Int J Pharm Bio Sci 2014 Jan; 5(1): (B) 353 - 360

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

DETERMINATION OF CORRELATION BETWEEN BIOFILM AND ESBL PRODUCERS OF *ENTEROBACTERIACEAE*

S.PRAMODHINI* AND S.UMADEVI

Department of Microbiology, Mahatma Gandhi Medical College & Research Institute, Pondicherry, India.

ABSTRACT

Antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronicity of infections. This study was aimed to find out the prevalence of biofilm producers among the microorganisms isolated from our set up and to find out their antimicrobial susceptibility pattern with special reference to ESBL and AmpC producers. Total 169 clinical isolates were tested for biofilm production. Any bacterial species which showed resistance to any of third generation cephalosporin was tested for ESBL production and AmpC production. Among 169 isolates, 100(59.2%) were biofilm producers. In our study, 44(26%) and 43(25%) isolates were ESBL and AmpC producers respectively. Of 44 ESBL producers, 42(95.4%) and of 43 AmpC producers 39(90.7%) were biofilm producers.. Hence,clearing of biofilm would enhance the bacteria to respond to the therapy,to which it was refractory.

KEY WORDS: **Biofilm, *Enterobacteriaceae*, ESBL producers, AmpC β lactamase, Tube adherence method**

S.PRAMODHINI
Department of Microbiology, Mahatma Gandhi Medical College & Research Institute, Pondicherry, India.

INTRODUCTION

A biofilm is a complex aggregate of microorganisms in which cells are adhere to each other and to a surface in a self-produced matrix of extracellular polymeric substance (EPS)/slime. In a biofilm, bacteria communicate with one another using chemical signal molecules, termed auto-inducers. This process of chemical communication, called quorum sensing, allows bacteria to monitor the environment for other bacteria and to alter the behavior in response to changes in a community [1]. Bacteria within biofilms are intrinsically more resistant to antimicrobial agents than planktonic ells. Antimicrobial concentrations sufficient to inactivate planktonic organisms are generally inadequate to inactivate biofilm organisms. Antibiotic resistance can increase 1000 fold [2]. According to a research, more than 60% of all infections involve biofilms [3].Both the Gram positive and Gram negative bacteria have the capability to form biofilms. In gram-negative bacteria, one of the important mechanisms of resistance is the production of beta-lactamases, which inhibit protein transpeptidases participating in bacterial cell wall synthesis [4,5].Of particular clinical and epidemiological importance are extended spectrum β lactamases (ESBLs) and AmpC β lactamases, capable of inactivating the effects of broad-spectrum cephalosporins and penicillins. Production of these enzymes in clinically significant *Enterobacteriaceae* represents an increasing problem resulting in higher patient morbidity and mortality.This study was aimed to find out the prevalence of biofilm producers among the microorganisms isolated from our set up and to find out their antimicrobial susceptibility pattern with special reference to ESBL and AmpC producers.

MATERIALS AND METHODS

Bacterial strains

This prospective, analytic study included 169, non-repeat, clinical isolates of *Enterobacteriaceae* collected over a period of 6 months. The isolates were obtained from clinical specimens from the NICU, the ICU, inpatient units and the outpatient department. Isolates identified with standard biotyping methods [6] were tested for biofilm production by tube adherence methods. Antimicrobial susceptibility testing was carried out with the disc diffusion method using current CLSI recommendations[7]. The antimicrobial susceptibility profiles against Amoxycillin-Clavulanic acid], gentamicin, amikacin, ceftriaxone, ciprofloxacin, piperacillin-tazobactum and imipenem were studied. Any bacterial species which showed resistance to any of third generation cephalosporin was tested for ESBL and AmpC β lactamases production.

Detection of Biofilm production

(i) Tube adherence method:

Biofilm production was estimated qualitatively for all the isolates by Tube adherence method by Christensen et al [8]. Suspension of tested strains was incubated in the glass tubes containing Brain Heart Infusion Broth (broth) aerobically at the temperature of 35°C for the period of 2 days. Then the supernatant discarded and the glass tube was stained by 0.1% safranin solution, washed with distilled water three times and dried. A positive result is defined as the presence of a layer of stained material adhered to the inner wall of the tubes. The exclusive observation of a stained ring at the liquid-air interface should be considered negative.

Screening for ESBL producing isolates

(i) Screening by standard disk diffusion method:

Screening for ESBL production was done according to criteria recommended by CLSI[7]. Two discs, ceftazidime (30 µg) and cefotaxime (30 µg) were used for *in vitro* sensitivity testing by Kirby-Bauer disk diffusion method. Zone diameters were read using CLSI criteria. An inhibition zone of ≤ 22 mm for ceftazidime and ≤ 27 mm for cefotaxime indicated a probable ESBL producing strain requiring phenotypic confirmatory testing [9].

(ii) Phenotypic confirmatory tests for ESBL production

The CLSI advocates use of cefotaxime (30 µg) or ceftazidime disks (30 µg) with or without

clavulanate (10 µg) for phenotypic confirmation of the presence of ESBLs in *Klebsiellae*and *Escherichia coli* (Esch.coli). A difference of >5 mm between the zone diameters of either of the cephalosporin disks and their respective cephalosporin/ clavulanate disk is taken to be phenotypic confirmation of ESBL production [7].

Screening for AmpC producing isolates

Screening for the inducible AmpC β -lactamase was done by the disc antagonism test[10] by placing cefoxitin disc (30 µg) at a distance of 20 mm from ceftazidime (30 µg) on the surface of MHA. Isolates showing blunting of the ceftazidime zone adjacent to cefoxitin disc were considered "screen positive" and selected for detection of AmpC β lactamases.

(iii) Detection of AmpC β lactamases by AmpC Disk Test[11]

Here, a lawn culture of *Escherichia coli* ATCC 25922 was prepared on MHA plate. Sterile disks (6 mm) were moistened with sterile saline (20 µl) and inoculated with several colonies of test organism. The inoculated disk was then placed beside a cefoxitin disk (almost touching) on the inoculated plate. The plates were incubated overnight at 35°C. A positive test appeared as a flattening or indentation of the cefoxitin inhibition zone in the vicinity of the test disk. A negative test had an undistorted zone.

Statistical analysis

Statistical analysis was done by taking percentage and simple ratios.

RESULTS

The study included 169 non-repeat, clinical isolates of *Enterobacteriaceae* collected over a period of 6 months. The organism included *Klebsiella pneumoniae* (68 isolates), *Escherichia coli*(.52 isolates), *Citrobacter spp* (23isolates), *Proteus spp* (15 isolates), *Enterobacter spp* (8 isolates) and *Morganella spp* (3 isolates). Among 169 isolates, 100(59.2%) were biofilm producers by Tube adherence method. The majority of organism were associated with biofilm production which included *Klebsiella pneumoniae* (55.9%), followed by *Escherichia coli*(57.7%), *Citrobacter spp* (73.9%), *Proteus spp* (53.3%), *Enterobacter spp* (50%), *Morganella spp* (100%) as shown in Table 1.

Table 1
Distribution and Biofilm production of the isolates

Isolates	Total no (%)	Biofilm producers (%)
Klebsiella	68(40.2%)	38(55.9%)
Escherichia coli	52(30.8%)	30(57.7%)
Citrobacter	23(13.6%)	17(73.9%)
Proteus sp	15(8.9%)	8(53.3%)
Enterobactersp	8(4.7%)	4(50%)
Morganellaspp	3(1.8%)	3(100%)
Total	**169(100%)**	**100(59.2%)**

In our study, ESBL production were seen in 44(26%) isolates of Enterobacteriaceae by phenotypic confirmatory tests for ESBL production and 43(25%) isolates were AmpC β –lactamase producers by AmpC disk test (Table 2). ESBL production was higher in E. coli followed by Klebsiella pneumoniaeand Citrobacter spp. Whereas AmpC producers were higher in Klebsiella pneumoniaefollowed by Escherichia coli and Enterobacter spp.

Table 2
ESBL and AmpC production in Enterobacteriaceae

ISOLATE	ESBL Positive No (%)	AmpC Positive No(%)
Klebsiella(68)	18(26.5%)	24(35.3%)
Escherichia coli(52)	22(42.3%)	15(28.9%)
Citrobacter(23)	4(17.4%)	2(8.7%)
Proteus sp (15)	-	-
Enterobactersp (8)	-	2(25%)
Morganellaspp (3)	-	-
Total 169	**44(26%)**	**43(25%)**

Table 3
Biofilm production in ESBL and AmpC producers

Isolate	Biofilm in ESBL producers(%) n=44	Biofilm in AmpC producers(%) n=43
Klebsiella(68)	17(94.4%)	21(87.5%)
Escherichia coli(52)	22(100%)	15(100%)
Citrobacterspp(23)	3(75%)	2(100%)
Proteus spp (15)	-	-
Enterobacterspp (8)	-	1(50%)
Morganellaspp (3)	-	-
Total 169	42(95.4%)	39(90.7%)

Of 44 ESBL and 43 AmpCβ -lactamases producers, 42(95.4%) and of 39(90.7%) were biofilm producers respectively.(Table 3).

Table 4
Antibiotic sensitivity pattern of biofilm producers

Antibiotics	Klebsiella (38)	Escherichia coli (30)	Citrobacter (17)	Proteus (8)	Morganella (3)	Enterobacter (4)
Amoxyclav	-	-	-	-		-
Gentamicin	18	6	6	4	-	4
Amikacin	33	22	13	6	2	4
Ceftriaxone	12	2	5	3	-	4
Ciprofloxacin	21	5	6	4	-	4
Piperacillin-Tazobactum	34	28	16	8	3	4
Imipenem	34	28	16	8	3	4

The overall percentage of sensitivity observed among all the isolates of biofilm producers for antibiotics tested is given in Table 4.

Sensitivity pattern of biofilm positive isolates showed 38%, 80%, 26%, 40%, 97%, 97% sensitivity to Gentamicin, Amikacin, Ceftriaxone, Ciprofloxacin, Piperacillin-Tazobactum and Imipenem respectively, compared to 71%, 88.4%, 62.3%, 75.4%, 98.6%, 97.1% sensitivity shown by biofilm non -producers for the same antibiotics.(Table 5).

Table 5
Comparison of sensitivity pattern of biofilm producers and Non biofilm producers

Antibiotics	Biofilm Producers % (n=100)	Biofilm Non-Producers% (n=69)	P value
Amoxyclav	1(1)	1(2%)	0.646851
Gentamicin	38(38%)	49(71%)	0.000048
Amikacin	80(80%)	61(88.4%)	0.217141
Ceftriaxone	26(26%)	43(62.3%)	0.000005
Ciprofloxacin	40(40%)	52(75.4%)	0.000012
Piperacillin-Tazobactum	97(97%)	68(98.6%)	0.890979
Imipenem	97(97%)	67(97.1%)	0.671888

The sensitivity pattern of non biofilm producers for gentamicin, ceftriaxone and ciprofloxacin was statistically significant than biofilm producers. (p value <0.0001).

DISCUSSION

A biofilm is a population of cells growing on a surface and enclosed in an exopolysaccharide matrix. Biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections. One can find a list of factors considered to be responsible for biofilm resistance which includes restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of possible resistance genes [12, 13].A total 169 isolates of *Enterobacteriaceae* were tested for biofilm production by tube adherence method which can be indicated for the routine detection of biofilm production because of its easy application and low cost and because it guarantees reliable results with excellent sensitivity and specificity [14, 15]. In our study, 59.2% of the tested organisms have shown the potential to make biofilms in comparison to similar study [16] which reported 54%.Extended spectrum β-lactamases (ESBLs) are plasmid mediated enzymes inactivating β-lactam antibiotics containing oxyimino group such as oxyimino-cephalosporins and oxyimino-monobactam, except cephamycins and carbapenems[17]. They are derived from the point mutation of plasmid determined TEM or SHV β-lactamases[17, 18]. ESBLs are inhibited by clavulanic acid and placed under Bush's functional class 2be [19]. Till date more than 200 different types of ESBLs have been described. In recent years, there is a dramatic increase in the prevalence of CTX-M type of ESBLs among clinical isolates of *Enterobacteriaceae*in Europe and Asia [20].ESBLs are the most evolving mechanism of antibiotic resistance among the family *Enterobacteriaceae*due to the selective pressure imposed by inappropriate use of third generation cephalosporins, most often encountered in ICU settings[18]. Plasmids coding for ESBL enzymes may carry co-resistance genes for other non-β-lactam antibiotics, thus limiting the number of useful drugs against these bacteria [21].

The prevalence of ESBL-producing enterobacterial isolates evaluated in the present study was 26% in comparison to a similar study [22] which showed 29%. However, in our study analysis of the 44 confirmed ESBL isolates revealed that ESBLs were predominantly present among *Esch. coli* (42.3%) compared to *K. pneumoniae*(26.5%) and other *Enterobacteriaceae spp.* Our findings are similar to that of several studies which reported a high prevalence of ESBLs among *E. coli* [23-26]. AmpC β-lactamases are Group I cephalosporinases that confer resistance to a wide variety of β -lactam antibiotics including alpha methoxy β -lactams such as cefoxitin, narrow and broad spectrum cephalosporins, aztreonam, and are poorly inhibited by β -lactamase inhibitors such as clavulanic acid [19]. Genes for AmpC β -lactamases are commonly found on the chromosomes of the several members of the family *Enterobacteriaceae.* Plasmid mediated AmpC β -lactamases has arisen through the transfer of chromosomal genes for the inducible AmpC β-lactamases on to plasmids [27]. This resistance mechanism has been found around

the world, can cause nosocomial outbreaks, appears to be increasing in prevalence, and merits further study to define the best options for detection and treatment [28].AmpC β -lactamases producers were 25% in our study; 20.7%, 24 % and 37.50% have been reported from several other similar studies [29-31]. Maximal incidence of AmpCβ -lactamases producers were found among *Klebsiella pneumoniae* (35.3%) followed by *Esch. Coli* (28.9%) in concordance with study by Manchanda *et al*[29] where they showed maximal incidence of AmpC producers among *Acinetobacter spp*. (42.8%) followed by *Klebsiella pneumoniae* isolates (33.3%). In contrary, Subha *et al* [31] in their study showed 24.1% of *Klebsiella* spp. and 37.5% of *Esch. Coli* were found to be AmpC β -lactamase producers.

In our study, 95.4% of ESBL producers and 90.7% of AmpC β -lactamases producers showed biofilm production. well in comparison to other studies conducted by Hemachandran *et al* [32] and Bhaskaran *et al* [33]where they showed high rate of biofilm production among ESBL producers than non β –lactamases producers. Among which, *Esch.coli* was found to be the good biofilm producer followed by the *Klebsiellaspp* and other *Enterobacteriaceae* in comparable to a similar study [26].The clinically relevant observation noted in our study was high resistance of biofilm producers to commonly used antibiotics than biofilm non producers similar to other studies [14, 34]. It was also observed that most of ESBL and AmpC β lactamase producers were sensitive to broad spectrum antibiotics like imipenem and piperacillin-tazobactum.

CONCLUSION

To conclude, among 169 isolates, 26% and 25% were ESBL and AmpC producers. Of which, more than 90% were biofilm producers which showed high resistance to commonly used antibiotics. There was sigmificant correlation between ESBL producers and biofilm production in our study. Currently, ESBL and AmpC β lactamase producers are becoming a major threat for patients in the hospital, long-term care facilities, and community. These bacteria have not only caused outbreaks but have become endemic in many hospitals throughout the world. In addition, antibiotic resistance of bacteria in the biofilm mode of growth contributes to the chronicity of infections such as those associated with implanted medical devices. The protective mechanisms at work in biofilms appear to be distinct from those that are responsible for conventional antibiotic resistance. Disabling biofilm resistance may enhance the ability of existing antibiotics to clear infections involving biofilms that are refractory to current treatments [35].

REFERENCES

1. Waters CM, Bassler BL. Quorum Sensing: cell to cell communication in bacteria. Annual Review of Cell and Developmental Biology 2005:21; 319-346.
2. Stewart PS, Costerton JW. Antibiotic resistance of bacteria in biofilms.*Lancet* 2001: 358; 135-138.
3. Kim L. Riddle of biofilm resistance. Antimicrobial Agents and Chemotherapy 2001:45; 999-1007.
4. Bradford PA. Extended-spectrum-beta-lactamases in the 21st century: characterization, epidemiology,and detection of this important resistance threat. Clinical Microbiology Reviews 2001: 14; 933–951.
5. Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. Clinical Microbiology Reviews 2005: 18; 657–686.
6. Crichton, P. B. Enterobacteriaceae: Escherichia, Klebsiella,Proteus and other genera. In Collee JG, Fraser AG, Marmion BP, Simmons A (eds). Mackie & McCartney Practical Medical Microbiology, 14th edn .Churchill Livingstone,Edinburgh, UK; 1996 : pp. 361–384.

7. Clinical Laboratory Standards Institute, 2012. Performance standards for antimicrobial susceptibility testing: Twenty second informational supplement ed. CLSI document M100S-S22 CLSI: Wayne, PA.
8. Christensen GD, Simpson WA, Bismo AL, Beachery EH. Adherence of slime – producing strains of staphylococcus epidermidis to smooth surfaces. Infect immune1982; 37: 318-326.
9. Jain A, Roy I, Gupta MK, Kumar M, Agarwal SK. Prevalence of extended spectrum β-lactamase producing gram negative bacteria in septicaemic neonates in a tertiary care hospital. J Med Microbiol 2003; 52:421-425.
10. Sanders CC, Sanders WE, Goering HV. *In vitro* antagonism of β-lactam antibiotics by cefoxitin.J AntimicrobChemother1982; 21: 968-975.
11. Singhal S, Mathur T, Khan S, Upadhyay DJ, Chugh S, Gaind R, *et al* . Evaluation of methods for AmpC Beta -lactamase in gram negative clinical isolates from tertiary care hospitals. Indian J Med Microbiol 2005; 23:120-124.
12. Costerton, J. W., P. S. Stewart, and E. P. Greenberg. Bacterial biofilms: a common cause of persistent infections. Science 1999:284; 1318–1322.
13. Gilbert, P., J. Das, and I. Foley. Biofilm susceptibility to antimicrobials.Adv. Dent. Res1997: 11; 160–167.
14. Prasad SV, Ballal M, Shivananda PG. Slime production a virulence marker in *Pseudomonas aeruginosa* strains isolated from clinical and environmental specimens: A comparative study of two methods. Indian J PatholMicrobiol 2009; 52:191-193.
15. Oliveira A, Cunha MdeL.Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Res Notes.2010; 14: 260.
16. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, IqbalM.Detection and antibiotic susceptibility pattern of biofilm producing Gram positive and Gram negative bacteria isolated from a tertiary care hospital of Pakistan Malaysian Journal of Microbiology 2011: 7; 57-60.
17. Paterson DL, Bonomo RA. Extended-spectrum β-lactamases: a clinical update. ClinMicrobiol Rev 2005; 18: 657-686.
18. Bradford PA. Extended-spectrum beta-lactamases in the 21 st century: characterization, epidemiology and detection of this important resistance threat. ClinMicrobiol Rev 2001; 14: 933-951.
19. Bush K, Jacoby GA, Medeiros AA. A functional classification 3.scheme for β-lactamases and its correlation with molecular structure. *AntimicrobAgents Chemother*1995; *39:* 1211-1233.
20. Bonnet R. Growing group of extended-spectrum β-lactamases: 4. the CTX-M enzymes. AntimicrobAgents Chemother2004; *48:* 1-14.
21. Agrawal P, Ghosh AN, Kumar S, Basu B, Kapila K. Prevalence of extended-spectrum β-lactamases among *Escherichia coli* and *Klebsiellapneumoniae*isolates in a tertiary care hospital. Indian J PatholMicrobiol2008; *51:* 139-142.
22. Silva Dias RC, Borges-Neto AA, D'AlmeidaFerraiuoli GI, Oliveira MP, Riley LW, Moreira BM. Prevalence of AmpC and other beta-lactamases in enterobacteria at a large urban university hospital in Brazil.DiagnMicrobiol Infect Dis2008; 60: 79–87.
23. Rudresh SM, Nagarathnamma T. Extended spectrum β-lactamase producing *Enterobacteriaceae*& antibiotic co-resistance. Indian J Med Res 2011; 133: 116-118.
24. Kumar MS, Lakshmi V, Rajagopalan R. Occurrence of extended spectrum beta-lactamases among *Enterobacteriaceae* spp. isolated at a tertiary care institute. Indian J Med Microbiol 2006; 24:208-211.
25. Ananthakrishnan AN, Kanungo R, Kumar A, Badrinath S. Detection of Extended spectrum β-lactamases producers among surgical wound infections and burns patients in JIPMER. IndianJ Med Microbiol 2000;18:160-165.

26. Hemachandran K, Bharathi S, Radhakrishnan M , BalagurunathanR.Studies on extended beta lactamase producing,biofilm forming clinical bacterial pathogens and its invitro inhibition by Actinobacterial extracts. Journal of Applied Pharmaceutical Science 2011; 1: 210-213.
27. Medeiros AA. Evolution and dissemination of β-lactamases, accelerated by generations of β-lactam antibiotics.Clin InfectDis1997; 24: S 19-45.
28. Philippon A, Arlet G, Jacoby GA. Plasmid determined AmpC type β-lactamases. Antimicrob Agents Chemother2002; 46*:* 1-11.
29. Manchanda V, Singh NP.Occurrence and detection of AmpC β-lactamases among Gram-negative clinical isolates using a modified three-dimensional test at Guru TeghBahadur Hospital, Delhi, India.Journal of Antimicrobial Chemotherapy2003; 51:415–418.
30. Sinha P, Sharma R, Rishi S, Sharma R, Sood S, Pathak D. Prevalence of extended spectrum beta lactamase and AmpC beta lactamase producers among *Escherichia coli* isolates in a tertiary care hospital in Jaipur. Indian J PatholMicrobiol 2008; 51:367-369.
31. Subha A, Devi VR, Ananthan S. AmpC beta-lactamase producing multidrug resistant strains of *Klebsiella* spp. and *Escherichia coli* isolated from children under five in Chennai. Indian J Med Res 2003; 117:13-18.
32. .Hemachandran. K, Bharathi S, Radhakrishnan, Balagurunathan R.Studies on extended beta lactamase producing, biofilm forming clinical bacterial pathogens and its invitro inhibition by Actinobacterial extracts..Journal of Applied Pharmaceutical Science 2011; 01 (08) : 210-213
33. Bhaskaran T Nair, Kishore G Bhat, Manjula Shantaram .In Vitro Biofilm Production and Virulence Factors of Uropathogenic *Escherichia Coli* .Int J Pharm Bio Sci 2013; 4(1): 951 - 956
34. Donlan, R. M. and Costerton, W. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiological Reviews 2002:15; 167-193.
35. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms.Int J Med Microbiol.2002; 292:107-113.