ANTIBIOTIC RESISTANCE AND BIOFILM FORMATION AMONG NOSOCOMIAL PATHOGENS IN A TERTIARY CARE HOSPITAL

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ABSTRACT

BACKGROUND

Biofilm formation is an important mode of growth because it is associated with the chronic nature of the subsequent infections, and it helps in inherent resistance to antibiotic chemotherapy. Various nosocomial infections which are associated with the indwelling devices are the source as their surfaces become a niche for biofilm producing microorganisms.

MATERIALS AND METHODS

Various tip cultures received in microbiology lab from OPD and IPD patients were processed for isolation, identification and antimicrobial sensitivity. Different tip cultures which were included in the present study were urinary catheter tip, tracheostomy tip, endotracheal tip, chest tube tip and central line tip. After identification, the different isolates were investigated for antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) and the zones were interpreted as per CLSI guidelines (4, 5). Multi-drug Resistant (MDR) organisms viz. *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii* were then further tested for biofilm production by microtiter plate method and tube method.

RESULTS

Highest percentage of biofilm producing organisms was of *K. pneumoniae*, followed by *A. baumannii* and *P. aeruginosa* whereas least strains were of *S. aureus*. Tube adherence method detected 2.33% strong producers whereas microtiter plate method detected 3.33% strong producers, 45% moderate biofilm producers were produced by tube adherence method and 58.33% moderate biofilm producers were produced by microtiter plate method. 52.67% and 38.33% non-biofilm producers were produced by tube adherence and microtiter plate method respectively. The total biofilm producing percentage came out to be 62%.

CONCLUSION

Nosocomial (hospital-acquired) infections continue to be a burden on the economy and on the life expectancy of patients. The increasing evidence on ability of nosocomial pathogens to form biofilms on various indwelling devices makes it imperative for the clinicians for early detection of biofilms and setting up of antibiotic policy to treat these pathogens in the hospital settings.

KEYWORDS

Antibiotic Resistance, Biofilm, Nosocomial Infections.

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BACKGROUND

Biofilm formation is an important mode of growth because it is associated with the chronic nature of the subsequent infections, and it helps in inherent resistance to antibiotic chemotherapy. The bacteria which adhere to the indwelling devices become the reason for persistent infections. These bacteria encase themselves in a proteinaceous matrix called exopolysaccharide (EPS) material. Various nosocomial infections which are associated with the indwelling devices are the source of nosocomial infections as their surfaces become a niche for biofilm producing microorganisms. These pathogens are specifically dangerous as they evade host defences and combat antimicrobial therapy.⁽¹⁾

Antibiotic resistance can be attributed to many mechanisms which are responsible for antibiotic resistance. The familiar mechanisms of antibiotic resistance, such as efflux pumps, modifying enzymes, and target mutations do not seem to be responsible for the protection of bacteria in a biofilm. In fact, there are evidences that show that there may be different mechanisms associated with antibiotic resistance in biofilms.

There are many hypotheses elucidated for the mechanism of antibiotic resistance in biofilms. First is the slow or incomplete penetration of antibiotics in bacterial biofilms and the second is the altered chemical microenvironment within the biofilm which prevents the access of antibiotics to the bacterial cells embedded in the community. Third is the presence of a subpopulation of persisters which forms a unique and highly protected phenotypic state which is similar to spore formation. Fourth is the slow growth of the bacteria that has been observed in mature biofilms as the cells growing in biofilms are expected to experience some form of nutrient limitation, that can account for the resistance of biofilms to antimicrobial agents.⁽²⁾ Another factor that plays an important role in increasing the resistance among biofilms

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is the general stress response which is initiated by growth within a biofilm. The general stress response brings about many physiological changes like the heat shock, cold shock, changes in pH, and many other chemical changes.

Hence, this study emphasises on the antibiotic resistance of organisms *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii* which were isolated and tested for biofilm production and their antibiograms were analysed.

MATERIALS AND METHODS

The present study was conducted in the Department of Microbiology, on different isolates from various clinical samples of IPD and OPD patients of all age groups after the clearance from Institutional Ethical Committee of the research committee. The various clinical samples received in microbiology lab from OPD and IPD patients were processed for isolation, identification and antimicrobial sensitivity. Different tip cultures which included were the urinary catheter tip, tracheostomy tip, endotracheal tip, chest tube tip and central line tip. To isolate the bacteria from the tips, both extra-lumen and intra-lumen of tip were injected with the nutrient broth so that the microflora is easily isolated. The broth was then cultured on to blood agar and MacConkey's agar plates which were incubated at 37°C for 24 hours. After 24 hours, the plates were examined for any bacterial growth. If growth appeared, colonies were identified by colony characters, Gram's staining and biochemical tests.⁽³⁾ After identification, the different isolates were investigated for antibiotic susceptibility testing by Kirby-Bauer disc diffusion method on Mueller-Hinton Agar (MHA) and the zones were interpreted as per CLSI guidelines.^(4,5) Multi-drug Resistant (MDR) organisms viz. *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* and *A. baumannii* were then further tested for biofilm production by the following enumerated methods.

Detection of Biofilm

Tube Adherence Method (TA)

The quantitative assay for Biofilm formation was performed according to the method described by Christensen et al.⁽⁶⁾ The test tubes were filled with 3 mL of Brain-Heart Infusion medium (HiMedia) which were inoculated with a loop full of a pure culture of a strain of *K. pneumoniae* grown overnight from blood agar plate. After 48 hours of incubation at 37°C, the content of each tube was decanted. The tubes were then stained with 0.1% crystal violet for 8 min. Then the tubes were washed with phosphate buffer saline pH 7.2 for 5 min. A positive result was indicated by the presence of an adherent film of stained material. The liquid-air interface alone was not regarded as indicative of slime production. Tubes containing BHI only were included in the test as negative controls.

Microtiter Plate Method (MTP)

Organisms were isolated from fresh agar plates and were inoculated in Brain-Heart infusion broth for 24 hours. The cultures were diluted 1:100 with fresh BHI broth. Wells of a sterile 96-well flat-bottomed plastic tissue culture plate (Genaxy) were filled with 200 µL of bacterial suspension in Brain-Heart infusion (BHI) broth and incubated at 37°C for 24 hours. Negative control wells contained broth only. The plates were covered and incubated aerobically for 24 hours at

37°C. Then the contents of each well were washed three times with 200 µL of PBS with pH 7.2 to remove non-adherent bacteria. Biofilm formed by bacteria adherent to the wells were fixed with 2% sodium acetate and stained by 0.1% crystal violet. Excess stain was washed by deionised water and later air dried. The optical density (O.D.) of each well was measured at 570 nm using ELISA reader. The experiment was done in triplicates.⁽⁷⁾

For the purpose of comparative analysis of test results, the adherence capabilities of the test strains were classified according to Mathur et al.⁽⁸⁾ Strains were classified as follows:

OD value of Biofilm Formation Microtiter Plate Method	
Less than 0.120	Non-biofilm producer
In the range of 0.120 – 0.240	Moderate Biofilm Producer
Greater than 0.240	Strong Biofilm producer

RESULTS

A total of 300 Multi-drug Resistant (MDR) strains were isolated from various indwelling devices from a period of July 2014 to December 2016. Out of the isolated strains, one group of strains of Gram-positive cocci, three groups of strains of Gram-negative bacilli and one group of strains of Gram-negative coccobacilli were classified on the basis of their biofilm production. A total of 20 *S. aureus*, 117 *K. pneumoniae*, 29 *E. coli*, 60 *P. aeruginosa* and 74 *A. baumannii* were isolated. *K. pneumoniae* was the maximum isolated microorganism followed by *A. baumannii*, *P. aeruginosa* and *E. coli*. *S. aureus* was the least isolated organism from various microorganisms.

The microbial strains were isolated from various indwelling devices, which were mostly left inside the body to maintain drainage, prevent obstruction, or provide a route for administration of food or drugs. The various indwelling devices from which organisms were isolated were central venous catheter, urinary catheter, endotracheal tubes, tracheostomy tubes and chest drain tube tips. Maximum numbers of organisms were isolated from the endotracheal tube tips followed by tracheostomy tube tips. *K. pneumoniae* was the organism which was maximum isolated from endotracheal tube tip, urinary catheter tip, central line tip and tracheostomy tube tip whereas only 2 strains of *S. aureus* were isolated from perisplenic drain tip which was the least among all.

In the current study, Tube adherence method and Microtiter plate method were used to examine the biofilm production. Maximum positivity was seen by Microtiter plate method with 58.33% moderate biofilm producers and 3.33% strong biofilm producers whereas tube method showed only 2.33% strong biofilm producers and 45.00% moderate biofilm producers.

Sl. No.	Type of Biofilm Producers	Percentage of Biofilm Production	
		Tube Adherence Method	Microtiter Plate Method
1	Strong biofilm producers	2.33	3.33
2	Moderate biofilm producers	45.00	58.33

3	Non-biofilm producers	52.67	38.33
	Total	100	100
Table 1. Percentage of Biofilm Production			

Individually different organisms were also classified on the basis of the biofilm detection methods in which out of 74 isolates of *A. baumannii*, 7 were strong biofilm producers by MTP method and 6 were strong biofilm producers by TA method, 49 were moderate biofilm producers by MTP method and 34 were strong biofilm producers by TA method. (Table-2). Similarly rest of the organisms also showed the same difference between detection methods of biofilm production. Highest numbers of moderate biofilm producers were observed in *K. pneumoniae* followed by *A. baumannii* whereas only 6 isolates were moderate biofilm producers of *S. aureus*.

Organism	No. of Isolates	Type of Biofilm Producers	Tube Adherence Method	Microtiter Plate Method
<i>A. baumannii</i>	74	Strong biofilm producers	6	7
		Moderate biofilm producers	34	49
		Non-biofilm producers	34	49
<i>E. coli</i>	29	Strong biofilm producers	0	0
		Moderate biofilm producers	7	6
		Non-biofilm producers	22	23
<i>K. pneumoniae</i>	117	Strong biofilm producers	2	3
		Moderate biofilm producers	55	76
		Non-biofilm producers	60	38
<i>P. aeruginosa</i>	60	Strong biofilm producers	0	1
		Moderate biofilm producers	33	38
		Non-biofilm producers	27	21
<i>S. aureus</i>	20	Strong biofilm producers	0	1
		Moderate biofilm producers	5	6
		Non-biofilm producers	15	14
Total isolates	300			
Table 2. Bacterial Strains Classified on the basis of Biofilm Production				

Once the biofilm detection was done and the organisms were classified, their antibiotic sensitivity was compared. Antibiotic resistance was one of the critical factors which were to be considered in the current study. Multi-drug resistant strains were picked up for investigating the biofilm production and its relation with biofilm formation was determined. In *K. pneumoniae* strains, biofilm producers were less sensitive to the panel of drugs recommended by the CLSI guidelines than the non-biofilm producers. Most of the 1st and 2nd generation cephalosporins were resistant to biofilm producers whereas carbapenem, polymyxin and tetracycline came out to be effective drugs against biofilm producers. According to the current study, Tigecycline was recommended as a drug of choice against biofilm producing *K. pneumoniae* strain. (Figure: 1).

Figure 1. Representation of Antibiotic Sensitivity of *K. pneumoniae*

In *E. coli* strains, 1st, 2nd and 3rd generation cephalosporins were not at all effective against biofilm producing strains whereas Gentamicin, Amikacin, Piperacillin + Tazobactam, Imipenem Chloramphenicol, Ertapenem and Doripenem were wee bit sensitive to the biofilm producers. The best drugs which were active against biofilm producing *E. coli* were Polymyxin B and Colistin showing 100% sensitivity against them. (Figure: 2).

Figure 2. Representation of Antibiotic Sensitivity of *E. coli*

P. aeruginosa was another Gram-negative organism which was highly prevalent as a nosocomial pathogen. Ceftazidime and Cefepime were 2.5% and 17.5% sensitive to biofilm producing strains whereas Piperacillin + Tazobactam was 35% sensitive and Tigecycline was 32.5% sensitive. Doripenem and Imipenem were showing similar sensitivity with 37.5% sensitivity towards biofilm producing strains. Polymyxin-B and Colistin were the best drugs which came out with highest sensitivity towards biofilm producing strains. (Figure: 3).

Figure 3. Representation of Antibiotic Sensitivity of *P. aeruginosa*

Acinetobacter baumannii was other Gram-negative coccobacilli whose biofilm production was tested and effective antibiotics for biofilm producing strains and non-biofilm producing strains were also tested. Doripenem and Tigecycline were the sensitive drugs towards biofilm producing strains with 26.92% and 65.38% sensitivity. Cefepime was 1.92% sensitive to biofilm producing strains whereas it had 0% sensitivity towards Non-biofilm producing strains. Highest sensitivity was shown by Polymyxin-B and Colistin with 98.08% and 78.85% sensitivity respectively. (Figure: 4).

Figure 4. Representation of Antibiotic Sensitivity of *A. baumannii*

Staphylococcus aureus was the only Gram-positive cocci whose biofilm production was studied as it is one of the leading nosocomial pathogen in the hospital settings. Linezolid and Vancomycin were the drugs of choice as they were 100% sensitive to biofilm producing strains. Chloramphenicol was 66.67% sensitive and Oxacillin, Cefoxitin and Doxycycline were 16.67% sensitive only. (Figure: 5).

Figure 5. Representation of Antibiotic Sensitivity of *S. aureus*

DISCUSSION

The structure of biofilm communities provides several advantages to the members forming biofilm including easy access to food and nutrients and resistance to antibiotics. Biofilms cause one of the most devastating infections as they are very difficult to eradicate. During chronic infections biofilms form the nidus of acute infections and can reoccur at any one time during the infections. Indwelling medical devices are the most vulnerable surfaces for the formation of biofilms. They are favourable surfaces because they can easily provide nutrients and a surface to the growing organism for adherence and biofilms are formed in very high shear environments i.e. rapidly flowing milieus. Once the organisms adhere to the surface it starts secreting the exopolysaccharides and the biofilm formation starts. It is observed that biofilms formed in low-shear environments have low tensile strength and break easily, but biofilms formed at high shear environment are highly strong and resistant to mechanical breakage. In the current study, different microorganisms were isolated from various indwelling devices and after investigating their antibiogram, biofilm production was tested of the concerned microorganisms. A total 300 microorganisms were isolated from various indwelling devices out of which 117 were *K. pneumoniae*, 74 were *A. baumannii* strains, 60 *P. aeruginosa*, 29 *E. coli* and 20 were *S. aureus* strains (Table:1) Highest percentage of biofilm producing organisms was of *K. pneumoniae*, followed by *A. baumannii* and *P. aeruginosa* whereas least strains were of *S. aureus*. On the contrary in a study done by Ruchi et al in 2016, it was shown that maximum biofilm forming percentage was of *E. coli* which was just 2% in the ongoing study. Similar findings were also reported in the studies conducted by Behzadi et al, Jain et al, Subramanian et al, and Noor et al.^(9,10,11,12) The high prevalence of *K. pneumoniae* in biofilm formation and as a nosocomial pathogen in the hospital settings can be due to its various virulence factors which contribute to the invasiveness and its inherent adhesiveness property. The considerable efficiency of colonisation when accompanied with acquired resistance to antibiotics has enabled *K. pneumoniae* to persist and spread rapidly in healthcare settings. The most common healthcare-associated infections caused by this strain involves the urinary tract infection, wound infections, lungs infection, colonisation of intra-vascular devices, surgical site and soft tissues infection and subsequent bacteraemia.⁽¹³⁾

In the prevailing study, it was found that *K. pneumoniae* was maximum isolated from the endotracheal tube tips and the tracheostomy tube tips followed by *A. baumannii* and *P. aeruginosa*. Since these organisms are highly linked as oral pathogens their association with biofilm formation on these tips can be a reason behind their high prevalence. Commensal oral bacteria like *S. aureus* colonise the tracheostomy tube lumen and may lead to Ventilator associated pneumonia (VAP).⁽¹⁴⁾

In the current ongoing study, a comparison has been done between antibiotic sensitivity of biofilm producing and non-biofilm producing microorganisms. In biofilm producing *K. pneumoniae*, cephalosporins, aminoglycosides, few of the carbapenems and fluoroquinolones were resistant and the most effective drug came out to be Tigecycline and Colistin (Figure: 1). These results were slightly contradictory as

compared to the study done by Ruchi et al 2016 who stated that Amikacin and Imipenem are effective drugs whereas in the current study Amikacin is 9.8% sensitive and Imipenem is 25.49% sensitive. Similar results were also stated by Abdallah et al who, in 2011, reported same efficacy of Imipenem and Amikacin. Tigecycline is a new glycylcycline derivative of tetracycline, which is bacteriostatic in nature and effective against both Gram-positive as well as Gram-negative organisms. This can be used against multi-drug resistant organisms.^(15,16)

The second biofilm positive strain isolated i.e. *E. coli* was totally resistant to 1st, 2nd and 3rd generation cephalosporins which is in correlation with a study done by Kazi et al⁽¹⁷⁾ whose study also investigated that *E. coli* as a uropathogen was resistant to Cefuroxime, Ciprofloxacin, Ceftazidime and Amoxicillin; however, Gentamicin, Amikacin, Piperacillin + Tazobactam, Imipenem, Chloramphenicol, Ertapenem and Doripenem were more sensitive which was again in accordance with the findings of drug efficacy of Kazi et al. The drug of choice in the present study came out to be Polymyxin B and Colistin with 60% sensitivity against biofilm producers. (Figure: 2)

Nonfermenters i.e. *A. baumannii* and *P. aeruginosa* which were biofilm positive showed a significant sensitivity towards Polymyxin B and Colistin. Similar result was also shown by Bose et al who showed that they were the drug of choice for the treatment of biofilm producing *A. baumannii* and *P. aeruginosa*. It is also found that while using Colistin and Polymyxin B, one has to be careful, because of side effects, such as nephrotoxicity, it is one of the most common kidney problem that occurs when your body is exposed to a drug or toxin that causes damage to your kidneys. (Figure: 3 and 4) When kidney damage occurs, one is unable to rid the body of excess urine and wastes. *S. aureus* was the Gram-positive biofilm producer which showed 100% sensitivity to Vancomycin which can be a drug of choice for its treatment. This result was similar to the results shown by Bose et al who also reported the efficacy of Vancomycin as a drug of choice for the treatment of biofilm producing *S. aureus*.⁽¹⁸⁾ (Figure: 5)

With the ever-increasing cases of Health Care Associated Infections (HCAI), early detection of biofilms is an important task which can help the clinicians to curb the high antibiotic resistance related to biofilm formation. In the current ongoing study, two basic methods i.e. Tube adherence method and Microtiter plate method were used to examine the biofilm production. Maximum positivity was seen by Microtiter plate method with 58.33% moderate biofilm producers and 3.33% strong biofilm producers whereas tube adherence method showed only 2.33% strong biofilm producers and 45.00% moderate biofilm producers. Similar results were also produced by Mathur et al and Panda et al.⁽¹⁹⁾ Also, all the above observers found the biofilm production in one type of strain of bacteria, but in the current study it was proved in five different strains of bacteria.

CONCLUSION

At the end, it was concluded that *K. pneumoniae* was the highest isolated organism with maximum percentage of biofilm production. A significant correlation was found between biofilm production and multi-drug resistance as

62% of organisms were biofilm producers. Therefore, as biofilm production was detected in many of our isolates, it is necessary to establish standard guidelines on the care of use of indwelling devices for all units in the hospital settings with a view to prevent nosocomial infections associated with the devices in patients.

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