Microbiology at Himalayan Institute of Medical Sciences, Dehradun, UK. A total of two hundred and twenty non repetitive isolates of *P.aeruginosa* were recovered from various clinical specimens. All the culture isolates obtained were identified as *P.aeruginosa* by routine bacteriology methods (5)and further processed for antibiotic sensitivity testing and MBL detection , AmpC detection and ESBL detection. Antibiotic sensitivity testing was performed on Mueller-Hinton Agar (MHA) plates with commercially available antibiotic discs (Hi Media, Mumbai) like aminoglycosides [amikacin (30 µg) , gentamicin (10 µg) , netilmycin (30 µg) , tobramycin (10mµg)] , cephalosporins [ceftriaxone (30µg) , ceftazidime (30 µg) , cefipime (30 µg)] , Betalactamase inhibitor combinations [cefoperazone-sulbactum (75-30 µg), piperacillin-tazobactum (100-10 µg)], fluoroquinolones [ciprofloxacin (5 µg), ofloxacin (5 µg), Levofloxacin (5 µg)] , Antipseudomonal drugs [piperacillin (100 µg), aztreonam (30 µg)], chloramphenicol (30 µg), imipenem (10 µg), polymyxin-B (300 µg), colistin (10 µg) by Kirby Bauer disc diffusion method and interpreted as per CLSI guidelines (6). *P.aeruginosa* ATCC 27853

DETECTION OF METALLO BETA LACTAMASE (MBL)

The screening of MBL production was done by using imipenem disk of 10 µg. All isolates of *P.aeruginosa* found resistant to imipenem (≤ 13 mm zone size) were further subjected to phenotypic conformation of MBL production by the Disk PotentiationTest (DPT) (4), Modified Hodge Test (MHT) (7) and E Test (8)

DISC POTENTIATION TEST

1. The test organisms were inoculated on to plates of Mueller-Hinton Agar (opacity adjusted to 0.5 Mcfarland opacity standards.)
2. A 0.5-M EDTA solution was prepared by dissolving 186.1 g of disodium EDTA $2H_2O$ in 1000 ml of distilled water and pH adjusted to 8.0 by sing NaOH. The mixture was sterilized by autoclaving.

3. Two 10 mg imipenem disc and two meropenem discs were placed on the plate atlas 10 mm apart. 5µl of EDTA solution was then added to one of the discs each.
4. The inhibition zones of imipenem and imipenem-EDTA discs and meropenem and meropenem-EDTA discs were compared aster 16-18 hrs of aerobic incubation at $35^{O}C$.
5. An increase in zone size of at least 7mm around the imipenem-EDTA and meropenem-EDTA disc was recorded as metallo beta lactamase positive strains.

MODIFIED HODGE TEST

1. The surface of Mueller Hinton Agar plate was introduced evenly using a cotton swab with an overnight suspension of *Escherichia coli* (ATCC 25922). This was adjusted to one-tenth turbidity of McFarland no.0.5.
2. After brief drying, an imipenem disc was placed at the centre of the plate, and imipenem - resistant test strains from overnight incubated culture plates were streaked heavily from the edge of disc to periphery.
3. The presence of distorted inhibitory zone after overnight incubation was interpreted as Modified Hodge Test positive.

E -test

E-test metallo-beta-lactamase strips (Bio-Merieux) with a double sided seven dilution range of imipenem IP (4 to 256 mg/ml) and IP (1 to 64 mg/ml) overlaid with a constant gradient of EDTA was taken.(The test was carried out as per the manufacturers instructions.)

Those isolates which were found to be imipenem resistant on initial screening and positive by any of the phenotypic conformation tests were considered as metallo betalactamase producing *P.aeruginosa*.

ESBL DETECTION

The extended spectrum beta-lactamase (ESBL) status of these strains was established by combined disk diffusion method per CLSI recommendations (9) using Cefotaxime (30µg) and Ceftazidime (30µg) disks alone and in combination with clavulanic acid.

Amp C Disk Test

AmpC disk test was done for the imipenem resistant strains for detection of AmpC beta-lactamases (10).

1. On a MHA plate, lawn culture of *E.coli* (ATCC 25922) was made from an overnight culture suspension adjusted to 0.5 Mcfarland's standards.
2. A 30mg cefoxitin disc was kept on the surface of the agar. a blank disk (6 mm in a diameter, whatmann filter paper no.1) was moistened with sterile saline and inoculated with a few colonies of the test strain.
3. The inoculated disc was then placed besides the cefoxitin disk almost touching it.
4. The plate was incubated overnight at 37^{o} C.
5. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain was interpreted as positive for the production of AmpC beta lactamase. An undistorted zone was considered as negative.

RESULTS

Table 1
Detection of MBL Production by Different Phenotypic Tests amongst Isolated Strains of Pseudomonas aeruginosa from different Clinical Samples

S.No.	Samples	*P. aeruginosa Isolates* n= 220 (%)	*Imipenem resistant*	Phenotypic Detection Tests			MBL Producers n= 14 (%)
				DPT	HT	E- Test	
1.	Pus	76 (34.54)	3	**Positive**	Negative	**Positive**	**3(21.44)**
2.	Urine	45 (20.45)	7	**Positive**	**Positive**	**Positive**	**7 (50.00)**
3.	Sputum	32 (14.54)	1	**Positive**	**Positive**	**Positive**	**1 (7.14)**
4.	Endotracheal tube	30 (13.63)	1	**Positive**	**Positive**	**Positive**	**1 (7.14)**
5.	Blood	9 (4.09)	-	-	-	-	-
6.	Tracheal Tube	6 (2.72)	-	-	-	-	-
7.	Pleural fluid	5 (2.27)	-	-	-	-	-
8.	BAL	4 (1.82)	-	-	-	-	-
9.	Foley's catheter	3 (1.36)	1	**Positive**	**Positive**	**Positive**	**1 (7.14)**
10	Vaginal swab	3 (1.36)	-	-	-	-	-
11.	Semen	2 (0.90)	-	-	-	-	-
12.	Nasal swab	2 (0.90)	-	-	-	-	-
13.	Ascitic fluid	1 (0.45)	-	-	-	-	-
14.	Bile	1(0.45)	1	**Positive**	**Positive**	**Positive**	**1 (7.14)**
15	Drain fluid	1(0.45)	-	-	-	-	-
	Total	**220**	**14 (6.36%)**	**14**	**11 (5%)**	**14**	**14**

Out of 220 isolates of *P.aeruginosa* ,14 were found to be Imipenem resistant(6.36%). 11 out of 14 were found to be MBL positive (5%) by using MHT. MBL production was confirmed in all 14 isolates by MBL E test using imipenem (IP) and imipenem +EDTA (IPI) combination and DPT(6.36%). 8 out 14 showed MIC ratio of 64 , while 2 isolates showed MIC ratio of 48, 2 isolates showed MIC ratio of >32 and 2 isolates showed MIC ratio of >96 and >128 respectively. Out of 14 MBL positive isolates 12 (85.71%) were from males and 2 (14.29%) were from females. Maximum no. of isolates were obtained from patients in the age group of 50-59years (28.57%), followed by 60-69 years (21.43%). Median age group of patients was 50 years .13 (85.17%) were obtained from the IPD patients and 2(14.29%) were obtained from OPD patients. Highest isolation rate of MBL positive *P.aeruginosa*, was from general surgery and general medicine ward (28.57% each)and mainly from urine (50.00%) . The maximum associated risk factor with MBL production was found to be cancer (21.34%). On comparison between MBL and non MBL producing *P.aeruginosa* isolates no significant statistical association could be elicited for any risk factor.

Table 2
Comparison of antibiotic sensitivity pattern in MBL positive and non MBL isolates

Antibiotics	Resistance in MBL positive isolates (%)	Resistance in Non MBL isolates (%)	P value
Amikacin	71.43	31.07	0.001
Gentamicin	85.72	38.35	0.001
Tobramycin	85.72	36.41	0.0007
Netilmicin	78.57	31.07	0.0008
Ciprofloxacin	78.57	39.81	0.01
Ofloxacin	92.86	47.09	0.002
Levofloxacin	85.72	40.78	0.002
Ceftazidime	85.72	43.20	0.004
Ceftriaxone	100.00	52.43	0.001(NP)
Cefepime	92.86	53.88	0.01
Chloramphenicol	85.72	56.80	0.06 (NS)
Cotrimoxazole	92.86	64.08	≤ 0.05
Aztreonam	85.72	37.38	0.001
Piperacillin	71.43	32.04	0.006
Polymyxin-B	0.0	1.46	0.46(NP)
Cefoperazone+Sulbactum	85.72	29.61	0.00005
Piperacillin+Tazobactum	78.57	23.30	0.00002
Colistin	0.0	0.0	NP

(NS) Non Significant (NP)# Not Producible

In MBL positive isolates, the antimicrobial sensitivity was best observed with colistin and polymyxin B (100% each). Poor sensitivity was seen for all other drugs. On comparing the antimicrobial sensitivity pattern of non MBL and MBL producers, MBL producers were more resistant to non MBL producers for all drugs. On using p value and fisher exact test, the difference in sensitivity was found to be significant for all the drugs except chloramphenicol. The data of comparison was non producible for ceftriaxone, polymyxin-B and colistin .(Table 2) Out of 14 MBL producing *P.aeruginosa* isolates, 8 were AmpC producers (57.14%). All the MBL producers were also ESBL producers. All the MBL producing isolates were multidrug resistant isolates as they were resistant to a variety of antibiotic classes mainly aminoglycosides, antipseudomonal, penicillins, cephalosporins, carbapenems and fluoroquinolones.

DISCUSSION

MBL associated isolates are associated with a higher morbidity and mortality. Given the fact that MBLs hydrolyze all beta lactamase and that we are several years away from the development of a safe therapeutic antibiotic; their continued spread would be a clinical disaster. MBL producing *P.aeruginosa* was first reported in 1991 from Japan (11). Since then, several reports from Asia, Europe, Australia, India, South America, North America have been published (4, 12). In India, it was first reported by Navneeth et al (13) to be 12%. Since then MBL production in *P.aeruginosa* has been reported from across India and its prevalence ranged from 6.89% to 75% (14-17). In the current study the prevalence of MBL production in *P.aeruginosa* was quite low (6.36%) as compared to the majority of the

Indian study probably due to restricted use of carbapenems at our center. Variations in prevalence can be also due to different study groups and different testing methodologies used by various authors. For the purpose of MBL screening in *P.aeruginosa*, Sugino and associates from Japan and Lee et al from Korea used carbapenem resistant isolates for the screening MBLs (7,18). Similarly at our centre we screened Imipenem sensitivity for MBL production. The DDST and Hodge test are often difficult and are subjective to interpret and are thus unsuitable for clinical laboratories to perform on a routine basis .The current study showed 3 additional imipenem resistant *P.aeruginosa* strains to be MBL producers by DPT and MBL E-test, which were not detected by MHT. Other studies are in accordance to our study which indicates that false negative results were more commonly seen with MHT however, the reason is unclear (19). Yong et al and other workers (20,21) found imipenem disc with EDTA simple to perform and highly sensitive in different MBL producing isolates with excellent specificity for *P.aeruginosa*. We have found that all 14 isolates (100%) which were MBL positive by DPT were also positive by MBL E test and 3 of which were negative by MHT were also MBL positive by MBL E test. Results of our study were comparable to various other published studies. Marchiaro et al. (2005) showed that MBL E Test had a sensitivity of 92% in identifying MBL positive isolates (22). Pitout (2005) reported 96% sensitivity of MBL E Test in detecting MBL positives among imipenem resistant *P.aeruginosa* (23). Behera et al. (2008) found 100% of all the carbapenem resistant *P. aeruginosa* strains to be MBL positive by MBL E Test (24). A recent study conducted by Bashir and co workers in 2011 found that Combined Disc Test (same as DPT that we have used in the current study), MIC reduction and E test to be equally sensitive in detecting MBL production (8). Our results are in concordance to their findings.

MBL E test could be used in clinical laboratories to monitor the emergence of MBL in a range of clinically significant bacteria and by surveillance network to establish the spread of enzyme. However, Yan et al. (21) have reported that E Test is insensitive in detecting carbapenem sensitive MBL organism furthermore given the cost constraints of E test, a simple screening method like DPT can be used. This technique is very easy, economical and can be incorporated in to routine testing of any busy Microbiological laboratory as has been done for ESBL screening (17). Recent studies done in 2010, 2011 also recommended use of imipenem and imipenem + EDTA combined disc test (8,25-28). The current study demonstrates that carbapenem resistance was due to MBL production and this was found in concordance to another Indian study conducted by Navneeth et al. (13). In the present study MBL producers were mainly obtained from males (85.71%), patients belonging to the age group 50-59 years (28.57%) with a median age of 50 years. 85.71% were obtained from IPD mainly from general surgery and general medicine wards, source were urine (50%) and pus (21.44%) samples. All urine samples with MBL producing *P.aeruginosa* were from patients on urinary catheters. This is consistent with other studies (8, 25). The clinical data concerning risk factors and treatment outcomes of infections caused by MBL producing strains are few. Reports suggested carbapenem use, ICU stay > 20 days, underlying neurologic disorder, urinary tract infection, renal failure, indwelling devices and neutropenia as contributing factors (29). We failed to identify any significant risk factor.

The comparison of antimicrobial sensitivity testing between MBL producers and non-MBL producers was found to be statistically significant. In the current study, MBL producers showed 100% sensitivity to colistin and polymyxin-B. It is well known that colistin and polymyxin-B are effective drugs against MBL's. They are expensive and toxic which limits their use. However reports support the use of these drugs under proper monitoring and supervision (30). Overall sensitivity of MBL producers was poor to all other drugs. Sensitivity to combination drugs piperacillin-tazobactam and cefoperazone-sulbactam, 3rd and 4th generation cephalosporins and anti-pseudomonal drugs like piperacillin was poor.

This is consistent with the findings reported previously (8,26-28). Out of 14 imipenem resistant *P. aeruginosa* 57.16% were AmpC producers. Similarly Upadhaya and coworkers found a prevalence of co-production of AmpC in MBL producers to be 46.6 %. An increase in AmpC producing isolates may be indicative of the ominous trend of more and more isolates acquiring resistance mechanisms rendering the antimicrobial armamarium ineffective. The only β-lactam active against co-AmpC and ESBL producers are carbapenems; however, recently resistance to carbapenems has been increasing, which is mostly due to the production of MBL(31). The same was noted by us too. The present study emphasizes on the appropriate use of antibiotics since all the isolates found to be MBL producers, were also ESBL producers and some were AmpC producers too. All 14 were multi drug resistant leaving no option but to use only available toxic drugs like polymyxin-B and colistin. Due to indiscriminate use of antimicrobials, the only available drugs for multi drug resistant isolates would also become resistant, leaving no therapeutic options and resulting in therapeutic catastrophy. DPT is a more sensitive and specific test to detect MBL production than Modified Hodge test. DPT was found to be equally sensitive for MBL detection as the MBL E Test. Furthermore it is more economical as compared to MBL E Test. Since DPT is easy to perform and economical; it can be carried out routinely even in a busy microbiology laboratory. All imipenem resistant *P.aeruginosa* should be routinely screened for MBL production using DPT. Co existence of MBL,ESBL and AmpC production was common. It is necessary to strengthen the surveillance of drug resistance and to gain an understanding of the characteristics and mechanisms of multidrug resistance infections among the local population. These efforts are of extreme importance for reducing the occurrence of drug-resistant strains and for effective control of infections. Good sensitivity to polymyxin B and colistin maybe life saving in serious infections; however they should be used with discretion and as a part of a combination therapy. Aztreonam was not found to be a very effective drug for *P. aeruginosa.* ISurveillance should be carried out more intensively to monitor the spread of these multi dug resistant bacteria and strict Infection control strategies and antibiotic stewardship programmes should be implemented for limiting their spread,

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