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Comparative study of Virulence factors among MBL and Non MBL producing Urinary isolates of *Pseudomonas aeruginosa* in a tertiary care hospital

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Abstract

Background: The study was done to detect various virulence factors of urinary isolates of *Pseudomonas aeruginosa* with special reference to MBL producing strains and to find out its significant association with the virulence factors.

Materials & Methods: This study was done in a tertiary care teaching hospital in Pondicherry, which included 50 urinary isolates of *Pseudomonas aeruginosa.* All isolates subjected to routine antibiotic susceptibility testing by Kirby Bauer Disc Diffusion method and screened for Metallo β-lactamase (MBL) by Imipenem-EDTA disc method. Phenotypic detection of virulence factors like phospholipase, hemolysin, gelatinase and DNAse were done for the same.

Results: Out of 50 isolates of *Pseudomonas aeruginosa*, 26% were MBL producers and 74% were non MBL producers by Imipenem-EDTA disc method. Virulence factors like hemolysin, gelatinase, phospholipase and DNAse were shown in 88%, 78%, 76% and 50% respectively. The association with the production of Virulence factors and MBL production were found to be statistically significant only in case of DNAse production.

Conclusion: This study concluded that *P. aeruginosa* is a pathogen able to accumulate various virulence factors which are often accompanied by multidrug resistance and pan-resistance, making the treatment of infections difficult for the clinicians.

Keywords: DNAse, Gelatinase, Hemolysin, Mettalo β-lactamase, Phospholipase

Introduction

Pseudomonas aeruginosa as an ubiquitous pathogen present in the hospital environment, can cause severe nosocomial infections which involves a broad spectrum of infections including respiratory, gastrointestinal, and urinary tracts as well as wound infections, sepsis and others.[1,2] One of the reasons that *P. aeruginosa* is a successful opportunistic pathogenic organism is due to the production of multiple virulence factors, which may be several cell-associated and secreted extracellular virulence factors.[3]

Metallo-β- lactamases are metalloenzymes which hydrolyze Carbapenems, which are β-lactam antibiotics and are clavulanic acid resistant, belongs to Ambler class B. These enzymes require divalent cations of zinc as a co-factors for its acivity and are inhibited by ethylenediamine tetra acetic acid (EDTA).[4,5] The strains which produces MBL enzymes are resistant to broad spectrum β-lactams, aminoglycosides agents and fluoroquinolones group of drugs which are used as major antitherapeutic agents.[5]

MBL producing *P. aeruginosa* was first reported from Japan[6] and since then its incidence and occurrence have been reported from various other parts of the world including India.[7-10]

This study was done to detect various virulence factors of urinary isolates of *Pseudomonas aeruginosa* which special reference to MBL producing strains and to find out its significant association with the virulence factors.

Materials & Methods

A prospective analytical study was done in a tertiary care teaching hospital in Pondicherry, which included 50 urinary isolates of *Pseudomonas aeruginosa*. Identification was done by conventional biochemical test using standard methods.[11] All isolates will be subjected to routine antibiotic susceptibility testing by Kirby Bauer Disc Diffusion method for various antibiotics, namely: Amikacin (30µg), Gentamicin (10µg), Tobramycin (30),Ciprofloxacin (5µg), Nitrofurantoin(3000µg), Ceftazidime (30µg), Imipenem (10µg), Piperacillin/ tazobactam (100µg/10µg) according to CLSI guideline.[12] Isolates will be further screened Metallo β-lactamase (MBL) by Imipenem-EDTA disc method/ Disk potentiation test.[13]

Detection of MBL production by Disk potentiation test: Metallo β-lactamase production by *Pseudomonas aeruginosa* was detected by Disc potentiation test (Imipenem-EDTA disk test). Two imipenem disks of concentration 10 µg were placed on the plate, to one of the disk 10 µl of 50mM zinc sulphate was added after drying, 5µl of 0.5M EDTA solution was then dispensedn (930 µg per disc). The inhibition zones of imipenem and imipenem-EDTA disks were compared after 35˚C incubation for 16 to18 hours. An increase in zone size ≥ 7 mm with imipenem and EDTA disk combination than with imipenem disk alone was considered positive for MBL producer.

Detection of virulence factors: Phenotypic detection of various virulence factors like Phospholipase,

Hemolysin, Gelatinase and DNAse were done by the following methods.

1. **Detection of Hemolysin production:** Sheep blood agar Plates inoculated with the colonies were incubated at 37ºC for 24 h and then checked for zone of haemolysis around them. The results were recorded as α-haemolysis (greenish zones), β-haemolysis (clear zone) or γ-haemolysis (no haemolysis).[14]
2. **Detection of Phospholipase production:** Egg yolk agar was inoculated with colonies from 18-24 hour culture, and incubated at 35^0C for 24-48. Colony which shows a milky white opaque halo around it was considered as positive for phospholipase C production.[15]
3. **Detection of Gelatinase production:** Gelatin production were determined by inoculating the character was tested by bacterial inoculation tubes containing nutrient gelatin medium(Fig. 1). The tubes were incubated for 48 h at 37ºC. Uninoculated tubes were kept as negative control. At the end of incubation period, liquefaction of the culture medium by placing the culture tube at 4°C overnight were observed positive for gelatinase production.[16]

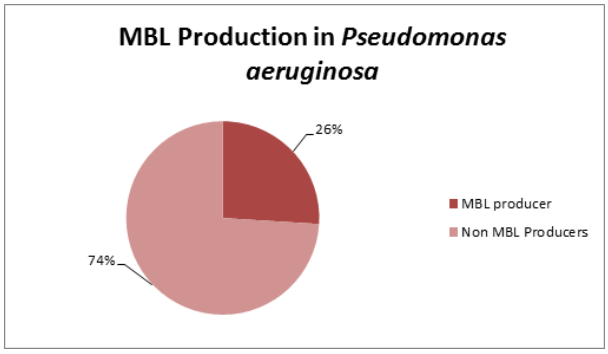

Fig. 1: Percentage of MBL production in urinary isolates of *Pseudomonas aeruginosa*(n=50)

4. **Detection of DNAase production:** Bacterial colonies were spot inoculated in a DNase test agar plates and incubated at 37°C for 24 to 48 hours after which it was flooded 1.0 N HCl. Bacterial colonies that secrete DNase hydrolyze the DNA in the medium resulting in clearance around the bacterial growth.[17]

Statistical Analysis: A percentage was calculated for categorical variables. The difference in production of the virulence factors among MBL and non MBL producing *Pseudomonas aeruginosa* was compared using Chi-square test or Fisher's exact test.

Results

In our study, among 50 urinary isolates of *Pseudomonas aeruginosa*, highest resistance were observed for Gentamicin (58%), Ciprofloxacin (44%), followed by Cetazidime(32%), Amikacin (32%) & Tobramycin(30%). Higher sensitivity were shown for Nitrofurantoin(92%) followed by Imipenem(82%) and Piperacillin – tazobactum(76%) [Table 1]. Those strains showed resistance to Ceftazidime, and Imipenem were subjected to MBL detection test.

Table 1: Antibiotic susceptibility pattern of urinary isolates of *Pseudomonas aeruginosa*(n=50)

Antibiotics	Sensitive(%)	Intermediate(%)	Resistant(%)
Amikacin(30µg)	23(46%)	1(2%)	16(32%)
Gentamicin(10µg)	17(34%)	4(8%)	29(58%)
Tobramycin(30µg)	31(62%)	4(8%)	15(30%)
Ciprofloxacin(5µg)	13(26%)	5(10%)	22(44%)
Nitrofurantoin(300µg)	46(92%)	1(2%)	4(8%)
Ceftazidime(30µg)	32(64%)	2(4%)	16(32%)
Piperacillin–tazobactum((100µg/10µg)	38(76%)	7(14%)	5(10%)
Imipenem(30µg)	41(82%)	0	4(8%)

Metallo β-lactamase (MBL) by Imipenem-EDTA disc method/ Disk potentiation test showed 26% were MBL producers and 74% were non –MBL producers as shown in Fig. 2.

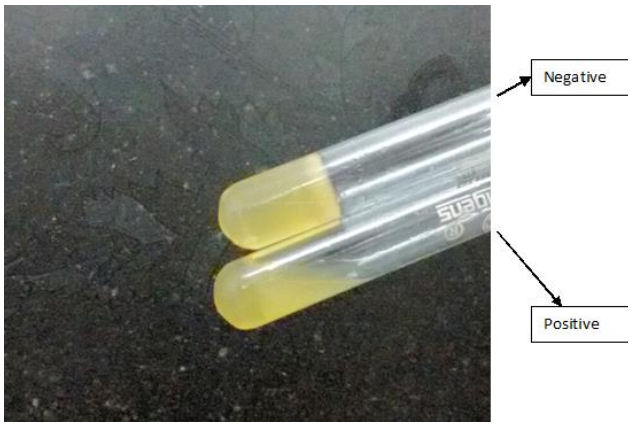

Fig. 2: Gelatinase Production of *Pseudomonas aeruginosa*

Table 2: Distribution of Virulence factors in urinary isolates of *Pseudomonas aeruginosa*(n=50)

Virulence factors	Positive (%)	Negative (%)
Hemolysin	44(88%)	6(12%)
Gelatinase	39(78%)	11(22%)
DNAse	25(50%)	25(50%)
Phospholipase	38(76%)	12(24%)

Virulence factors production by phenotypic methods in the current study showed, 88% of the isolates demonstrated hemolytic activity, 78% of the isolates produced gelatinase, 50% of the isolates produced DNAse and 76% were positive for. Phospholipase.

Out of 13(26%) MBL producers, 11(84.6%) were positive for hemolysin production, 10 (76.9%) were positive for Gelatinase and DNAse production, 12 (92.3%) showed positivity for phospholipase production. Out of 37(74%) MBL producers, 33(89.1%) were positive for hemolysin production and 29(78.4) were positive for Gelatinase production. 15(40%) and 27(72.9%) showed positivity for DNAse and Phospholipase production. (Table 3)

Table 3: Virulence factors production in MBL and Non MBL producing *Pseudomonas aeruginosa* (n=50)

Virulence factors	MBL Producers(n=13)		Non MBL Producers(n=37)		χ^2
	Positive	Negative	Positive	Negative	p value
Hemolysin	11(84.6%)	2(15.4%)	33(89.1%)	4(10.8%)	p = 0.66
Gelatinase	10(76.9%)	3(23.1%)	29(78.4%)	8(21.6%)	p = 0.91
DNAse	10(76.9%)	3(23.1%)	15(40.5%)	22(59.5%)	p = 0.02
Phospholipase	12(92.3%)	1(7.7%)	27(72.9%)	10(27.1%)	p = 0.14

Discussion

Among the most common infectious diseases, urinary tract infections (UTIs) are more frequently encountered diseases in developing countries with an estimated annual global incidence of about 250 million.[18,19]

UTIs are classified as uncomplicated and complicated urinary tract infections. Uncomplicated UTIs means that occur in a normal genitourinary tract with no prior instrumentation. Complicated infections are common in genitourinary tracts with structural or functional abnormalities, as well as following instrumentation such as indwelling urethral catheters.[20,21] *Enterococcus faecalis* and highly resistant Gram-negative rods including *Pseudomonas spp*. are more commonly encountered organism in complicated UTIs.

The incidence of antibiotic resistance pattern among uropathogens has been increasing worldwide. The most difficult situation that is accomplished during treatment of such infection is that, bacteria resistant to single antibiotic are also likely to develop resistant to other antibiotics, thereby reducing the chances of second empirical treatment.[22]

In current study, production of MBL by Disk potentiation test showed 26% in uropathogenic *Pseudomonas aeruginosa.* MBL productions in clinical isolates were reported to be around 19.15% and 15.38% in various other studies.[23,24] Nitrofurantoin (92%), Imipenem (82%) and Piperacillin –tazobactum (76%) and has got the better antipseudomonal activity in this study.

Pathogenesis of *P. aeruginosa* is multifactorial, which involves various virulence factors that include structural components, toxins, and enzymes[25]. Some of the various virulence factors were selected in our study based on the importance of their role in disease production and to establish the infection of *P. aeruginosa.* Extracellular enzymes alter microbial behavior by promoting invasiveness, serum resistance, and evasion of host immune mechanisms.[26]

Hemolysin production is an important virulence property of urinary tract infections. Hemolysins inflict direct cytotoxic effects on renal epithelium resulting in scaring. Also, hemolysins destroy various host tissues and cells including RBCs, leucocytes, epithelial and endothelial cells.[27]

Extracellular protease plays an important role in the cell survival and cell-cell communication.[28] The ability of proteases as a virulence factors is partly determined by exo-products such as alkaline protease and elastase. These enzymes brings about damage to the tissues, by degrading elastin, collagen, proteoglycans and also bring about proteins degradations that function in host defense mechanism in vivo.[29]

Another virulence factor, Phospholipase C produced by *P. aeruginosa* which catalyzes the hydrolysis of phosphatidylcholine which constitute the important component of surfactant of the lung. It destroys the pulmonary surfactant and plays an important role in establishing infections in cystic fibrosis patients.[30]

In present study, 88% of the isolates showed hemolytic activity. Thirty nine (78%) of the isolates produced gelatinase. 76% and 50% of the isolates were positive for Phospholipase and DNAse. Similar to our study Mittal et al [31] reported high level of haemolysin production in uroisolates. Another study[32] done on virulence factors of *Pseudomonas aeruginosa* showed 93.3% hemolysin,80% gelatinase comparable to our study. Finlayson et al [33] reported 75.8% positivity for DNAse in pigmented Pseudomonas, higher than our study. Several other studies have reported higher percentage of Phospholipase production comparable to our study[34,35]. Study by Mohammad *et al* have shown 87.5% and 81.25% positivity for phospholipase C and gelatinase.[36]

The association with the production of virulence factors and MBL production were investigated in this study. We found that there were no significant difference in either of the two cases for hemolysin, gelatinase and Phospholipase except for DNAse production which was statistically significant ($p < 0.05$). In the current study, there is not much significant association between virulence factors expression and metallo-beta -lactamase production in *Pseudomonas aeruginosa*, in concordance to study conducted by Aoki et al.[37]

The association between resistance and virulence may either have a positive effect (increased resistance plus increased virulence) or negative effect (i.e., increased resistance correlated with diminished virulence). The opposite may also occur, so that increased virulence may also lead to decreased resistance. In such situation, compensatory mutations may arise to equilibrate the balance and finally proceed together to confer the bacteria with a selective advantage. In a normal clinical situation all the virulence factors in conjunction may decide the probable outcome of an infection and hence all the factors should be considered.[38]

Our study concluded that as in other infections, uropathogenicity of *P. aeruginosa* was also multifactorial. *P. aeruginosa*, as an opportunistic nosocomial pathogen accumulates several virulence factor which are often accompanied by multidrug resistance and pan-resistance, making the treatment of infections caused by this bacterium difficult.

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