International Journal of Microbiological Research 2 (3): 208-212, 2011
ISSN 2079-2093
© IDOSI Publications, 2011

Concurrent Determination of Collagenase and Biofilm Formation Activities in Metallo-Beta-Lactamase Producing *Pseudomonas aeruginosa*

[1]Debasrita Chakraborty, [1]Saikat Basu, [2]Payel Chatterjee, [3]Subrata Kumar Dey and [1]Satadal Das

[1]Research Fellow, West Bengal University of Technology, Kolkata-70006
[2]Department of Microbiology and Serology,
Peerless Hospital and B.K. Roy Research Centre, Kolkata-700094, India
[3]The School of Biotechnology, West Bengal University of Technology,
BF-142, Salt Lake City, Kolkata-700064, India

Abstract: *Pseudomonas aeruginosa*, an opportunistic pathogen causes community acquired infections and also hospital acquired infection. Metallo beta lactamase (MBL) producing *P. aeruginosa* are very difficult to exterminate, as a result they cause high morbidity and mortality among patients infected by them. Strains of *P. aeruginosa* exhibiting biofilm forming characteristic have been found to resist different antibiotic therapy and also their ability of collagenase formation showed increased pathogenicity. In this study, the difference of collagenase activity and degree of biofilm forming ability was analyzed using both MBL producing and MBL non-producing strains of *P. aeruginosa*. The study showed that MBL producers have strong Biofilm forming ability and higher collagenase activity and also we found out a correlation existing between these two virulence factors.

Key words: Collagenase % Biofilm % Metallo-Beta-Lactamase % *Pseudomonas aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is a gram-negative rod that belongs to the family Pseudomonadaceae which are widespread in nature, inhabiting soil, water, plants and animals, including humans. It is an opportunistic pathogen, implicating its exploitation on intermission in the host defense mechanisms to instigate infection. It causes community-acquired infections, for instance otitis externa, ulcer, skin and tissue infections and also causes hospital-acquired infections including urinary tract infection, pneumonia, bacteremia and burn infection [1].

Metallo beta lactamases (MBL) is a group of beta lactamase producing bacteria that requires zinc ion as cofactor for their activity. Carbapenems are the most potent beta lactams because of its strong affinity to penicillin binding proteins, stability against most serine beta lactamases and high permeability across the outer membrane [2, 3]. The misuse and overindulgence of it causes production of metallo beta lactamase producing bacteria, those are resistant to most of beta lactam antibiotics including imipenem and meropenem [4]. It has been seen that mortality rate of patients infected with MBL producing bacteria is high, mainly due to multi organ failure and mostly are male patients with old age group[5].

A biofilm is an aggregate of microorganisms in which cells of one or more species are attached with each other on a surface to form a self produced matrix of extracellular polymeric substances [6]. In the biofilm, cells communicate via quorum sensing [7] and they may alter in shape and size. Biofilm bacteria display particular phenotypes that distinguish them from their freely growing counterparts [8]. In the initial steps of biofilm formation different gene expressions are responsible [9], such as the up regulation of exopolysaccharides synthesis that causes cell adhesion [10].

One of the important characteristics of *P. aeruginosa* is biofilm formation that helps them to survive different antibiotic therapy and antibiotic resistant strains of *P. aeruginosa* have been found exhibiting better biofilm forming ability [11]. In chronic infection, *P. aeruginosa* generally grow in biofilm mode and causes persistent

Corresponding Author: Satadal Das, Department of Microbiology and Serology,
Peerless Hospital and B.K. Roy Research Centre, 360, Panchasayar, Kolkata-700094, India.
Tell: +91- 09831092130, 033-2462 2394, Fax: 91(33) 2462 0766, E-mail: drsatdas@hotmail.com.

infection [12]. This nature of biofilm formation of *P. aeruginosa* gives trouble to clinicians to treat infections, as they colonize on the surface of human tissues or catheters or other devices, from which they cause very serious infections.

P. aeruginosa produces several extracellular proteases including elastase and collagenase. Collagenase is a zinc metalloproteinase that catalyses the hydrolysis of native collagens, requires zinc and calcium ions as enzyme cofactors for its optimum activity with the pH of 6.3-7.5. They normally target the connective tissue in muscle cells and other body organs. Collagen, an inert, rigid protein found predominantly in skin, tendon, blood vessels, ligaments and bones, a key component of the animal extracellular matrix, is made through cleavage of procollagen by collagenase [13].

The aim of our study was to analyze the differences of collagenase activity as well as degree of biofilm forming ability between both of the MBL producing and non-producing strains of *P. aeruginosa* and find out whether there is a correlation between these two activities.

MATERIALS AND METHODS

MBL Producing and MBL Non-Producing Strains Selection: Strains of *P. aeruginosa* were collected from different clinical specimens of critically ill patients, admitted in ICCU and ITU wards of a tertiary care hospital of Kolkata, India. Specimens like blood, pus, stool, sputum, urine, throat swab and pleural fluid were the sources of the bacterial strains. With those collected strains MBL producers were selected by three phenotypic methods; imipenem-EDTA combined disc test [14], imipenem-EDTA double disc synergy test [15] and E-test [16]. Eight MBL producing and six MBL non-producing bacteria were selected.

Biofilm Production Determination: In this method, we used tryptic soy broth with 1% glucose. In each test tube, containing 10mL of this media one loopful of 18hrs fresh culture of *P. aeruginosa* was inoculated and incubated for 24 hrs at 37°C. The cell suspensions were poured out off the tubes and washed with PBS of pH 7.3 and dried, keeping the tubes in inverted state. The walls of the tubes were stained with 1% safranin for 5 min and then washed with deionized distilled water. The tubes were again dried and the viscid layer produced on the walls were interpreted as biofilm production. Ring formation only at the liquid interface should not be considered as biofilm formation, it should be a visible ring along with the film lined the wall and the bottom of the test tube. Biofilm production was scored as negative (-), weak positive (1+), moderate positive (2+) or strong positive (3+) [17].

Tendon Supernatant Preparation: Since the composition of normal tendon is made of about 86% of collagen, 2% elastin, 1-5% proteoglycan and remaining inorganic components such as ions of copper, magnesium, calcium, hence goat (*Capra indica*) tendon was used as the source of collagen in our study. Freshly bought goat tendon was washed thoroughly with deionized distilled water thrice and then it was sterilized under UV light of 275 nm wave length for 15 min. It was then chopped into very fine slices under sterile conditions. Again 15-20 min UV exposure was provided to those tendon slices which then were added to 10mL deionized water in sterile centrifuged tubes. The mixture was then thoroughly vortex mixed for 15 min and then centrifuged for 10 min at 3000 rpm to get well separated supernatant. The supernatant was used for further work.

Collagenase Production Determination: Eight MBL producing and six MBL-non producing *P. aeruginosa* strain suspensions were prepared keeping the turbidity at 0.5 McFarland standard. The tendon supernatant was thawed after 24 hrs and then 100ìl of it was loaded into U.V sterilized microtiter wells and the absorbance of the supernatant in micro wells was adjusted ~ 0.200 at 620 nm. 10ìl each of *P. aeruginosa* strain broth culture was loaded into wells containing the supernatant. A single strain was added to the suspension of three wells followed by the addition of another strain into the successive three wells and this was repeated. Immediately after the completion of the additions the absorbance was measured at 620 nm, this was again followed after every 30 min intervals. We considered the initial O.D value along with the value of the first 30 min interval [18]. Since the generation time of *P. aeruginosa* is 45 min so after 1 h they would get doubled in number and error may occur in O.D value recordings, hence the rest of the O.D values after the first 30 min were not considered for interpretation.

RESULTS

Biofilm Formation: It was found that MBL producing *P. aeruginosa* could produce increased amounts of biofilms in comparison to MBL non-producing *P. aeruginosa.* Thus 62.5% of MBL producing *P. aeruginosa* could produce markedly higher amounts of biofilms while 37.5% of this category was moderately

Table 1: Biofilm formation of MBL producing and MBL non producing *P. aeruginosa*

		Biofilm Production			
Species	Number of isolates	3(+)	2(+)	1(+)	0
MBL producing *P. aeruginosa*	8	5	3	-	-
MBL non producing *P. aeruginosa*	6	-	1	4	1

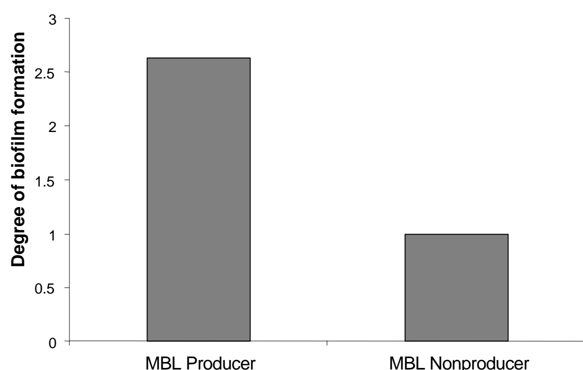

Fig. 1: Degree of biofilm formation of MBL producing and MBL nonproducing *P.aeruginosa*

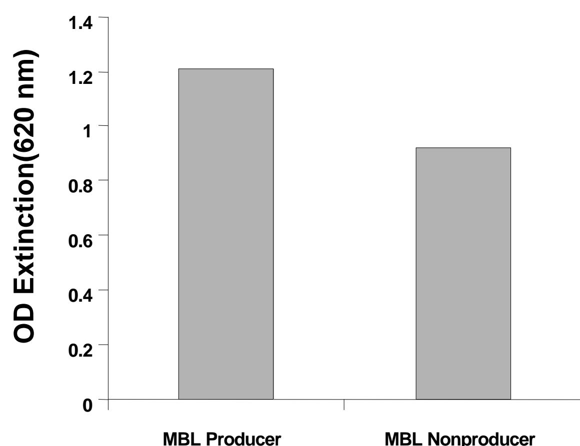

Fig. 2: Collagenase activities of MBL producing and MBL non-producing *P. aeruginosa*

positive biofilm producers and there were no weakly positive biofilm producers or biofilm non producers. In case of MBL-non producing *P. aeruginosa*, only 16.6% were moderately positive biofilm producers, while 66.6% and 16% were weakly positive biofilm producers and biofilm non producers respectively (Table 1, Fig. 1).

This difference in the degree of biofilm formation in between MBL producing and MBL non-producing *P. aeruginosa* was found highly significant after statistical analysis. In MBL producers the mean value of degree of biofilm production with SD and SEM was, 2.63± 0.52± 0.18 and in case of MBL non producers these values were 1± 0.63± 0.26. The t-value was found to

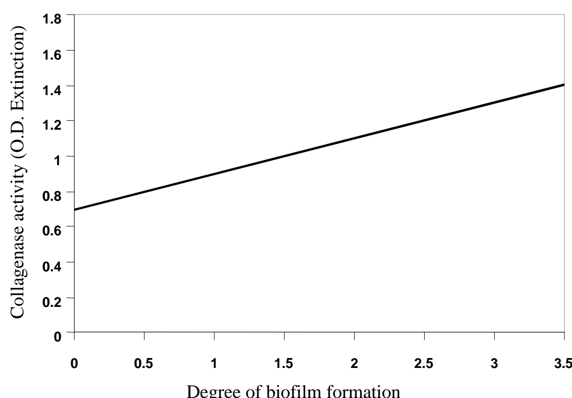

Fig 3: Correlation between biofilm formations and collagenase activities P.aeruginosa

be 5.29 and the two tailed P value was 0.0002, that was considered to be highly statistically significant.

Collagenase Activities of *P. aeruginosa*: In MBL producers the mean value of collagenase activity with SD and SEM were 1.20±0.225±0.0797 (OD extinction values at 620 nm 30 min as the ratio between the initial and final values) and in case of MBL non producers these values were 0.925±0.145±0.059. The t-value was found to be 2.67 and the two tailed P value was 0.0203, that was considered to be statistically significant. The difference between the mean collagenase activities was shown in fig. 2. When collagenase activities were correlated with degree of biofilm formations, it was found that there was a very significant positive correlation in between the two (Spearman rho correlation value was +0.92, Fig. 3).

DISCUSSION

P. aeruginosa is an important opportunistic pathogen that causes life threatening infections in immunocompromised patients and also causes outbreaks of hospital acquired infections [19]. MBL production is a very significant problem in intensive care unit isolates of *P. aeruginosa.* Recent upsurge of multi drug resistance bacteria throughout the globe also accentuates number of MBL producing bacteria which leads to significant increase in morbidity and mortality in hospitals.

Biofilm production has been reported in strains of *P. aeruginosa* and most Pseudomonas infections are difficult to eradicate because they have a tendency to produce biofilms which confers efficient protection of bacteria against activities of host defense system. On the surface of many biomedical devices *P. aeruginosa* grow in biofilm mode [20].

Production of collagenase and biofilms are considered to be the important virulence factors for *P. aeruginosa*.In this study we observed that collagenase activities and degree of biofilm formations are correlated in *P. aeruginosa*. Thus we find that in those bacteria in which collagenase activities were high, strongly or moderately positive biofilms were produced by them.

When we compared the differences of the degree of biofilm formations between MBL producing and MBL non-producing *P. aeruginosa*, we found that MBL producers are able to form thick and very prominent biofilms while MBL non-producers either can not form biofilm or form very weakly positive biofilms. MBL producers had also higher collagenase activities compared to MBL non-producers. It can be concluded that MBL production has some relationship with collagenase activity and degree of biofilm formation. If we consider the virulence, biofilm formation and toxin secretion are the important factors. Higher degree of biofilm formation and collagenase production make those bacteria highly pathogenic and because of MBL secretion, it is very difficult to kill them. In conclusion we may say that multidrug resistant and MBL producing bacteria are more capable of producing collagenase and degree of biofilm formation is also high in them.

REFERENCES

1. Pierce, G.E., 2005. *Pseudomonas aeruginosa*, Candida albicans and device-related nosocomial infections: implications, trends and potential approaches for control. J. Ind. Microbiol. Biotechnol., 32: 309-318. Make references like this style.
2. Livermore, D.M., 2001. Of Pseudomonas, porins, pumps and carbapenems. J. Antimicrob. Chemother., 47: 247-250.
3. Livermore, D.M., 1995. $-lactamases in laboratory and clinical resistance. Clin. Microbiol. Rev., 8: 557-584.
4. Walsh, T.R., M.A. Toleman, L. Poirel and P. Nordmann, 2005. Metallo-$-lactamases: the quiet before the storm? Clin. Microbiol. Rev., 18: 306-325. Make references like this style.
5. Chakraborty, D., S. Basu and S. Das, 2010. A Study on Infections Caused By Metallo Beta Lactamase Producing gram Negative Bacteria in Intensive Care Unit Patients. American J. Infect. Dis., 6: 34-39. DOI:10.3844/ajid sp.2010.34-39.
6. Samaranayake, L.P., 2006. Essential microbiogy for dentistry. 3rd edition, Edinburgh. Churchill Livingstone.
7. Liu, Z., F.R. Stirling and J. Zhu, 2007. Temporal quorum sensing induction regulates *Vibrio cholerae* biofilm architecture. Infect. Immun., 75: 122-126.
8. Watnick, P. and R. Kolter, 2000. Biofilm, city of microbes. J. Bacteriol., 182: 2675-2679.
9. Prigent-Combaret, C., O. Vidal, C. Dorel and P. Lejeune, 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol., 181: 5993-6002.
10. Davies, D.G., A.M. Chakrabarty and G.G. Geesey, 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol., 59: 1181-1186.
11. Drenkard, E. And M.F. Ausubel, 2002. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. Nature, 416: 740-743. DOI:10.1038/416740a.
12. Schaber, A., T. Jeffrey, J. Sang, J. Oliver, C. Hastert, A. Griswold, Manfred, Auer., Abdul, Hamood and R. Kendra, 2007. Pseudomonas aeruginosa forms biofilms in acute infection independent of cell-to-cell signaling. Infection and Immunity, 75: 3715-3721. DOI:10.1128/IAI.00586-07.
13. Lodish, H., A. Berk, S.L. Zipursky, *et al*., 2000. Molecular Cell Biology. 4th edition, New York, W.H. Freeman.
14. Yong, D., K. Lee, J.H. Yum, H.B. Shin, G.M. Rossolini *et al.,* 2002. Imipenem-EDTA disk method for differentiation of metallo-$-lactamases producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J. Clin. Microbiol., 40: 3798-3801. DOI:10.1128/JCM.40.10.37983801.2002.
15. Lee, K., Y.S. Lim, D. Yong, J.H. Yum and Y. Chong, 2003. Evaluation of the Hodge test and the imipenem-EDTA double disc synergy test for differentiationof metallo-$-lactamases producing clinical isolates of Pseudomonas spp. and Acinetobacter spp. J. Clin. Microbiol., 41: 4623-4629. DOI:10.1128/JCM.41.10.4623-4629.2003.

16. Walsh, T.R., M.A. Toleman, L. Poirel and P. Nordmann, 2005. Metallo-beta-lactamase: The quiet before the storm? Clin. Microbiol. Rev., 18: 306-325.DOI:10.1128/CMR.18.2.306-325.2005.
17. Basu, S., D. Chakraborty, S.K. Dey and S. Das, 2011. Biological Characteristics of Nosocomial *Candida tropicalis* Isolated from Different Clinical Materials of Critically Ill Patients at ICU. Int. J. Microbiol. Research, 2: 112-119.
18. Nagaveni, S., H. Rajeswari, A.K. Oli, S.A. Patil and R.K. Chandrakanth, 2010. Evaluation of biofilm forming ability of mutidrug resistant Pseudomonas aeruginosa., The Bioscan, 5: 563-566.
19. Sader H.S., A.C. Gales, M.A. Pfaller *et al.,* 2001. Pathogen frequency and resistance patterns in Brazilian hospital: Summery of results from three years of the SENTRY Antimicrob. Surveillance Program. Braz. J. Infect Dis., 5: 200-14.
20. Donlan, R.M. And J.W. Costeron, 2002. Biofilms:Survival mechanisms of clinically relavant microorganisms. Clin. Microbiol. Rev., 15: 167-193.