

## PLASMID PROFILE AND CURING, BIOFILM DETECTION AMONG ESBL PRODUCING ISOLATES OF *ACINETOBACTER* SPECIES

E. KUMAR<sup>1</sup>, K. USHA<sup>1</sup>, BV. RAMANA<sup>2</sup>, A. CHAUDHURY<sup>2</sup>, DVR. SAI GOPAL<sup>\*1</sup>

<sup>1</sup>Department of Virology, Sri Venkateswara University, Tirupati, India, <sup>2</sup>Department of Microbiology, Sri Venkateswara Institute of Medical Sciences, India  
Email: dvrsaigopal@gmail.com

Received: 28 Oct 2015 Revised and Accepted: 11 Feb 2016

### ABSTRACT

**Objective:** The aim of the present study is to evaluate the plasmid profile and curing in extended spectrum beta-lactamases (ESBL) producers and know the association of biofilm formation among ESBLs.

**Methods:** Standard disk diffusion method (Kirby Bauer's method) was performed to know the sensitivity and resistance patterns for commonly used antibiotics and clinical laboratory standard institute (CLSI) recommended confirmatory phenotypic method was used to confirming ESBL producers. 56 ESBL producers were subjected to plasmid profiling and curing. The tube (qualitative) and microtitre (quantitative) methods were performed to detect the biofilm formation.

**Results:** In all ESBL producers, a single plasmid was found with approximately 21, 226 bp. After plasmid curing, the resistance patterns against the third generation cephalosporin group of antibiotics were transformed into susceptible for all bacterial isolates. In tube method, 11 (19.64%) isolates were strong adherent, 08 (14.28%) were either weakly adherent or non-biofilm producers. In the microtitre plate method, 09 (16.07%) were considered as positive, and 47 (83.92%) were considered as negative for biofilm formation.

**Conclusion:** We found a single resistant plasmid in all isolates and after curing of plasmids, the transformation of resistance to susceptibility against third generation drugs were observed. So, we concluded that ESBL mediated antimicrobial resistance mechanisms in *Acinetobacter* spp. is maybe plasmid-mediated in the tested isolates. However, there was no association found between biofilm producers and ESBLs, this baseline information will be helpful to study more in this area.

**Keywords:** *Acinetobacter* spp, Biofilm detection, ESBL, Plasmid profile and curing

© 2016 The Authors. Published by Innovare Academic Sciences Pvt Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

### INTRODUCTION

*Acinetobacter* spp. is one of the emerging opportunistic and multidrug-resistant (MDR) bacteria among NFGNB (Non Fermentative Gram Negative Bacteria) causing various infections such as pneumonia, urinary tract infection, endocarditis, surgical site infection, meningitis, and septicaemia, particularly in immunocompromised patients [1]. Extended-spectrum beta-lactamases (ESBLs) are enzymes produced by gram negative bacteria including *Acinetobacter* spp. that inactivates extended-spectrum cephalosporins and other beta-lactam antimicrobial agents and are inhibited by clavulanic acid, sulbactam or tazobactam [2]. ESBLs can be plasmid mediated resistance mechanism. The plasmid may spread antibiotic resistance genes to their host bacteria by various mating processes and replicates independently of the host chromosome. Their size varies from 1 to over 1,000 Kb. The number of plasmids can range from one to thousands under some circumstances [3]. Biofilm is the surface-attached (sessile) community of microorganisms growing embedded in a self-produced matrix of extracellular polymeric substances (EPS). Typically biofilm shows an increased antimicrobial resistance as compared to planktonic bacteria [4]. Frequently, the biofilm production should be found in the clinically relevant infections, and the biofilm producers should be resistance to the commonly used antibiotics and also a variety of disinfectants [5]. This study was designed to know the nature of plasmid as an antibiotic resistance marker in ESBLs and biofilm detection among ESBL producing *Acinetobacter* species.

### MATERIALS AND METHODS

During 8 mo period (May 2013-December 2013), 56 ESBL producing *Acinetobacter* spp. (isolated from the clinical specimens of routine diagnosis) were obtained in the Department of Microbiology, Sri Venkateswara Institute of Medical Sciences, Tirupati. Kirby Bauer's

method [6] was performed to know the resistance pattern. The CLSI recommended confirmatory phenotypic method [7] was performed to confirm the presence of ESBL production. *K. pneumoniae* ATCC 700603 (positive control) and *E. coli* ATCC 25922 (negative control) were used as quality control.

### Isolation and detection of molecular weight of plasmid DNA

The plasmid DNA from ESBL producing isolates of *Acinetobacter* spp. were isolated using alkaline lysis method described by Sambrook *et al.* [8] The bacterial cultures were grown in LB medium with appropriate antibiotic at 37 °C, 150 rpm for 16-18 h. After nucleic acid precipitation, plasmid pellet was dissolved in 50 µl of TE (10 mM tris, 1 mM EDTA, pH 8) buffer. Agarose gel electrophoresis was performed on 0.8 per cent (w/v) agarose prepared in TBE buffer. The plasmid profiles were documented under UV light in Gel Documentation System. (fig. 1)

### Curing of plasmid DNA

The curing of plasmid DNA was performed in ESBL producing strains of *Acinetobacter* spp. by the method as described by Deshpande *et al.* [9]. The efficiency of plasmid curing was expressed as a number of colonies with cured phenotype per 100 colonies tested. In cured strains, the antibiotic sensitivity test (disc diffusion method) was repeated to check the presence or loss of resistance character and additionally, agarose gel electrophoresis was performed with the plasmid DNA preparation of respective culture to confirm the physical loss of plasmids.

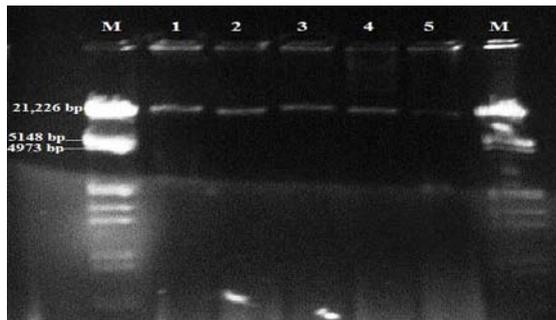
### Biofilm detection by tube method

We screened all isolates for the biofilm formation by tube method (qualitatively) as described by Christensen *et al.* [10]. Brain-heart infusion (BHI) was inoculated with loopful of test culture from overnight culture plates and incubated for 24 h at 37 °C. Then, tubes

were decanted and washed with PBS (pH 7.3) and dried than tubes were stained with crystal violet (0.1%). Excess stain was removed with PBS and tubes were washed with deionized water. Tubes were than dried in inverted position and observed for biofilm formation. Biofilm formation was considered positive when a visible film lined the wall and bottom of the tube. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was recorded as strongly adherent (strongly positive), weakly adherent (week positive) and non-adherent (negative). Experiments were performed in triplicate and repeated three times.

#### Biofilm detection by microtitre method

Micro-titre method (quantitatively) [10] was performed to screen the biofilm formation. A 1:100 dilution of overnight broth culture of the test organism was inoculated in BHI broth with 2% sucrose and transferred to 96 well polystyrene flat-bottomed microtitre plates.

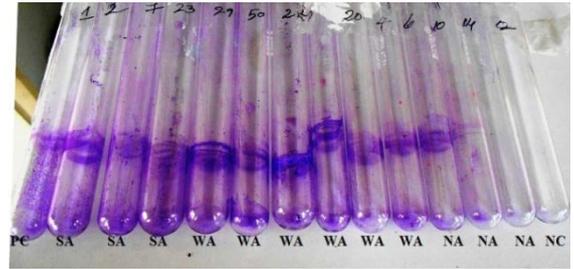


**Fig. 1: Plasmid profile of ESBL producing *Acinetobacter* spp M:  $\lambda$  DNA (EcoRI+Hind III digested); 1,2,3,4 and 5: presence of plasmid DNA**

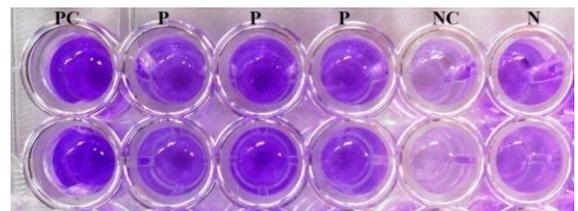
After incubation at 37 °C, washed and air dried then stained with crystal violet solution, the glacial acetic acid was added to the wells and optical density was read at 490 nm. The optical density (OD) greater than 0.120 was considered for biofilm formation. The biofilm positive control strain was maintained [11].

All ESBL positive samples were sensitive to tegecyclin (100%) followed by polymixin-B (91.07%) and imipenem (66.66%) and

100% resistance to third generation cephalosporins which includes cefotaxime, ceftazidime, cefepime and monobactam includes aztreonam and resistance pattern for other antibiotics were shown in table 1.



**Fig. 2: Showing biofilm detection in tube method, PC: Positive Control; NC: Negative Control; SA: Strong Adherent; WA: Weak Adherent; NA: Non-Adherent**



**Fig. 3: Showing biofilm detection in microtitre method, PC: Positive Control; NC: Negative Control; P: Positive; N: Negative**

The analysis of plasmid in ESBL producing strains revealed the presence of plasmid DNA with an approximate molecular weight of 21,226 bp in all isolates (fig. 1). Most of the isolates showed thick blue ring at the liquid-air interfaces (fig. 2). In tube method, 11 (19.64%) isolates were strong positive, 08 (14.28%) were weakly adherent and 37 (66.07%) were non-biofilm producers. Isolates that showed weakly adherent were considered as non-biofilm producers. In the micro-titre plate method, the 09 (16.07%) were showed OD values greater than 0.120 and remaining isolates 47 (83.92%) were considered as negative for biofilm formation. were considered as negative for biofilm formation (fig. 3).

**Table 1: Antibiotic sensitivity pattern of 56 ESBL producing *Acinetobacter* spp**

Serial number	Antibiotic (concentration)	Sensitive (%)	Intermediate (%)	Resistance (%)
1.	amikacin (30 µg)	16 (28.57)	03 (5.35)	37 (66.07)
2.	ampicillin (10µg)	00 (0.00)	03 (5.35)	53 (94.64)
3.	aztreonam(30µg)	00 (0.00)	00 (0.00)	56 (100)
4.	cefepime(30µg)	00 (0.00)	00 (0.00)	56 (100)
5.	cefotaxime (30µg)	00 (0.00)	00 (0.00)	56 (100)
6.	cefoxitin (30µg)	08 (14.28)	03 (5.35)	45 (80.35)
7.	ceftazidime (30µg)	00 (0.00)	00 (0.00)	56 (100)
8.	chloramphenicol (30µg)	08 (14.28)	04 (7.14)	44 (78.57)
9.	ciprofloxacin (5µg)	09 (16.07)	04 (7.14)	43 (76.78)
10.	co-trimoxazole (25µg)	24 (42.85)	05 (8.92)	27 (48.21)
11.	gentamicin (30µg)	09 (16.07)	04 (7.14)	43 (76.78)
12.	imipenem (10µg)	37 (66.07)	08 (14.28)	11 (19.64)
13.	meropenem (10µg)	40 (71.42)	08 (14.28)	08 (14.28)
14.	nitilmicin (30µg)	20 (35.71)	00 (0.00)	36 (64.28)
15.	polymixin-B (300 units)	51 (91.07)	00 (0.00)	05 (8.92)
16.	tegecyclin (15 µg)	56 (100)	00 (0.00)	00 (0.00)
17.	piperacillin tazobactam (100/10µg)	13 (23.21)	08 (14.28)	35 (62.5)
18.	cefaperazone sulbactam (75/10µg)	29 (51.78)	07 (12.5)	20 (35.71)
19.	amoxy clav (20/10µg)	02 (3.57)	14 (25.00)	40 (71.42)

µg: microgram; %: percentage.

#### DISCUSSION

Most commonly, extended spectrum beta lactams and other beta lactam group of antibiotics have been used to treat gram negative

bacterial infections including *Acinetobacter* spp. But the emergence of ESBL producing and another beta-lactamase producing organisms has been posed a serious threat for their continuing use [12, 13]. The nosocomial infections are becoming rising trouble for hospitalized

patients [14]. If the antibiotic resistant bacteria, unluckily spread to patients in any transmission mode, subsequently susceptible microbial flora may also be transformed into the resistant strain or susceptible bacteria into resistant, as a result, the risk factors are directly or indirectly increases treatment failure. *Acinetobacter baumannii* were highly resistant to a  $\beta$ -lactam group of antibiotics which were isolated from the ICU patients [15]. In our study, gentamicin resistance was found to be 76.78% which was high as compared to the previous study reported by Shafaq Aiyaz Hassan et al., [16] (60%) and low (92.6%) when compared with Iroha et al., [17].

The resistance profile of ciprofloxacin among ESBL producers varies with geographic regions 13.7% in Canada, 34.8% in France, 34.2% in Europe to 65.5% in Western pacific [18]. In our study, 76.78% of resistance pattern was documented which was close prevalence to (76.5%) reported in Bangladesh [19].

Treatment options for ESBL producing isolates are available, but they are limited. The risk factor of treatment failure is more in patients infected with ESBL producers than ESBL non-producers [20]. In a previous studies from Pakistan [21] and Bangladesh [19], all ESBL producers were sensitive (100%) to carbapenems. The carbapenems are used to treat the infections caused by ESBL producers, but carbapenem-resistant ESBL isolates have been reported from various parts of the world including United States, Greece, Korea, Israel, and China [22]. In our study, 19.64% and 14.28% of resistance were found against imipenem and meropenem respectively.

In a study from Pune, *Acinetobacter* spp. isolated from human skin showed a maximum of three plasmids ranging from 1.5 to 40 kb [23] but in our study only single plasmid (approximately 21,226 bp) was observed among all ESBL strains isolated from clinical specimens. The physical loss of plasmid (after curing) is the evidence for the nature of antibiotic resistance marker in the ESBL strains. The cured isolates were found sensitive to third generation cephalosporins.

Microbial biofilms are considered as the virulence factors. In this study, the detection of biofilm production in *Acinetobacter* spp. was aimed to verify any association between the biofilm formation and ESBLs. The device related nosocomial infection also causes the organisms to resist physical and chemical disinfection, often by forming a biofilm. Therefore, infections due to bacteria that form biofilms are a persistent clinical problem. In a study, both qualitative (tube method) and quantitative (microtitre plate) method showed 30 isolates (60%) as biofilm producers [24]. In the present study, 11 (19.64%) isolates were considered as strong adherence, 08 (14.28%) isolates were weak adherence and 37 (66.07%) were showed non-adherence by tube method whereas in micro-titre plate, 09 (16.07%) were considered as biofilm producers and 47 (83.92%) were considered as non-bio film producers. However, as adherence alone may not complete the cycle of the process of biofilm formation and there might be many other mechanisms that could explain adherence [25]. So, we categorized isolates that were weakly adherent as biofilm negative and this report was suggested that low prevalence then Dheepa et al., a study [24]. In another study, it was observed that 44% of positive biofilm isolates were isolated from wound infections and 30% of biofilm producers were identified from patients suffered from pneumonia. This indicates that in the wound and respiratory infections, the biofilm may contribute to antibiotic resistance [25] and in the same study, both the qualitative and quantitative method showed 62% of isolates as strong biofilm producers. Fourteen isolates, which were weakly adherent, were additionally picked up by the quantitative microtitre assay asserting its sensitiveness.

We concluded that the nature of plasmid plays a major role in antibiotic resistance with reference to ESBL mediated resistance. In this regard, furthermore, molecular studies to be needed to check whether the prevalence of resistance was due to a specific gene or multiple genes.

#### CONFLICT OF INTERESTS

Declared none

#### REFERENCES

- Bergogne-Berezin E, Towner KJ. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin Microbiol Rev 1996;9:148-65.
- Payne DJ, Cramp R, Winstanley DJ, Knowles DJ. Comparative activities of clavulanic acid, sulbactam, and tazobactam against clinically important beta-lactamases. Antimicrob Agents Chemother 1994;38:767-72.
- Lipps G. Plasmids, Current Research, and Future Trends. Horizon Scientific Press UK; 1008. p. 263.
- Donlan RM, Costerton JW. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 2002;15:167-93.
- Saitou K, Furuhashi K, Kawakami Y, Fukuyama M. Biofilm formation abilities and disinfectant resistance of *Pseudomonas aeruginosa* isolated from cockroaches captured in hospitals. Biocontrol Sci 2009;14:65-8.
- Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standard disk diffusion method. Am J Clin Pathol 1966;45:493-6.
- Clinical Laboratory Standards Institute: Performance standards for antimicrobial susceptibility testing. Twenty-second informational supplement. Wayne, PA, USA: CLSI; 2012. p. M100-S22.
- Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press: Cold Spring Harbor; 1989.
- Deshpande NM, Dhakephalkar PK, Kanekar PP. Plasmid mediated-dimethoate degradation in *Pseudomonas aeruginosa* MCMB-427. Lett Appl Microbiol 2001;33:275-9.
- Christensen GD, Simpson WA, Younger JA, Baddour LM, Barrett FF, Melton DM, et al. Adherence of coagulase negative *Staphylococci* to plastic tissue culture: a quantitative model for the adherence of *staphylococci* to medical devices. J Clin Microbiol 1985;22:996-1006.
- Chaudhury A, Nagaraja M, Kumar AG. Potential of biofilm formation by *Staphylococci* correlation with methicillin susceptibility. Indian J Med Microbiol 2009;27:377-8.
- Ndugulile F, Jureen R, Harthug S, Urassa W, Langeland N. Extended-spectrum beta-lactamases among Gram-negative bacteria of nosocomial origin from an intensive care unit of a tertiary health facility in Tanzania. BMC Infect Dis 2005;5:86.
- Kumar E, Usha K, Chaudhury A, Ramana BV, Sai Gopal DVR. Detection of AmpC  $\beta$ -lactamases production in *Acinetobacter* species by an inhibitor (disk) based & modified three-dimensional (enzyme extraction) methods. Indian J Med Res 2014;140:688-90.
- Tullu MS, Deshmukh CT, Baveja SM. Bacterial profile and antimicrobial susceptibility pattern in catheter-related nosocomial infections. J Postgrad Med 1998;44:7-13.
- Kumar E, Usha K, Ramana BV, Chaudhury A, Gopal DVR. Molecular characterization of plasmid-mediated *bla*CTX-M15 extended spectrum  $\beta$  lactamase (esbls) in *Acinetobacter* spp. Isolates from intensive care unit patients, at a tertiary care hospital, South India. Indian J Med Microbiol 2015;33:601-2.
- Shafaq Aiyaz Hassan, Syed Asfar Jamal, Mustafa Kamal. The occurrence of multidrug resistant and ESBL producing *E. coli* causing urinary tract infections. J Basic Appl Sci 2011;7:39-43.
- Iroha IR, Oji AE, Afiukwa TN, Nwuzo AC, Ejikegwu PC. Extended spectrum beta-lactamase (ESBL) mediated resistance to antibiotics among *Klebsiella Pneumoniae* in enugu metropolis. Maced J Med Sci 2009;2:196-9.
- Winokur PL, Canton R, Casellas JM, Legakis N. Variations in the prevalence of strains expressing an extended-spectrum beta-lactamase phenotype and characterization of isolates from Europe, the Americas, and the Western Pacific region. Clin Infect Dis 2001;2(Suppl 2):94-103.
- Iraj Alipourfard, Nilufar Yeasmin Nili. Antibiogram of extended spectrum beta-lactamase (ESBL) producing *Escherichia coli* and *Klebsiella pneumoniae* isolated from hospital samples. Bangladesh J Med Microbiol 2010;4:32-6.
- Hirakata Y, Matsuda J, Miyazaki Y, Kamihira S, Kawakami S, Miyazawa Y, et al. Regional variation in the prevalence of

- extended-spectrum beta-lactamase producing clinical isolates in the Asia-pacific region (SENTRY 1998-2002). *Diagn Microbiol Infect Dis* 2005;52:323-9.
21. Jabeen K, Zafar A, Hassan R. Frequency and sensitivity pattern of extended spectrum beta-lactamase producing isolates in a tertiary care hospital laboratory of Pakistan. *J Pak Med Assoc* 2005;55:436-9.
  22. Sader HS, Hsiung A, Fritsche TR, Jones RN. Comparative activities of cefepime and piperacillin/tazobactam tested against a global collection of *Escherichia coli* and *Klebsiella* spp. with an ESBL phenotype. *Diagn Microbiol Infect Dis* 2007;57:341-4.
  23. Karishma R Pardesi, Supriya P Yavankar, Balu A Chopade. Plasmid distribution and antimicrobial susceptibility patterns of *Acinetobacter* genospecies from healthy skin of a tribal population in western India. *Indian J Med Res* 2007;125:79-88.
  24. Dheepa M, Vinitha L Rashme, Appalaraju B. Comparison of biofilm production and multiple drug resistance in clinical isolates of *Acinetobacter baumannii* from a tertiary care hospital in South India. *Int J Pharm Biomed Sci* 2011;2:103-7.
  25. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. *Science* 1999; 284:1318-22.