

Original article:

Prevalence of Extended spectrum beta lactamase (ESBL) producing *Pseudomonas aeruginosa* strains recovered from human patients in Himachal Pradesh

Bharti and P.C.Sharma*

Dept. of Microbiology, School of Biotechnology, Shoolini University of Biotechnology and Management Sciences, Bajhol, Solan (H.P.), India.

*Corresponding author's E-mail: dr.sharmapc@gmail.com

Abstract

Introduction: Extended spectrum beta lactamases are on the rise in nosocomial settings across the globe. Hence, it is necessary to know the prevalence of ESBL so as to formulate a policy of empirical therapy in high risk units. Present study aims at assessing the prevalence of ESBL producing *P. aeruginosa* in Shimla region of Himachal Pradesh.

Methods: A total of 200 isolates of *P. aeruginosa* were obtained from Indira Gandhi Medical College, Shimla, Himachal Pradesh. Of these, 180 isolates were confirmed as *P. aeruginosa* on the basis of morphology, Gram's staining and biochemical tests. For preliminary screening of ESBL producing *P. aeruginosa*, the susceptibility of the isolates was determined against third generation cephalosporins following the method of Kirby Bauer (1966)¹. These antibiotics are most commonly used to treat *P. aeruginosa* infections. ESBL positive isolates were confirmed by Double disc diffusion synergy test (DDST) and E test.

Observations and Results: 95% (171/180) isolates were ESBL producers in the preliminary screening, 56 /171 (32.75%) isolates were confirmed as ESBL producers by DDST method. But only 26.67% (8 out of 30 DDST positive isolates) were ESBL producers by E test also. The DDST thus, was more sensitive test as compared to E test for detecting ESBL producers. MIC values as determined by E test were recorded as >16µg/ml for mix and 0.442µg/ml for mix⁺ respectively.

Conclusion: The present study reveals moderate prevalence of ESBL producing *P. aeruginosa* in the state of Himachal Pradesh which might have implications in treating infections due to this organism.

Keywords- Extended spectrum β – lactamases, Double disc diffusion synergy test, E – test

Introduction

The need for antimicrobial susceptibility testing is increasing day by day with rising emergence of multidrug resistant microorganisms especially *Pseudomonas aeruginosa*². This organism is an aerobic gram negative rod, usually 1.5-5µm in length and 0.5 to 1.0 µm in width and is motile due to the presence of flagella³. According to the Centres for Disease Control and Prevention (CDC), *P. aeruginosa* is the fourth most commonly isolated nosocomial pathogen accounting for 10.1 percent of all hospital-acquired infections⁴. This

organism is an opportunistic pathogen with innate resistance to many antibiotics and disinfectants. Infections due to this organism are seldom encountered in healthy adults; but this bacterium has been implicated in hospitalized patients of cancers, cystic fibrosis and burns etc. during last two decades.

Pseudomonads are more versatile than members of *Enterobacteriaceae* in acquiring drug resistance by various mechanisms. The production of extended-

spectrum β - lactamases (ESBLs) confers resistance at various levels to expanded spectrum cephalosporins. ESBLs are encoded by different genes located either on chromosome or plasmids. These enzymes mediate resistance to extended-spectrum cephalosporins (ESCs), such as cefotaxime (CTX), ceftriaxone, ceftazidime (CAZ) and the monobactam aztreonam (ATM). They belong to the Ambler class A and D beta-lactamases. ESBLs are most commonly found in *Klebsiella pneumoniae* and *Escherichia coli* and have been detected in *P. aeruginosa* recently at low frequency⁵. Early detection of occurrence and types of multiple β -lactamase enzymes is crucial for the implementation of proper antibiotic therapy and infection control policy⁶.

Various tests have been developed for detecting β -lactamases. In the present study, occurrence of different β - lactamase producing *P. aeruginosa* isolates recovered from pus, blood, urine, sputum specimens of human patients suffering from various disease conditions at Indira Gandhi Medical College, Shimla, Himachal Pradesh during one year period has been investigated. The susceptibility and resistance patterns of these isolates against different cephalosporins have been determined, so as to select most resistant phenotypes for further molecular characterization.

Materials and Methods

Confirmation of isolates of *P. aeruginosa*

A total of 200 isolates of *P. aeruginosa* recovered from human patients at Indira Gandhi Medical College, Shimla, Himachal Pradesh were collected during the period December, 2012 to December, 2013.

Of these, 180 isolates were confirmed as *P. aeruginosa* on the basis of morphology, microscopic examination of Gram's stained preparations and standard biochemical tests.

The isolates were maintained on slants of

Pseudomonas isolation agar (Himedia Mumbai) and preserved in 80% glycerol at -80°C . Subculturing was done on regular basis in order to maintain fresh cultures for the experiment. *P. aeruginosa* strain ATCC 27853 was used as quality control strain.

Screening of isolates for ESBL production

Screening of all the isolates of *P. aeruginosa* for ESBLs production was done by *in vitro* antibiotic culture sensitivity assay using the following discs of cephalosporins: ceftazidime (30 μg), cefotaxime (30 μg), ceftriaxone (30 μg), cefuroxime (30 μg), aztreonam (30 μg) and cefpodoxime (10 μg) (Himedia, Mumbai, India). The disc diffusion method of Kirby Bauer (1966)¹ was followed. The diameters of zones of inhibition were measured after 24h of incubation at 37°C . The results were interpreted according to recommended ESBL screening criteria as specified in CLSI (M100-S22)⁷.

Confirmatory methods

Double disc diffusion synergy test (DDST)

ESBL production was confirmed by double disc synergy test, a phenotypic test⁸. Synergy was determined between a disc of amoxycylav (20 μg amoxicillin and 10 μg clavulanic acid) and a 30 μg disc of cefotaxime (3rd generation cephalosporin). Plates were incubated at 37°C for 24 h and the diameters of zones of inhibition were recorded. The isolates that exhibited a distinct shape/size with potentiation towards amoxycylav disc were considered potential ESBL producers (CLSI, 2013)⁷.

ESBL E – test

E - test ESBL strips are double ended strips with antibiotic and antibiotic/inhibitor gradients. The upper half has mixture of ceftazidime, cefotaxime and cefepime plus clavulanic acid and tozobactam (mix⁺) with highest concentration tapering downwards, whereas lower half is similarly coated

with mixture of ceftazidime, cefotaxime and cefepime (mix) in a concentration gradient in reverse direction. These strips yield the MIC as well as the MIC ratio, which determines the presence of an ESBL.

The results were interpreted as follows as specified by the manufacturers: if the value of the ratio between MIC for mix and MIC for mix⁺ was equal to 8 or more, the isolate was considered ESBL producer.

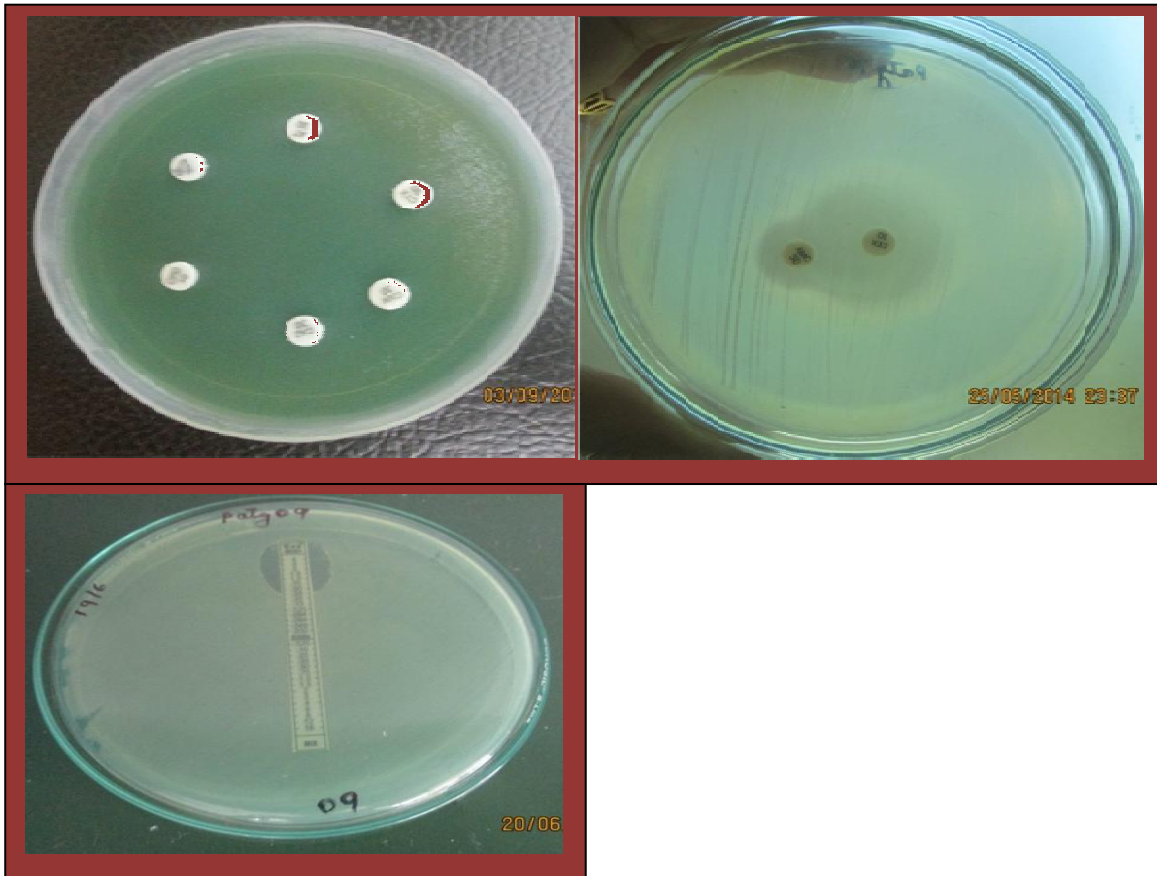


Figure 1. A Isolate no. Paig - 11 showing complete resistance towards all the third generation cephalosporins;
B Isolate no. Paig - 76 showing increase of zone of inhibition towards agumentin disc in Double disc diffusion test;
C Ellipse formation by isolate no. Paig -09 in E - Test.

Statistical analysis

Chi square (χ^2) test was applied to find the significance of difference between the susceptibility of the ESBL producing strains to third generation cephalosporins using SPSS (Statistical Package for the Social Sciences) software, version 20. p value \leq 0.05 was considered significant.

Results

Confirmation of *P. aeruginosa* isolates

A total of 180 isolates were confirmed as *P. aeruginosa* isolates and screened for ESBL production.

Phenotypic detection of ESBL producers and their confirmation

The results of *in vitro* culture sensitivity to different cephalosporins are presented in (Table 1). Majority of isolates (89.44%) were resistant to cefpodoxime followed by cefuroxime (78.33%), ceftriaxone (61.66%) and cefotaxime (41.66%). The proportion of isolates resistant to aztreonam and ceftazidime was however lower and ranged from 31.11% to 32.77% respectively (Table 1, Fig. 2). A total of 171 (95%) isolates of *P. aeruginosa* were resistant to one or more third generation cephalosporins used in the screening test. Following DDST, ESBL as a mechanism of resistance was confirmed in 56/171 (32.75%) isolate. Majority isolates, 67.25% were non ESBL producers. The potentiation of augmentin disc towards third generation cephalosporin is presented through Figure 1B.

Comparison of DDST and E – test

In E test, only 30 DDST positive isolates were tested. Only 8 / 30 (26.67%) isolates were ESBL positive by this test (Table 2, Fig. 1C). The mean MIC values in the E test were recorded as $>16\mu\text{g/ml}$ for mix and $0.442\mu\text{g/ml}$ for mix⁺ (Table 3).

Discussion

The prevalence of extended spectrum beta lactamases is on increase all around the world. ESBL producing strains are usually found in those areas of hospitals such as ICUs where use of antibiotics is frequent and the patient's condition is critical. In India, prevalence rate of ESBLs ranging from 28% to 84% has been reported from various parts of the country: Bakshi *et al.* 2013, reported high prevalence of (50%) ESBL among *P. aeruginosa* at Patiala (Punjab) ⁽⁹⁾. 22.22% ESBL producing *P. aeruginosa* have been reported from Karnataka ⁽¹⁰⁾ and 40% from Coimbatore ⁽¹¹⁾, 20.27% from Haryana ⁽¹²⁾. However higher incidence of ESBL among *P.aeruginosa* (42.31%) has been reported by researchers at AIIMS, New Delhi ⁽¹³⁾ and even higher rate to the tune of 64% has been reported by Mathur *et al* (2002) from South India ⁽¹⁴⁾. In the present study, we observed 32.75% ESBL positive *P. aeruginosa* isolates.

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conducted the study during a period of one year, regular studies over a longer period of time on larger number of isolates might present a better picture. Jacobson *et al* (1995)¹⁵ depicted a very low rate (7.7%) of ESBL production in *P. aeruginosa*.

Cephalosporins are known antipseudomonal drugs; especially the third generation ceftazidime. 72.1% isolates were found to be susceptible to this drug¹⁶. We observed 171 (95%) resistance of *P. aeruginosa* isolates to at least one or more 3rd generation cephalosporins used in the antibiotic culture sensitivity assay. The lowest resistance was against aztreonam (31.11%) and highest resistance to cefpodoxime (89.44%) (Table 1). Only 9/180 (5%) isolates of *P. aeruginosa* were non ESBL producers in screening test which suggests that it is necessary to perform sensitivity assay prior to initiating therapy for effective treatment of *P. aeruginosa* infections. High prevalence of resistant strains of *P. aeruginosa* has been reported from other parts of India as well as other countries: 78.4% of *P. aeruginosa* were resistant to third generation cephalosporins at Pondicherry, India¹⁷, 73.4% of *P. aeruginosa* were resistant to ceftazidime in Iran¹⁸. In the present study, we observed a moderate 32.77% isolates resistant to ceftazidime.

Certain problems are associated with ESBL detection such as: large number of enzymes with different substrate affinities, the presence of other co-expressed resistance mechanisms (cephalosporinases or reduction of permeability by porin modification) that can mask the inhibitory effect of clavulanic acid and interfere with synergy tests for detection. This increased prevalence of ESBLs creates a greater need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates.

The Double disc diffusion synergy test (DDST) is the most widely used test due to its simplicity and ease with which the results can be interpreted. It is reliable method of ESBL detection. However, sensitivity of DDST in different studies ranged from 79% to 96%. This variation in the sensitivity is due to the fact that DDST is not a standardized procedure¹⁹. The sensitivity of DDST varies with the distance between the discs.

E-test observes the synergistic effect between extended-spectrum cephalosporins and inhibitor. However, the antibiotic strips are expensive and the interpretation of results requires experience. The other limitation of the E - test is that this method is not suitable when the drug concentration on the strip is less than the MIC of cephalosporin. Accordingly, higher concentration might have an inhibitory action. This may be the reason for detecting less proportion of ESBL producers in E - test.

The comparative analysis of ESBL producing *P. aeruginosa* by Double disc diffusion synergy test and E test revealed a marked difference between the two methods as only 8 out of 30 ESBL positive isolates by DDST could only be detected by E test. DDST is therefore more sensitive as compared to E test.

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IC value : 5.49

(2013 updated value)

These results are contrary to those reported by others. Vercauteren *et al* (1997)²⁰ detected 81% of ESBLs by E test with ceftazidime. The ESBL E test was 98% sensitive with cefepime-clavulanate, 83% with cefotaxime-clavulanate ceftazidime-clavulanate strips, and 74% with ceftazidime-clavulanate strips at Safdarjung Hospital, New Delhi²¹. In the present study, we however, utilized a combination of cephalosporins for E – test as mentioned under material and methods section which is expected to provide better results. Garrec *et al* (2011)²² reported a sensitivity of 90.0% and a specificity of 89.0% among the strains of members of *Enterobacteriaceae* using E test. But in the present study we observed discordance between the two tests. E - test appears less reliable as compared to DDST.

Further DDST is cost effective as compared to E test strip. Although E test is simple and easy to perform, its cost and limited availability in India may limit its use. On the other hand, DDST is simpler and cost effective for detecting ESBL producing *P. aeruginosa*.

All ESBL positive and negative isolates in DDST and E test have been statistically tested for their resistance to 3rd generation cephalosporins by using Chi (χ)² square test.

All ESBL positive and negative isolates had higher resistance rate to to 3rd generation cephalosporins (95%) although this difference was statistically significant towards all the antibiotics used in the present study (Table 5).

The prevalence of 32.75% ESBL producing *P. aeruginosa* in the state might pose therapeutic challenge to treat infections due to *P. aeruginosa* particularly in hospital settings. The occurrence of ESBLs in the isolates emphasize the need to screen the isolates for their susceptibility to cephalosporins and to adopt effective therapeutic measures in order to reduce the ESBL burden as well the spread of drug resistant *P. aeruginosa* strains in the state. Constant monitoring and surveillance of *P. aeruginosa* strains is therefore, essentially required.

Acknowledgements: Authors are thankful to the Vice Chancellor, Dr. P.K. Khosla, Shoolini University of Biotechnology and Management Sciences and University Grant Commission, New Delhi for providing necessary facilities and financial support to carry out this piece of work. We also express our sincere thanks to Dr. Vanita Sharma, IGMC, Shimla for providing isolates of *P. aeruginosa*.

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Table 1. *In vitro* susceptibility of *Pseudomonas aeruginosa* isolates to 3rd generation cephalosporins

Antimicrobial Agent	Percentage (%) of isolates					
	ESBL			Non-ESBL		
	S	I	R	S	I	R
Cefotaxime	23.88	30.00	41.66	3.88	1.11	0.00
Ceftriaxone	18.33	17.22	61.66	4.44	0.00	0.00
Cefpodoxime	4.44	1.11	89.44	5.00	0.00	0.00
Ceftazidime	56.66	5.55	32.77	5.00	0.00	0.00
Aztreonam	48.33	15.55	31.11	5.00	0.00	0.00
Cefuroxime	10.55	6.11	78.33	4.44	0.55	0.00

Here, S= susceptible; I= intermediate; R= resistance

Table 2. E-test for clinical isolates

Organism	Number of isolates	Ratio of the E test (Mix/Mix ⁺)		
		≥8	<8	NC
<i>P. aeruginosa</i>	30	8	3	19

NC: Non-conclusive, no zone of inhibition was observed on either side of the disc

Table 3. MIC for mix and mix⁺ according to E test

MIC standard range (µg/ml)		MIC interpretation (µg/ml)	
Mix	Mix ⁺	Mix	Mix ⁺
(0.125-16)	(0.032 - 4)	>16	0.442

(Mix: Ceftazidime, cefotaxime and cefepime; Mix⁺: Ceftazidime, cefotaxime and cefepime + clavulanic acid and tozobactam)

Table 4. Values used in the χ^2 calculation

Antibiotics	ESBL negative n=115	ESBL positive n=56
	Resistance to 3 rd generation cephalosporins	Resistance to 3 rd generation cephalosporins
Cefotaxime	23	57
Ceftriaxone	33	75
Cefpodoxime	55	108
Ceftazidime	23	35
Aztreonam	17	39
Cefuroxime	48	94

Table 5. Yates correction applied and comparison of significant level of p- value at alpha.

χ^2 (calculated value)	Degree of freedom	Significant at alpha		
		0.05	0.01	0.001
2.35	5	11.1	15.1	20.5