Int J Pharm Bio Sci 2013 Oct; 4(4): (B) 203 - 208

Research Article **Microbiology**

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

STUDY OF BIOFILM FORMATION IN GRAM POSITIVE CLINICAL ISOLATES AND ASSOCIATED RISK FACTORS

MUKESH SWARNAKAR[1], KARUNA TIWARI[2] AND TUHINA BANERJEE[*2]

[1] Department of Botany, BHU, Varanasi, India
[2] Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India

ABSTRACT

Biofilm production in bacteria causing infections is of recent interest especially in relation to emerging drug resistance and increased use of medical devices. The present study was performed to determine biofilm production among pathogenic Gram positive cocci in association with antibiotic resistance and other risk factors. A total of 100 clinical isolates of *Staphylococcus aureus* (50) and enterococci (50) were included for antibiotic susceptibility testing and semi-quantitative biofilm production assay. Fischer's exact test was used to study the association of infection with biofilm producers and other risk factors. Biofilm production varied with drug resistance and use of indwelling urinary catheters, intravascular catheters and prolonged hospital stay were significant risk factors ($p<0.05$). Prudent use of antibiotics and invasive devices should be considered along with a better understanding of the mechanisms of biofilm formation for effective control of these biofilm producing organisms.

KEYWORDS: Gram positive cocci, biofilm, drug resistance, devices

TUHINA BANERJEE
Department of Microbiology, Institute of Medical Sciences, BHU, Varanasi, India

INTRODUCTION

With the widespread and ever increasing use of antibiotics along with invasive medical devices, microorganisms too have developed different adaptations to fight the adverse environment. Of the several such adaptations, biofilm production is perhaps one of the most troublesome to the medical community. By definition biofilms are microbial derived sessile communities attached to a surface and embedded in a self produced polymetric matrix [1]. Biofilms help bacteria to survive and persist at sites of infection or colonization and make them considerably resistant to host defence mechanisms and antibiotics, thus accounting for nearly 60% of the infections [2]. Though biofilm production is a property in most of the bacteria, majority of the studies have focused on those pathogens with remarkable ability to produce biofilm. Gram negative bacilli especially the nonfermenters are a prominent member of this group [3]. However, biofilm production is also being increasingly recognized as an important virulent trait in Gram positive organisms. The most noted in the latter group are staphylococci mostly *Staphylococcus aureus* and *Staphylococcus epidermidis* especially in association with medical devices usage [4]. Similarly, biofilm forming ability of enterococci, also a Gram positive cocci and a member of the normal body flora is basic in causing dental infections and urinary tract infections (UTI) [5]. With this background, this study was undertaken to determine biofilm production in staphylococci and enterococci isolated from clinical samples, their drug resistance and the risk factors associated with them.

MATERIALS AND METHODS

A total of 100 clinical isolates comprising of 50 *Staphylococcus aureus* and 50 *Enterococcus* spp were included in the study. Clinical samples were collected from different patients attending the various units of a tertiary care university hospital in north India. Primary isolation was done on cystine lactose electrolyte deficient (CLED) agar medium for urine samples and on blood agar plates for other samples. Preliminary identification of the isolates was done by Gram staining followed by characterization of species based on standard tests [6, 7]. Antimicrobial susceptibility testing was done by disc diffusion method based on Kirby Bauer method [8] using the following discs for enterococcal isolates ampicillin (30µg), ciprofloxacin (5µg), nitrofurantoin (300 µg)(for urinary isolates only), high strength gentamicin (120 µg),vancomycin (30 µg), linezolid (30 µg). For staphylococcal isolates, ciprofloxacin (5 µg), cotrimoxazole (7.5/2.5µg), gentamicin (10 µg), nitrofurantoin (300 µg) (for urinary isolates only), erythromycin (10µg) (Hi Media, India) was used. Screening for vancomycin resistant enterococci (VRE) was done by agar dilution method using 6 µg/ml vancomycin (Hi Media, India). Screening for MRSA was done by cefoxitin disc (30 µg) for the staphylococcal isolates [8] as per standard guidelines.Biofilm production was determined by semi-quantitative adherence assay as described elsewhere [9]. Briefly, an overnight culture grown in brain heart infusion broth (BHI) at 37°C was diluted to 1:100 with 2% glucose. A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate (Tarsons, India). The plates were incubated aerobically at 37°C for 24 hr followed by washing of the microtiter plate twice with phosphate buffered saline (PBS) and drying. Adherent bacteria were fixed with 95% ethanol and stained with 1% crystal violet solution for 15 min. This was followed by washing and drying of the microplates and reading the optical density (OD) of each well at 570 nm using an automated ELISA reader. Strong biofilm producers were considered in those with OD values >0.5. Moderate biofilm production was considered in those with OD values >0.2 but <0.5. Values lower than this was not taken positive for biofilm in this study. Wells with sterile BHI alone served as negative control and a known biofilm producing strain of *Candida albicans* served as a positive control.Infections with biofilm producing isolates (cases) were compared with non biofilm producing isolates

(controls) using the Fischer's exact test. Odds ratio was calculated to analyze the risk factors associated with infection due to biofilm producing Gram positive strains. For comparison, a p value <0.05 was considered significant.

RESULTS

A total of 100 isolates were studied comprising of 20 methicillin susceptible *Staphylococcus aureus* (MSSA), 30 methicillin resistant *Staphylococcus aureus* (MRSA), 28 *E.faecium* and 22 *E. fecalis.* On screen agar, 38 of the total 50 enterococci were high level gentamicin resistant enterococci (HLGRE) and 6 were VRE. The sources of the isolates are shown in Table 1. Majority of the enterococcal isolates were collected from patients with UTI whereas main source of staphylococcal isolates were from pus and wound infections. Of the total, 42 isolates were biofilm producers. Strong biofilm production was seen in 17 *Staphylococcus aureus* isolates and 13 enterococcal isolates (8 *E.faecium* and 5 *E.fecalis*). Moderate biofilm production was seen in 12 enterococcal isolates (6 *E.faecium* and 6 *E.fecalis*).

Table 1
Source of the isolates

Source	Enterococci n(%)	Staphylococci n(%)
Urine	32 (64)	7 (14)
Blood	2 (4)	15 (30)
Exudates	16 (32)	28 (56)

Drug resistance pattern of the isolates and biofilm production has been shown in Table 2. Among the enterococcal isolates, while resistance to 3 drugs was found in most of the isolates without biofilm production, resistance to 4 or 5 drugs including vancomycin was mostly noted amongst biofilm producers. Majority of the MSSA (80%) and MRSA (56.6%) isolates did not show biofilm formation. However, MRSA with multidrug resistance (MDR) were also biofilm producers in 26.6% of the cases.

Table 2
Resistance pattern and biofilm production

Resistance pattern	Biofilm producers	Biofilm nonproducers
Enterococci		
$Amp^{R}HSG^{R}Cip^{R}$	10 (10/25, 40%)	11 (11/25, 44%)
$Amp^{R}HSG^{R}Cip^{R}Nitro^{R}$	8 (8/25, 32%)	3 (3/25, 12%)
$Amp^{R}HSG^{R}Cip^{R}Nitro^{R}Van^{R}$	4 (4/25, 16%)	2 (2/25, 8%)
Staphylococci		
MSSA	4 (4/20, 20%)	16 (16/20, 80%)
MRSA	13 (13/30, 43.3%)	17 (17/30, 56.6%)
$MRSA^{+}Cip^{R}Gen^{R}$	8 (8/30, 26.6%)	3 (3/30, 10%)

Amp=ampicillin, HSG=high strength gentamicin, Cip=ciprofloxacin, Nitro=nitrofurantoin, Van=vancomycin, Gen=gentamicin, R=resistance

Analysis of the risk factors associated with infection due to biofilm producing isolates revealed that they were more common among the higher age group. Association with indwelling urinary catheters and prolonged duration of hospital stay were significant (Table 3).

Table 3
Risk factors associated with infection by biofilm producing isolates

Risk factors	Cases n=42	Controls n=58	Odds ratio (95% CI)	p value
Mean age (yrs)	42.4	22.3	-	-
Male sex	34	35	1.51 (0.70-3.22)	0.282
Indwelling catheters	17	8	4.25 (1.61-11.18)	0.003*
Intravascular catheters	9	2	7.6 (1.55-37.5)	0.012*
Prolonged hospital stay (> 7 days)	13	2	12.55 (2.6-59.4)	0.001*

p < 0.05

DISCUSSION

Bacterial adherence to host tissue is a very vital step in initiation of any infection process [10]. Biofilm formation helps in adhesion to host epithelial surfaces so that organisms can exert their toxin mediated damages. Additionally, these highly organized complex structures protect organisms from direct effects of antimicrobial agents and thus make infections unresponsive to treatment. Enterococci, the otherwise gut commensals are also equally important as nosocomial pathogens ranking amongst the most common causes of nosocomial blood stream infections, surgical sites infections and UTIs [11]. *E.fecalis* isolates from infection have been shown to form biofilms in vitro and also been seen to be formed on indwelling devices. Majority of the biofilm producing isolates of enterococci was isolated from urine. In UTI, biofilm production within the urinary tract helps in effective adherence of the organisms thus facilitating long term persistence. Similarly, *Staphylococcus aureus* is also noted for their ability to form biofilm helping in adherence of these organisms onto the surface of medical devices. Infections caused by them are troublesome not only in the healthcare settings but also in the community. Recently, increasing tendency to give rise to complications following trivial soft tissue infections has been noted in MRSA primarily due to increasing drug resistance [12]. The persistence of microorganisms and therefore the infections causes the requirement of prolonged therapy and emergence of antimicrobial resistance in biofilm producing organisms. UTI due to MRSA has also been increasing due to frequent use of various urinary stents and catheters [13].Drug resistance is an emerging problem associated with nosocomial pathogens. One of the major contributing factors to the emergence of drug resistance in enterococci is its ability to form biofilm. In this study, MDR enterococci were increasingly associated with biofilm production. However, majority of MRSA did not produce biofilm. Often it has been noted that the 'sessile forms' which are the major forms in a biofilm structure, are more drug resistant than their 'planktonic forms' which grow on artificial media in the laboratory [1]. Even if certain drugs show in vitro activity on these microorganisms, subpopulation of highly resistant biofilm producing organisms may remain undetected by the laboratory method. Later, in vivo this subpopulation may lead to treatment failure ultimately requiring removal of implanted devices or surgical removal of colonized surfaces [14, 15]. The study showed a significant association with use of catheters and intravascular devices and infection caused by biofilm producing organisms. Usage of central venous catheters, urinary catheters, prosthetic heart valves and orthopedic devices are often colonized or infected with different microorganisms, all sharing a common ability for biofilm formation. External colonization takes place with either skin flora or other organisms followed by internalization of the organisms in case of intravascular or intra luminal devices. Organisms may ultimately enter the bloodstream or invade tissues thus leading to severe infections [16, 17]. Even though the sites of infection are different, all these devices usually present with chronic infection unresponsive to

treatment. Moreover, prolonged hospital stay was recognized as an important risk factor. The hospital environment serves as an important reservoir for these pathogens under selective antibiotic pressure and easy dissemination from patient to patient due to lack of proper infection control practices [18]. Therefore, from better healthcare point of view, complete eradication of these biofilm producing organisms from the hospital environment often becomes demanding due to ineffective infection control strategies and indiscriminate antibiotic use [19] both in the hospitals and in the community.

CONCLUSION

Biofilm production in Gram positive cocci is equally effective virulent trait responsible for chronicity of infections and persistence of these organisms in infection sites and hospital environment. Due to their association with invasive devices, a judicious approach should be adopted regarding usage of these devices and administration of antibiotics, as these are the major factors responsible for their easy dissemination and survival.

REFERENCES

1. Flemming K, Klingenberg C, Cavanagh JP, Sletteng M, et al, High in vitro antimicrobial activity of synthetic antimicrobial peptidomimetics against staphylococcal biofilms. Journal of antimicrobial chemotherapy, 63(1): 136-145, (2009).
2. Spoering AM and Lewis K, Biofilm and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials, J Bacteriol,183:6746–51, (2001).
3. Abdi-Ali A, Mohammadi-Mehrb M,Agha Alaei Y, Bactericidal activity of various antibiotics against biofilm-producing Pseudomonas aeruginosa,International Journal of Antimicrobial Agents, 27:196–200, (2006).
4. Mathur T, Singhal S, Khan S, Upadhyay DJ, et al, Detection of biofilm formation among the clinical isolates of Staphylococci:an evaluation of three different screenig methods, Indian J Med Microbiol, 24(1):25-9, (2006).
5. Fisher K and Phillips C, The ecology, epidemiology and virulence of Enterococcus, Microbiology, 155: 1749–57, (2009).
6. Iorio NLP,Ferreira RBR, Schuenck RP, Malvar KL, et al, Simplified and Reliable Scheme for Species-Level Identification of Staphylococcus Clinical Isolates, J Clin Microbiol, 45(8): 2564–69, (2007).
7. Facklam RR and Collins MD, Identification of Enterococcus species isolated from human infections by a conventional test scheme, J Clin Microbiol, 27:731–34, (1989).
8. Clinical Laboratory and Standards Institute,Performance standard for antimicrobial susceptibility testing; Twenty first informational supplement M100:31(1), (2011).
9. Kouidhi B, Zmantar T, Mahdouani K, Hentati H, Bakhrouf A, Antibiotic resistance and adhesion properties of oral Enterococci associated to dental caries, BMC Microbiol,29;11:155, (2011).
10. Jett BD, Huycke MM and Gilmore MS, Virulence of enterococci, Clin Microbiol Rev, 7(4):462-78, (1994).
11. Sood S, Malhotra M, Das BK, Kapil A, Enterococcal infections & antimicrobial resistance, Indian J Med Res, 128: 111-21, (2008)
12. Kokai-Kun JF, Chanturiya T ,Mond JJ, Lysostaphin eradicates established Staphylococcus aureus biofilms in jugular vein catheterized mice, Journal of Antimicrobial Chemotherapy, 64: 94–100, (2009).
13. de Araujo GL, Coelho RL, de Carvalho CB, Maciel1 RM,et el, Commensal isolates of methicillin-resistant Staphylococcus epidermidis are also well equipped to produce biofilm on polystyrene surfaces,

Journal of Antimicrobial Chemotherapy, 57: 855–64, (2006).
14. Hill KE, Malic S, McKee R, Rennison T, et al, An in vitro model of chronic wound biofilms to test wound dressings and assess antimicrobial susceptibilities. J Antimicrob Chemother, 65(6):1195-206, (2010).
15. Kokare CR, Chakraborty S, Khopade AN, Mahadik KR, Biofilm: importance and applications. Indian J Biotechnol, 8(2): 159-68, (2009).
16. Rupp ME and Hamer KE, Effect of subinhibitory concentrations of vancomycin, cefazolin, ofloxacin, L-ofloxacin and D-ofloxacin on adherence to intravascular catheters and biofilm formation by Staphylococcus epidermidis, J Antimicrob Chemother, 41(2):155-61, (1998).
17. Sauer K, Steczko J, Ash SR, Effect of a solution containing citrate/Methylene Blue/parabens on Staphylococcus aureus bacteria and biofilm, and comparison with various heparin solutions, J Antimicrob Chemother, 63(5):937-45, (2009).
18. Karpanen TJ, Worthington T, Hendry ER, Conway BR, Lambert PA, Antimicrobial efficacy of chlorhexidine digluconate alone and in combination with eucalyptus oil, tea tree oil and thymol against planktonic and biofilm cultures of *Staphylococcus epidermidis*, J Antimicrob Chemother, 62(5):1031-6,(2008).
19. Lalan BK, Hiray RS, Ghongane BB, Drug prescription pattern of outpatients in a tertiary care teaching hospital in Maharashtra, Int J Pharm Bio Sci, 3(3):225-29, (2012).