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SHORT COMMUNICATION

Detection and antibiotic susceptibility pattern of biofilm producing Gram positive and Gram negative bacteria isolated from a tertiary care hospital of Pakistan

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ABSTRACT

Microorganisms adhere to non-living material or living tissue, and form biofilms made up of extracellular polymers/slime. Biofilm-associated microorganisms behave differently from free-floating bacteria with respect to growth rates and ability to resist antimicrobial treatments and therefore pose a public health problem. The objective of this study is to detect the prevalence of biofilm producers among Gram positive and Gram negative bacteria isolated from clinical specimens, and to study their antimicrobial susceptibility pattern. The study was carried out from October 2009 to March 2010, at the Department of Microbiology, Army Medical College/ National University of Sciences and Technology (NUST), Rawalpindi, Pakistan. Clinical specimens were received from various wards of a tertiary care hospital. These were dealt by standard microbiological procedures. Gram positive and Gram negative bacteria isolated were subjected to biofilm detection by congo red agar method (CRA). Antimicrobial susceptibility testing of those isolates, which showed positive results (slime production), was done according to the Kirby-Bauer disc diffusion technique. A total of 150 isolates were tested for the production of biofilm/slime. Among them, 81 isolates showed positive results. From these 81, 51 were Gram positive and 30 were Gram negative. All the 81(54%) slime producers showed reduced susceptibility to majority of antibiotics. Bacterial biofilms are an important virulence factor associated with chronic nosocomial infection. Detection of biofilm forming organisms can help in appropriate antibiotic choice.

Keywords: Antibiotic resistance, Biofilm, Congo red agar method

INTRODUCTION

A biofilm is a complex aggregate of microorganisms in which cells are adhere to each other and to a surface. These adherent cells are embedded within a self-produced matrix of extracellular polymeric substance (EPS)/slime. Slime is made up of proteins and polysaccharides. 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 (Waters and Bassler, 2005). Availability of key nutrients, chemotaxis towards surface, motility of bacteria, surface adhesins and presence of surfactants are certain factors which influence biofilm formation (Thomas and Day, 2007). Both the Gram positive and Gram negative bacteria have the capability to form biofilms. Bacteria commonly involved include *Enterococcus faecalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus viridans*, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis* and *Pseudomonas aeruginosa* (Donlan, 2001). 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 (Stewart and Costerton, 2001). According to a research, more than 60% of all infections involve biofilms (Kim, 2001). There are various methods to detect the biofilm producers among the microorganisms. Congo Red Agar (CRA) is a method that can be used to determine the ability of the organism to produce biofilms. 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. This will help our clinicians in prescribing an appropriate antibiotic against chronic infections or for patients having indwelling device which promote the chances of a biofilm production.

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MATERIALS AND METHODS

Clinical specimens of urine, blood, pus, sputum, catheter tips, central venous catheters, high vaginal swab, naso-bronchial lavage were received from patients admitted in tertiary care hospital over a period of six months. All these specimens were inoculated on appropriate culture media (blood agar, MacConkey's agar, chocolate agar, Oxoid, UK) and incubated for 24 h at 37 ℃. After incubation, organisms were identified by standard microbiological procedures (Gram's stain appearance, colonial morphology, catalase test, cytochrome oxidase reaction, motility, triple sugar iron test, urease test, citrate test, indole test, DNAase test). We tested the isolated organisms for their ability to form biofilm by the production of slime using the Congo red agar method (CRA). CRA medium was prepared with brain heart infusion broth (Oxoid, UK) 37 g/L, sucrose 50 g/L, agar No 1 (Oxoid, UK) 10 g/L and Congo red indicator (Oxoid, UK) 8 g/L. First congo red stain was prepared as a concentrated aqueous solution and autoclaved (121 ℃ for 15 min) separately from the other medium constituents. Then it was added to the autoclaved brain heart infusion agar with sucrose at 55 ℃ (Freeman *et al*., 1989). CRA plates were inoculated with test organisms and incubated at 37 ℃ for 24 h aerobically. *Staphylococcus epidermidis* ATCC 35984 (high slime producer) and *S. epidermidis* ATCC 12228 (non-slime producer) were used as control strain. Black colonies with a dry crystalline consistency indicated biofilm production (Freeman *et al*., 1989). The experiment was performed in triplicate and repeated three times. Antibiotic susceptibility test of Gram positive and Gram negative biofilm producers was performed by using the Kirby-Bauer disc diffusion techniques according to CLSI guidelines (Bauer *et al*., 1966; Wayne, 2009). Inocula were prepared by suspending the isolates in normal saline equal to the turbidity of 0.5 McFarland turbidity standard (10^6 CFU/mL) and applied on Mueller Hinton agar (Oxoid, UK) plates. All antibiotic discs were obtained from Oxoid, UK. Antibiotic discs were used depending on the type of microorganism and on the type of specimen (ampicillin 10 µg, cotrimoxazole 25 µg, ciprofloxacin 5 µg, aztreonam 30 µg, meropenem 15 µg, cefoperazone-sulbactam 105 µg, chloramphenicol, vancomycin 30 µg, erythromycin 15 µg, amoxicillin-clavulanic acid 20/10 µg, oxacillin 1 µg, linezolid 30 µg, penicillin 10 units, gentamicin 10 µg). These were incubated along with controls for 18-24 h at 37 ℃ aerobically. *Escherichia coli* ATCC 25922 was used as control strain. The results were interpreted according to criteria set by Clinical and Laboratory Standards Institute (CLSI) (Wayne, 2009).

RESULTS

A total of 150 organisms were tested in our study. Of them, 31 isolates showed black colonies with dry crystalline consistency (high biofilm producer), 50 showed black colonies with intermediate consistency (moderate biofilm producer),), and 69 showed pink/bordeux coloured colonies with mucoid appearance (weak/non-biofilm producers). Among those 81 (31 high and 50 moderate), 63% were Gram positive and 37% were Gram negative bacteria. Tables 1 and 2 show the Gram positive and Gram negative bacteria with potential of forming biofilm. Among the *Staphylococcus epidermidis,* maximum biofilm producers were from catheters (21 out of 27). Among 21 catheters from which *S. epidermidis* were isolated, 13 were from intravenous catheters and 8 were from foley's catheter. Table 3 shows the specimen from which Gram positive and Gram negative biofilm producers were isolated. A high antibiotic resistance pattern was seen in biofilm producers. Tables 4 and 5 show the antimicrobial resistance pattern of Gram positive and Gram negative biofilm producing bacteria in this study, respectively. Gram positive biofilm producer were more resistant to penicillin, rifampicin, oxacillin, ciprofloxacin, erythromycin and cotrimoxazole than non biofilm producer. All Gram positive biofilm producers were sensitive to linezolid and vancomycin. All Gram negative biofilm producers were more resistant to ampicillin, ciprofloxacin, cotrimoxazole, aztreonam, amikacin, ceftriaxone and cefoperazone and sulbactam as compared to non biofilm producing Gram negative bacteria. All Gram negative biofilm producing bacteria were sensitive to meropenem.

Table 1: Gram positive biofilm producers (n=51)

Organism	Number (%)
Staphylococcus epidermidis	27 (52.9)
Staphylococcus aureus	18 (35.2)
Enterococcus faecalis	6 (11.7)

Table 2: Gram negative biofilm producers (n=30)

Organism	Number (%)
Escherichia coli	14 (46.6)
Klebsiella pneumoniae	9 (30)
Enterobacter species	5 (16.6)
Citrobacter freundi,	2 (6.6)

Table 3: Sources of biofilm producing bacteria (n=81)

Specimen	Number (%)
Urinary catheter tips	24 (29.6)
Intravenous catheter tips	19 (23.4)
Pus	13 (16)
Urine	11(13.5)
Nasobronchial lavage	6 (7.4)
High vaginal swab	5 (6.1)
Sputum	3 (3.7)

DISCUSSION

In our study, 54% of the tested organisms have shown the potential to make biofilms. This highlights the high prevalence of resistant microorganism in our set up.

Table 4: Resistance pattern of biofilm producer *S. aureus* in comparison with non-biofilm producer *S. aureus*

Antimicrobial agent	Biofilm producer *S. aureus* %	Non-biofilm producer *S. aureus* %
Penicillin	100	100
Rifampicin	70	30
Ciprofloxacin	40	10
Erythromycin	40	20
Cotrimoxazole	30	25
Linezolid	0	0
Vancomycin	0	0

Table 5: Resistance pattern of Gram negative biofilm producers in comparison with non-biofilm producers

Antimicrobial agent	Biofilm producer Gram negative organisms %	Non-biofilm producer Gram negative organisms %
Ampicillin	100	100
Ciprofloxacin	95	50
Cotrimoxazole	90	83
Aztreonam	90	50
Amikacin	64	37
Ceftriaxone	58	33
Cefoperazone-sulbactam	36	0
Meropenem	0	0

Baqai *et al.* (2008) also reported high occurrence of biofilm producing bacteria (75% among the uropathogens, mainly from *S. aureus* (75 %), *E. faecalis* (75%) and *E. coli* (40%). *S. epidermidis* was the major isolate from clinical samples that formed biofilm. We found that after *S. epidermidis*, *S. aureus* (18 out of 51*)* are involved in large number in production of biofilm. Ammendolia *et al.* (1999) and Bose *et al.* (2009) also reported involvement of *S. aureus* in biofilm production. In this study, majority of the biofilm producers were isolated from catheter tips (intravenous and urinary, 29.6 and 23.4% respectively) followed by urine and pus specimens. In our study, antibiotic susceptibility pattern of biofilm producing organisms was obtained. The clinically relevant observation was high resistance of biofilm producers to commonly used antibiotics. This observation was also mentioned in another study (Donlan and Costerton, 2002). We have seen that Gram positive biofilm producers showed 100% sensitivity to vancomycin and linezolid. Among the Gram negative bacteria, most of them were sensitive to broad spectrum antibiotics like meropenem, imipenem and cefoperazone-sulbactam. We have performed CRA method to detect biofilm production. The CRA medium was prepared according to protocol by Freeman *et al.* (2009). Biofilm producers produce black colonies and non-producers form pink colored colonies on CRA. It is known that Congo red can directly interact with certain polysaccharide forming colored complexes. Jain and Agarwal (2009) also supported the use of CRA method for biofilm detection. In a country like ours, a low cost method for detection of biofilm is needed which require inexpensive equipment. CRA test is easy to perform and less time consuming.

Microbial biofilms lead to chronic infections. Such infections are a major challenge for the physicians and have economic relevance as well. Detection of biofilm producers and appropriate antibiotic doses can help prevent such problems.

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