Characterization, Antibiotic Resistance Pattern and Detection of Metalloß-lactamases and Amp C in Pseudomonas aeruginosa in a Tertiary Care Hospital

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ABSTRACT

Background and Objectives: *Pseudomonas aeruginosa is a common nosocomial pathogen. It is a major cause of multidrug resistance infections in hospitalized patients. Antibiotic resistance increases the morbidity and mortality associated with pseudomonal infections. This leads to rising costs of care resulting from prolonged hospital stay and the need for more expensive drugs. It therefore becomes imperative to study the antibiotic resistance pattern of P.aeruginosa. Objectives of this study were,*

1. *To isolate and identify Pseudomonas aeruginosa from various clinical samples.*
2. *To determine the antibiotic susceptibility pattern of these isolates.*
3. *To detect MBL and Amp C production in the isolates.*

Methods: *250 isolates of P.aeruginosa obtained from various clinical samples were identified using standard procedures. The antibiotic susceptibility patterns of the isolates were determined by disc diffusion method for the routinely used antibiotics as per the CLSI guidelines, Minimum inhibitory concentrations (MIC) of the isolates to meropenem was determined using micro broth dilution and agar dilution method. The isolates were also tested for the presence of AmpC (Disc antagonism) and MBL (Imipenem (IMP)-EDTA Combined disk test).*

Results: *Of the 250 P.aeruginosa included in the study, 238 were isolated from Pus/swab and 12 from urine samples. By disc diffusion method third generation cephalosporin resistance and carbapenem resistance was observed at 30.7% and 28.6% respectively in pus/swab isolates and 50% and 24% respectively among urine isolates. The MIC values of the tested isolates ranged from <0.5 to 16 µg/mL with a MIC50 and MIC90 values of 2 µg/mL and 16 µg/mL respectively. 12.8% of the strains produced AmpC and 18% produced MBL. One isolate was observed to produce both AmpC and MBL.*

Keywords: *Pseudomonas aeruginosa, Meropenem, AmpC, MBL*

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INTRODUCTION

Pseudomonas aeruginosa is an opportunistic bacterium which is ubiquitous in nature and in moist environmental hospital sites such as sinks, toilets, mechanical ventilators, dialysis equipment[1]. *Pseudomonas aeruginosa* are rod shaped, Gram negative bacteria, motile by means of one or more polar flagella[2]. The importance of this species derives from the widespread distribution of its strains in nature, their resistance too many antibiotics and the number of pathogenicity factors that they can possess. *P.aeruginosa* is one of the so called fluorescent species because they can produce pigments that fluoresce under UV light [2]. *P.aeruginosa* produces infections in patients with abnormal host defenses, and is a common nosocomial pathogen and a major cause of drug resistant infection in immune compromised and hospitalized patients[2].

P.aeruginosa exhibits resistance to a variety of antimicrobials and often involves more than one antimicrobial class[3]. Acquiredmetallo-β lactamases (MBL) have recently emerged as one of the most notorious resistance mechanisms owing to their capacity to hydrolyze all β-lactams except aztreonam. The occurrence of an MBL positive isolate in a hospital environment poses not only a therapeutic problem, but also a serious concern for infection control management[3].

MATERIALS AND METHODS

This study was conducted in the Department of Microbiology St. John's Medical College Hospital between September 2009 and January 2011. A total of 250 isolates of *Pseudomonas aeruginosa* obtained from various clinical samples received at the laboratory were included in this study. The information collected was computerized and analyzed by using Statistical Package for Social Science (SPSS 17th version) software.

Inclusion Criteria

1. *Pseudomonas aeruginosa* isolates from in-patient clinical samples received in the microbiology laboratory.

Exclusion Criteria

1. *Pseudomonas aeruginosa* isolated from out - patient clinical samples.
2. *Pseudomonas aeruginosa* isolated from urine with a colony count of $<10^5$cfu/ml.
3. *Pseudomonas aeruginosa* isolated from pus samples without pus cells in the Gram stained direct smear.
4. *Pseudomonas aeruginosa* isolated from a single blood culture.

Sample Processing

Pseudomonas aeruginosa from various clinical samples were identified using standard procedures[4].

Antibiotic Susceptibility Testing

Drug susceptibility tests were done for commonly used antibiotics against *P.aeruginosa* by Kirby Bauer- Disk Diffusion method[5] and the MIC for meropenem was ascertained by micro broth dilution and agar dilution method[5].

Disc Diffusion Method

Kirby Bauer- Disk Diffusion method was performed for Gentamicin 10µg, Amikacin 30µg, Netilmicin 30µg, Ciprofloxacin 5µg, Cefoperazone 75µg, Ceftazidime 30µg, Piperacillin 100µg, Piperacillin-tazobactam 100/10µg & Meropenem 10µg. The isolates were interpretated as susceptible, intermediate & resistant according to CLSI guidelines[6].

Dilution Methods

Minimum inhibitory concentration for meropenem (obtained from HIMEDIA LTD) was determined by micro broth dilution and agar Dilution Method[5]. Meropenem drug dilutions from 0.5µg/ml to 32µg/ml were tested and Theisolates were interpretated as susceptible, intermediate & resistant according to CLSI guidelines[6].

Test to detect Amp C Production

Disk antagonism test (DAT)[7]: The disk antagonism tests were used to detect inducibility of β-lactamase. Disks of inducing agent cefoxitin and cephalosporins (Cefoperazone, Ceftazidime) were placed on the surface of the test bacterial lawn on MHA of the suspectedinducible Amp C β-lactamase producers separated by 15mm. The plates were examined after overnight incubation at 37°C. If blunting of the cephalosporin disks adjacent to the cefoxtin disks occurred, the organisms were considered to produce inducible AmpCβ-lactamase.

Test to detect MBL production:

Imipenem (IMP)-EDTA Combined disk test[8]: Test organisms were inoculated on to plates with Mueller Hinton agar as recommended by the CLSI. Two 10µg imipenem disks were placed on the plates and appropriate amounts of 10µl of EDTA solution was added to one of them to obtain desired concentrations (750µg). The inhibition zones of imipenem and imipenem EDTA disks are compared after 16-18 hrs of incubationin air at 25^0C. If the increase in inhibition zone with the imipenem and EDTA disk was ≥7mm than imipenem disk alone, test was considered as MBL positive.

RESULTS

Specimens and isolates: Of the 250 *P.aeruginosa* included in the study, 238 were Isolated from Pus/swab and 12 from urine samples.

Patient Demographics: The 250 clinical isolates were identified as Pseudomonas species using standard methods. They were obtained from patients, between the ages 6 years to 85years. Majority were isolated from patients in the age range of 15-30 years (29.2%), closely followed by 41-60 years (28.8%) and 31-40 years (29.2%). The male (70.8%) to female (29.2%) ratio observed was 2.4.

The distribution of the isolates from different clinical conditions is described in Table 1. Among *Pseudomonas aeruginosa* isolated from the Pus/swab samples, maximum isolation was from patients presenting with infected wound and/or gangrene (n=99) followed by patients presenting with diabetic foot ulcer (n=22). However no specific diagnosis could be obtained for 32.8% of patients (n=82). 50% of the *P.aeruginosa* isolated from urine was associated with UTI.

Table 1: Distribution of *P.aeruginosa* isolates with respect to clinical conditions in patients.

Diagnosis	Pus/Swab	Urine
No specific diagnosis (n=82)	78(32.8%)	4(33.3%)
Diabetic foot ulcer (n=22)	22(9.2%)	0
Burns (n=20)	20(8.4%)	0
Infected wound, gangrene (n=101)	99(41.6%)	2(16.6%)
Necrotising fasciitis (n=5)	5(2.1%)	0
UTI (n=6)	0	6(50%)
Pemphigus vulgaris (n=6)	6(2.5%)	0
CKD (n=2)	2(0.8%)	0
CSOM (n=5)	5(2.1%)	0
Corneal ulcer (n=1)	1(0.4%)	0
Total (n=250)	238	12

Among the Pus/swab samples most of the *P.aeruginosa* isolates were from Surgical wards

(General surgery, n=87 and Plastic surgery, n=44) followed by burns unit (8.8%). 3.36% of the isolated *Pseudomonas aeruginosa* were from patients admitted in the Intensive care unit. 66.6% of the urine isolates of *P.aeruginosa* were from urology ward (n=8).

The antibiotic susceptibility pattern of the *P.aeruginosa* isolates to routinely tested antibiotics based on Kirby Bauer's disc diffusion method is depicted in Table 2. Aminoglycoside, fluoroquinolone and third generation cephalosporin resistance was observed to be more in the urine isolates compared to the pus isolates, though the number of urine isolates is very less. Third generation cephalosporin resistance and carbapenem resistance was observed at 30.7% and 28.6% respectively in pus/swab isolates and 50% and 24% respectively among urine isolates.

Table 2: Susceptibility test results of 250 *P. aeruginosa* isolates for routinely used antibiotics.

	Pus/Swab(n=238)			Urine(n=12)		
Antibiotic	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Gentamicin	67.9	0	32.7	24	0	76
Amikacin	65.1	0	34.9	50	0	50
Nitilmicin	66.3	0	33.7	50	0	50
Ciprofloxacin	67.2	0	32.8	24	0	76
Cefoperazone	68.9	12.6	29.8	50	0	50
Ceftazidime	69.3	0	30.7	50	0	50
Piperacillin	71.4	0	28.6	76	0	24
Piperacillin-tazobactum	71.4	0	28.6	76	0	24
Meropenem	71.4	0	28.6	76	0	24

MIC for meropenem was determined by both micro broth dilution method and agar dilution method at MIC range of 0.5 to 32 µg/mL. A susceptibility breakpoint of $\leq$ 4 µg/ml was considered as per the CLSI guidelines. Concordant results were observed between both the methods, as well as with meropenem disc diffusion method. The MIC values of the tested isolates ranged from $\leq$0.5 to 16 µg/mLwith a MIC_{50} and MIC_{90} values of 2 µg/mL and 16 µg/mL respectively (Table 3).

Table 3: MIC for Meropenem by Micro broth and Agar dilution

	No of isolates with MIC (µg/mL) of						
	0.5	**1**	**2**	**4**	**8**	**16**	**32**
Pus/Swab(n=238)	18	74	56	19	0	71	0
Urine(n=12)	1	4	4	0	0	3	0
Total (n=250)	19	78	60	19	0	74	0

AmpC and MBL production among all the isolates was performed phenotypically using disc antagonism method and Imipenem-EDTA combined disc test respectively. 12.8% of the strains produced AmpC and 18% produced MBL. One isolate was observed to produce both AmpC and MBL. In 68.8% of the isolates, no AmpC and/or MBL production was detected (Table 4).

Table 4: Amp C and MBL production in *P.aeruginosa* isolates

Drug resistance mechanism	AmpC	MBL	Isolates
	-	-	172(68.8%)
	+	-	31(12.4%)
	-	+	46(18.4%)
	+	+	1(0.65%)
			Total n=250

Fig 1: AmpC Detection by Disk Antagonism Method

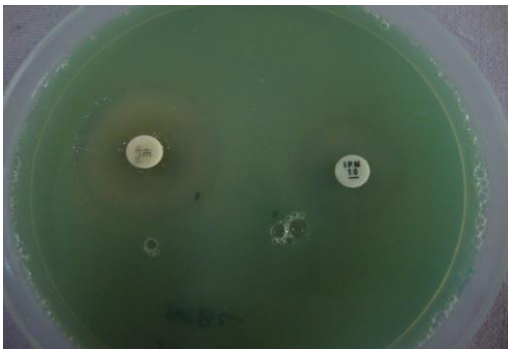

Fig 2: MBL detection by Imipenem (IMP)-EDTA Combined disk test

DISCUSSION

Pseudomonas aeruginosa is intrinsically resistant to many of the routinely used antibiotics in clinical practice. In this study we have studied the antibiotic susceptibility pattern of the 250 clinical isolates of *P.aeruginosa* by disc diffusion. MIC for meropenem was performed for all the isolates by micro broth and agar dilution methods.

The disc diffusion test was done for routinely used antibiotics. In the present study, aminoglycoside, fluoroquinolone and third generation cephalosporin resistance was observed to be more among the urine isolates compared to the pus isolates.

Third generation cephalosporin resistance and carbapenem resistance was observed at 30.7% and 28.6% respectively in pus/ swab isolates and 50% and 24% respectively among urine isolates. The *Pseudomonas aeruginosa* strains isolated from our tertiary care hospital was found to be more susceptible to Amikacin, fluoroquinolone, piperacillin/ tazobactum and meropenem compared to isolates from a multi-center study at Christian Medical College, Vellore[9]. While another study from Chandigarh, North India showed a lower level of resistance to aforementioned antibiotics compared to ours[10].

The Minimum inhibitory concentrations were determined for the 250 *P.aeruginosa* isolates for meropenem using CLSI guidelines by the microbroth and the agar dilution methods at MIC range of 0.5 to 32 µg/mL. A susceptibility breakpoint of $\leq$ 4 µg/ml was considered as per the CLSI guidelines[6]. Concordant results were observed between both the methods (micro broth dilution& agar dilution), as well as with meropenem disc diffusion method. The MIC values of the tested isolates ranged from $\leq$0.5 to 16 µg/mL with a MIC_{50} and MIC_{90} values of 2 µg/mL and 16 µg/mL respectively. Among 28% isolates which were resistant to meropenem, we observed a high level of resistance to meropenem compared to Manoharan et al. study from Vellore which showed a lower MIC_{50} and MIC_{90} values of 0.75 µg/mL and 12 µg/mL respectively[9].

AmpC beta-lactamases are cephalospor-inases, which belong to the molecular class C as classified by Ambler in 1983 and Group I under the classification scheme of Bush *et al*. These are clinically significant as they may confer resistance to a wide variety of b-lactam drugs, including α-methoxy-β-lactams, narrow, expanded and broad-spectrum cephalosporins, aztreonam, a monobactam and most significantly b-lactam plus b-lactamase inhibitor combinations (*viz.*, ampicillin-clavulanic acid, pipericillin-tazobactam, *etc*.)[7].

Among the 250 isolates 12.8% produced AmpC (n=32). A similar finding of 17.3% inducible AmpC production was observed by Arora and Bal using disc antagonism method[7].

Another study from Banaras Hindu University found inducible AmpC production in 7% of the isolates and coproduction of AmpC and MBL was observed in 46.6% of the isolates[11]. In the present study coproduction of AmpC and MBL was observed in only in 1(0.65%) isolate among the 250 *P.aeruginosa.*

The only β-lactam active against combined AmpC and ESBL producers are carbapenems; however, recently resistance to carbapenems has been increasing, which is mostly due to the production of MBL. Metallo β lactamases are β lactamases which hydrolyze all β lactam antibiotics including carbapenem with the exception of aztreonam. In the present study phenotypic detection of MBL was done on isolates using Imipenem- EDTA combined disc. 47(18.8%) isolates of *P.aeruginosa* produced MBL. In a study at JIPMER, Pondicherry among the 32 meropenem resistant isolates of *P.aeruginosa* 16(50%) produced MBL[12]. In another study done at CMC Vellore out of the 28 strains of *P.aeruginosa* 21(75%) produced MBL by Imipenem- EDTA combined disc[13]. Both these studies were from southern India showing a very high degree of MBL production among the *P.aeruginosa* isolates compared to ours. In the present study, number of urinary isolates was small and studies involving larger isolates have to be done, to draw any meaningful conclusions.

CONCLUSIONS

It is of utmost importance to monitor the drug resistance pattern of the *P.aeruginosa* isolates to understand the local epidemiology, which should dictate the empirical therapeutic intervention. Compared to our study third generation cephalosporin resistance and carpapenem resistance was more in few studies and less in few. Many of the *P*.aeruginosa isolates were susceptible to the commonly used antibiotics. Inappropriate use of carbapenems may contribute to increase in resistance among clinical isolates of *Pseudomonas aeruginosa*. There is a need to formulate an antibiotic policy to better utilize antibiotics in treatment of Pseudomonal infections and prevent evolution of drug resistant organisms in the hospital.

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