Original Research Article

Antimicrobial Susceptibility Pattern of Extended Spectrum Beta-lactamase (ESBL) and Non ESBL Producing *Pseudomonas aeruginosa*, Isolated from Pus Samples from a Tertiary Care Hospital in Bihar

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A B S T R A C T

Pseudomonas aeruginosa is a gram negative bacillus that colonizes in moist environment of the hospital settings with occurrence of antimicrobial resistance. Increasing resistance to beta-lactam drugs, especially the 3rd-generation cephalosporins, in *Pseudomonas aeruginosa* is predominantly due to the production of extended spectrum beta-lactamases (ESBLs). This was a laboratory based observational study carried out over a period of 7 months from April 2019 to December 2019. A total of 207 pus samples from different patient sources of the hospital were collected and processed in the microbiology laboratory. Among these samples, positive clinical isolates of *Pseudomonas aeruginosa* were recovered. Next, ESBL production in all these isolates was detected by using double disc synergy test (DDST). *P. aeruginosa* were also tested against different antibiotics for determination of the antibiogram of these isolates. Out of 207 pus samples, 45 (21.7%) clinical isolates of *P. aeruginosa* were detected. Out of these, 26.6 % of the *P. aeruginosa* isolates (n=12) were confirmed to be ESBL producers by the DDST method and rest 73.3%were non-ESBL producer (n=33). Our results showed that the ESBL strains were multi-drug resistant but colistin sensitive. Our results show that one-fourth of *P. Aeruginosa* clinical isolates express ESBLs; and were resistant to commonly used antibiotics inclusive of some beta-lactams and non-beta-lactam antibiotics. There is therefore need for proper monitoring and detection of the ESBL strain for guiding suitable therapy and to contain their nefarious effect on antibiotics.

Introduction

Pseudomonas aeruginosa is an important opportunistic nosocomial pathogen that causes a variety of infections such as otitis, mastitis, hemorrhagic pneumonia, urinary tract Infections, wound and burn infections in humans[1, 2]. Globally, the urgent problem inpublic health is the increasing resistance to commonly used antibiotics in potentially pathogenic *Pseudomonas aeruginosa*. ESBL production in *P. aeruginosa* has been reported previously; and show remarkable resistance to different classes of antibiotics, including penicillins and cephalosporins[3,4]. ESBLs are newer beta-lactamases that confer resistance to some of the latest beta-lactam antibiotics, especially the cephalosporins[2,5]. ESBLs are

encoded by genes located on bacterial plasmids which also carry genes responsible for resistance to many other antimicrobials such as aminoglycosides, tetracyclines and sulphonamides[6]. They are derived from the earlier beta-lactamases such as the TEM enzymes, SHV and OXA-beta-lactamases with a narrower-spectrum of activity in terms of the antibiotics they degrade; and ESBLs are largely responsible for the multidrug resistance amongst Gram negative bacteria[1,7,8].

All ESBL-type enzymes are categorized into two structural ambler classes, viz. A and D. In *P. aeruginosa* strains, the ESBL enzymes of both these classes are observed, primarily β-lactamases from the PER, GES[9], VEB[10,11], BEL[12,13], and PME[14] family (belonging to class A) and from the OXA family (class D), named the extended-spectrum class D β-lactamases (ES-OXAs) [15].

Additionally, in a few *P. aeruginosa* isolates, the presence of ESBLs similar to the *Enterobacteriaceae* family, such as TEM, SHV[16] and CTX-M-type, was described.

Resistance to antimicrobial agents is an increasing clinical problem and is a recognized global public health threat. *P. aeruginosa* shows a particular propensity for the development of resistance. The emergence of resistance in *P. aeruginosa* also limits future therapeutic choices and is associated with increased rates of mortality and morbidity and higher costs [17,18].

Failure to detect pathogenic ESBL-bacteria routinely in the clinical microbiology laboratory leads to treatment failure (especially with the cephalosporins and beta-lactams), prolonged illness, prolonged hospitalization, inappropriate antibiotic prescription etc. So it is important to identify ESBL-producing *P. aeruginosa* isolates and their antibiogram for lowering the rate of treatment failure in hospital settings. To the best of our knowledge, there is no study from Bihar state on ESBL-producing clinical isolates of *P.aeruginosa.* Therefore, our current study was taken up with the aim of identifying ESBL-producing clinical isolates of *P.aeruginosa* in pus samples acquired from OPD and IPD of a tertiary care hospital in Bihar and study their resistance pattern with different antibiotics.

Materials and Methods

This was a laboratory based observational study. *Pseudomonas aeruginosa* isolates were recovered from the pus sample collected at Microbiology department, AIIMS Patna during April 2019 to December 2019 (7 months). All study protocols were approved by the Institutional Review Boards and Ethical Committee.

Pus samples

Pus samples were collected from different departments (*i.e.* from Trauma & Emergency, Surgery, Orthopaedics, Gastroenterology, Pulmonary Medicine, Paediatrics and Medicine department) of AIIMS Patna during the study period.

Confirmation tests for *P. aeruginosa*

The collected pus samples were processed for identification and detection of *P. aeruginosa* by direct microscopy, gram's staining, inoculation in Blood Agar (BA), MacConkey Agar (MA) and Nutrient agar (NA) media and incubated at 37^0C for 24-48 hours under aerobic conditions. After growth of colonies, the samples were subjected to different biochemical tests for confirmation of *P. aeruginosa,* viz. catalase test, oxidase test, indole test, semisolid agar test, citrate test and TSI test.

Double disc synergy test (DDST)

Firstly, we aimed to investigate and identify ESBL producing clinical strains of *P.aeruginosa.* ESBL production in all these isolates are detected by using double disc synergy test as described by Jarlier[19]. Synergy was determined between a disc of amoxyclav 30μg (20μg amoxicillin & 10μg clavulanic acid) and Ceftazidime (30μg) disc, placed 15 mm apart on a lawn culture of the isolate, on Mueller Hinton agar (MHA). The plates were incubated at 37^0C for 18-24 hrs. The test organism was considered to produce ESBL phenotypically[20] as the zone size around the antibiotic disc increased towards the amoxyclav disc (Figure 1). *Escherichia coli* ATCC 25922 was used as a negative control for the ESBL and *P. aeruginosa* ATCC 27853 were used as a control strain for a positive ESBL[21].

Antimicrobial susceptibility testing (AST)

P. aeruginosa are intrinsic resistant[22] to Ampicillin, Amoxicillin, Ampicillin-subactam, Amoxicillin-clavunate, Cefotaxim, Ceftriaxone, Ertapenam, Tetracycline, Tigecycline, Trimethoprim, Trimethoprim-Sulfamethoxazole, Chloramphenicol. So, *P. aeruginosa* were tested against different susceptible antibiotics and commonly used discs was Amikacin (30 μg), Aztreonam(30 μg), Ceftazidime (30 μg), Ceftazidime-clavulanate (30/10 μg), Cefepime(30 μg), Cefoperazone-sulbactam (100/10 μg), Ciprofloxacin (5 μg), Colistin (10 μg), Cotrimoxazole (Trimethoprim-suifamethoxazole 1.25/23.75 μg), Gentamicin (10 μg), Imipenem (10 μg), Levofloxacin (5 μg), Meropenam(10 μg) and Piperacillin/Tazobactam (100/10 μg). Zone of inhibition was recorded as "Sensitive" or "Resistant" according to the Clinical and Laboratory Standards Institute (CLSI) guideline-2019[22] (Figure 2 and 3).

Furthermore, we have performed colistin MIC test by E-strip (Ezy[TM] CL, HIMEDIA) and macro-dilution method. For macro-dilution method, we made colistin drug of 1mg/ml concentration (by mixing 80ml distilled water in a 80mg dry powder colistin vial) and then made a serial dilution of drug of 8mcg/ml, 4mcg/ml, 2mcg/ml, 1mcg/ml and 0.5mcg/ml(by adding 2ml of 0.5 McFarland *P. aeruginosa* suspension to already taken 16 mcl colistin drug, thus achieving the final concentration of 8mcg/ml. Then, we again made serial double dilutions to reach up to 0.5mcg/ml concentration (Figure 4). MIC was the minimum drug concentration where no bacterial growth was found and designated as resistant if MIC is > or = 4 mcg/ml[22].

Results and Discussion

The total no. of 207 pus samples were collected from different departments of AIIMS, Patna. The distribution of samples per department was as follows: viz. from Trauma & Emergency 35.3%, Surgery 29.5%, Orthopaedics15.9%, Gastroenterology 9.2%, Pulmonary Medicine 4.3%, Paediatrics 3.4% and from Medicine 2.4%. We have processed all the 207 pus samples and the 45 (i.e. 21.7%) confirmed *P. aeruginosa* clinical isolates were further processed for antibiogram information. *P. aeruginosa* isolates that were obtained as a pure and predominant growth from the clinical specimens were only considered for the present study. *P. aeruginosa* was confirmed by examining the gram's staining morphology (presence of gram negative, non sporing bacilli), colony characteristics on MA (non-lactose fermenting colonies), BA (mostly hemolytic colonies), NA (colonies with diffusible pigment of bluish-green or greenish-yellow in colour, and emission of a characteristic fruity odour) and with the results of biochemical tests which were catalase test - positive, oxidase test - positive,

absence of indole ring, positive motility on semisolid agar, utilization of citrate and TSI-k/k reaction without H$_2$S production.

Out of 45 *P. aeruginosa* isolates, 12 (i.e.26.6%) were found to be positive for ESBL production according to the DDST method and rest 33 were non-ESBL producer (i.e. 73.3%) (Figure 5). Non ESBL *P. aeruginosa* were mostly sensitive to commonly used drugs (Ciprofloxacin, Gentamicin, Amikacin, Aztreonam, Cefepime, Ceftazidime, Imipenem, Meropenam, Piperacillin/Tazobactam, Cefoperazone- sulbactam) but most were resistant to Cotrimoxazole but still non of

them resistant to colistin (Figure 6 & summarized in Table 1, 2). The most of ESBL *P. aeruginosa* were multi-drug resistant (drug resistance to more than or equal to three different classes of drug) and maximum sensitivity (100%) was seen with Colistin followed by Piperacillin/ Tazobactam (66.66%), Ceftazidime/ clavulanate (66.66%), Imipenem (41.66%) and Cefoperazone-sulbactam (41.66%). Monotherapy by Ceftazidime shows 68.66% sensitivity but when used in combination with clavulanate-Ceftazidime/ clavulanate (30/10 μg) the overall sensitivity increased to 82.22% (Figure 6 & Table 1, 2).

Table.1 Sensitivity and Resistant pattern of *P. aeruginosa* isolates (both ESBL & NON-ESBL) with different antibiotics

Antibiotics	Number of Sensitive *Pseudomonas* Strains(Percentage) (n = 45)	Number of Resistant *Pseudomonas* Strains (Percentage) (n = 45)
Ciprofloxacin	29 (64.44%)	16 (35.55%)
Cotrimoxazole	16 (35.55%)	29 (64.44%)
Gentamycin	21 (46.66%)	14 (31.11%)
Amikacin	33 (73.33%)	12 (26.66%)
Aztreonam	33 (73.33%)	9 (20%)
Cefepime	30 (66.66%)	15 (33.33%)
Ceftazidime	31 (68.66%)	14 (31.11%)
Imipenem	30 (66.66%)	14 (31.11%)
Meropenam	29 (64.44%)	16 (35.55%)
Piperacillin-Tazobactam	37 (82.22%)	5 (11.11%)
Ceftazidime-clavulanate	37 (82.22%)	83 (17.77%)
Cefoperazone-sulbactam	35 (71.11%)	9 (20%)
Colistin	45 (100%)	0 (0%)

Table.2 Sensitivity and Resistant pattern of ESBL & NON-ESBL *P. aeruginosa* isolates with different antibiotics

Antibiotics	Number of strains(Percentage)						P Value
	ESBL *Pseudomonas* (n = 12)			NON-ESBL *Pseudomonas* (n = 33)			
	S	I	R	S	I	R	
Ciprofloxacin	5(41.66%)	-	7 (58.33%)	24(72.72%)	-	9 (27.27%)	0.054244
Cotrimoxazole	3(25%)	-	9 (75%)	13(39.39%)	-	20 (60.60%)	0.372382
Gentamycin	4(33.33%)	-	8 (66.66%)	27(81.81%)	-	6 (18.18%)	0.001891
Amikacin	5(41.66%)	-	7 58.33%)	28(84.84%)	-	5 (15.15%)	0.003771
Aztreonam	7(58.33%)	-	5 (41.66%)	26(78.78%)	3	4 (12.12%)	0.170022
Cefepime	4(33.33%)	-	8 (66.66%)	26(78.78%)	-	7 (21.21%)	0.004231
Ceftazidime	5(41.66%)	-	7 (58.33%)	26(78.78%)	-	7 (21.21%)	0.017376
Imipenem	4(33.33%)	1	7 (58.33%)	26(78.78%)	-	7 (21.21%)	0.004231
Meropenam	5(41.66%)	-	7 (58.33%)	24(72.72%)	-	9 (27.27%)	0.054244
Piperacillin-Tazobactam	7(58.33%)	1	4 (33.33%)	30(90.90%)	2	1 (3.03%)	0.011486
Ceftazidime-clavulanate	8(66.66%)	-	4 (33.33%)	29 (87.88%)	-	4 (12.12%)	0.099793
Cefoperazone-sulbactam	5(41.66%)	-	7 (58.33%)	30(90.90%)	1	2 (6.06%)	0.000442
Colistin	12(100%)	-	0 (0%)	33(100%)	-	0 (0%)	……

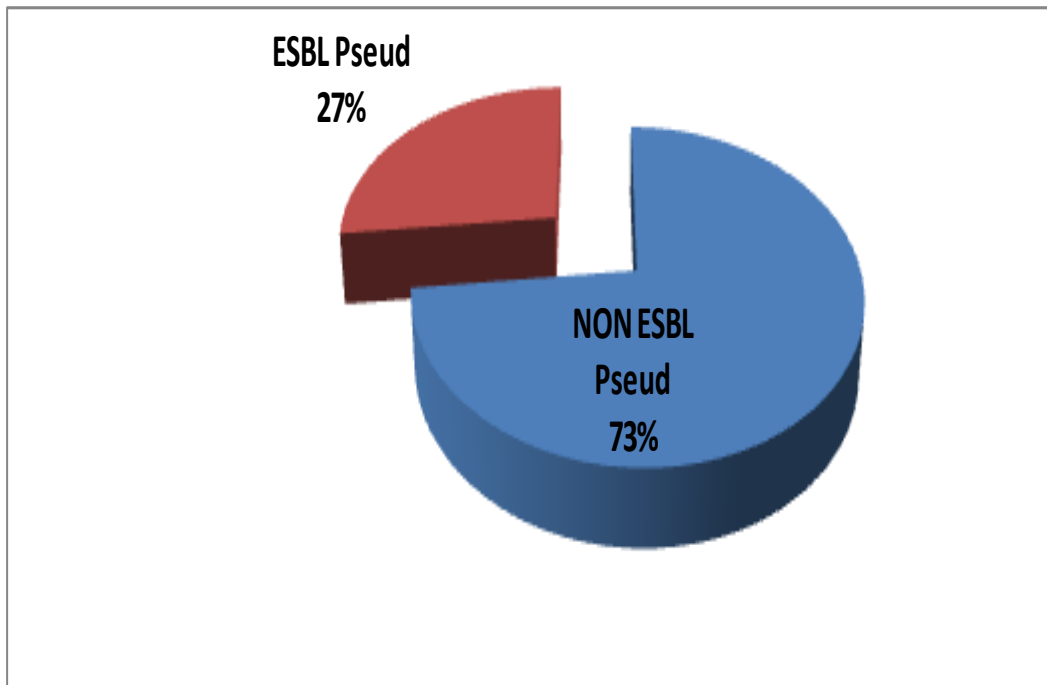

Fig 1: Double disc synergy test (DDST)
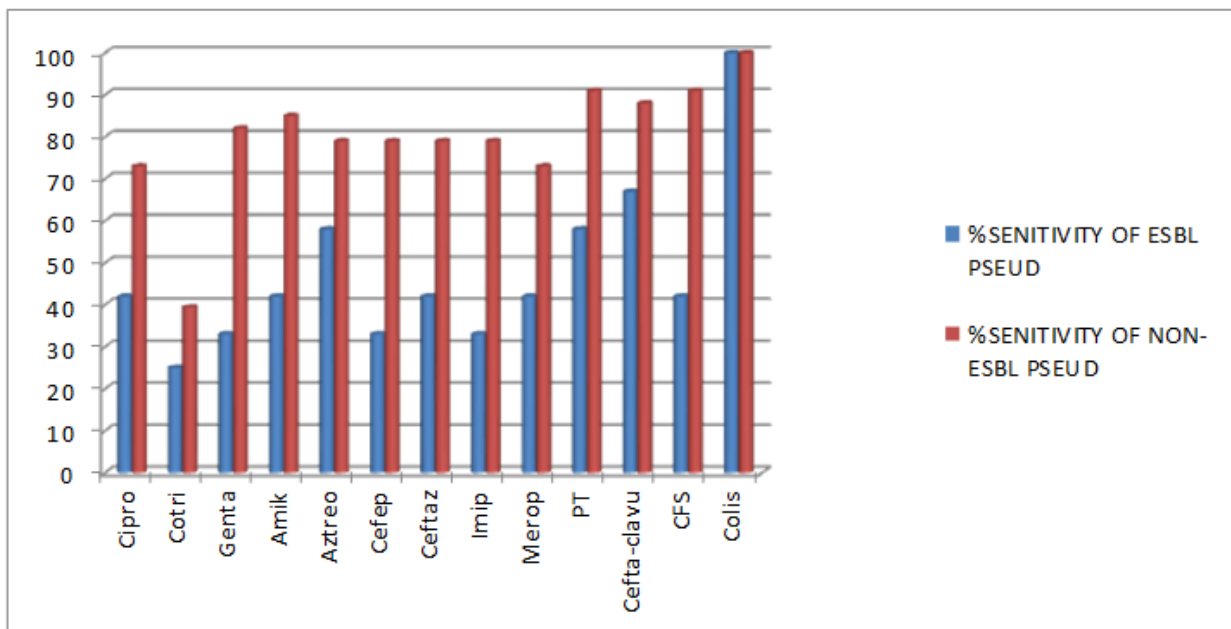

Fig 2: AST

Fig 3: AST 2

Fig 4: MIC Colistin by E-strip

Figure.5 Distribution of *P. aeruginosa* isolates according to ESBL production

Figure.6 Comparison of Sensitivity pattern between ESBL & NON-ESBL *P. aeruginosa* isolates with different antibiotics

The study results show 27% (12 out of 45) were ESBL producing strains among all *P. aeruginosa* isolates. The studies conducted by others depicted very lower rates of ESBL production in *P. aeruginosa* 3.7%, 4.2% and 7.7%, respectively by Lim KT *et al.,* in 2009[23], Woodford *et al.,* in 2008 [24] and Jacobson KL *et.al* in 1995[25]. A study conducted by Agrawal *et al.,*[26] in Haryana, India in 2018 found 20.27% ESBL producing strains. In addition, the results of others, showed more higher rates (20.3% to 46%) of ESBLs in *P. aeruginosa* isolated in their investigations of Mirsalehian *et al*[27], Ullah F *et.al*[28] and Shah Cheraghi *et al*[29]. In general, there is a considerable geographic difference in the prevalence of ESBLs in different countries.

This study reported the highest resistance rate to Cotrimoxazole, Gentamycin, Ciprofloxacin. However, resistance value of gentamicin was much lower than the values reported in other studies, viz. Fadeyi *et al.,*[30]; Ogundipeju *et al.,* [31]. In the present study,

Ciprofloxacin exhibited high susceptibility pattern (64.44%), while Gul *et al.*[32] reported that more than 90% of isolates were sensitive to ciprofloxacin. This might be a characteristic of the clinical strains of *P. aeruginosa* prevailing in Bihar state. Resistance to piperacillin-tazobactam as reported by Javiya *et al.,*[33] 73.21% is much higher than that reported in our study *i.e.* 9 (11.1%).

Reports present the resistance to carbapenems to be around 35%, results from reduced levels of drug accumulation or increased expression of pump efflux or production of ESBL[34,35,36]. Interestingly, carbapenems are still considered to be the treatment of choice against ESBL associated infections. Cephalosporins, especially the third-generation ceftadizime, are known as anti-pseudomonal drugs has demonstrated high susceptibility pattern about 68.66%. In contrast, cephalosporins tested in a study conducted in Ibadan, southwestern Nigeria[37], showed that 90% of the isolates were sensitive[38] but study conducted in

Malaysia[23] show 40% sensitivity. In present study 100% of isolates were found to be susceptible to colistin among all ESBL and Non ESBL strains. This colistin sensitivity found in our current study is comparable to results of Das *et al* [39]. That also showed 100% sensitivity.

In conclusion, *Pseudomonas aeruginosa* is one of the most important nosocomial pathogens in health care system. The presence of ESBL in *P. aeruginosa* tend to increase, resulting in ineffectiveness of many antimicrobial agents. The emergence of resistance in *P. aeruginosa* strains that had been consistently susceptible to standard antimicrobial therapy is of growing clinical concern, as is the alarming trend to multidrug resistance. With this in mind, the early detection of ESBLs, the judicious use of appropriate antibiotics and the implementation of infection control strategies are major concerns to avoid the spread of this threat in the hospital. In this study, *P. aeruginosa* showed resistance to various antimicrobial agents but was susceptible to colistin. This finding may help in deciding upon the definitive antimicrobial therapy as single drug or in combination therapy. Further studies are needed on better use of the existing antibiotics along with infection controls measures, antimicrobial stewardship programs and should fully focus on search for new antibiotics.

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