Biology



Biofilm Forming Abilities and Antibiotic Susceptibility Pattern of Clinical Isolates of Staphylococcus aureus

KEYWORDS	S. aureus, MDR, Cholic acid, Biofilm producers.				
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ABSTRACT Microorganisms growing in a biofilm are associated with chronic and recurrent human infections and are highly resistant to antimicrobial agents. The objective of this study was to observe the biofilm forming abilities, which is an important virulence factor and to study antibiotic susceptibility pattern of clinical isolates of Staphylococcus aureus. Clinical specimens received from various hospitals and pathological laboratories of Akola city. Out of 38 isolates of S. aureus 20 (52.63%) isolates were found to be biofilm producers by Standard tube (ST) method and 11 (28.94%) were by Congo red agar (CRA) method. All the 38 isolates were checked for their antibiotic susceptibility by Kirby- Bauer method. It was found that multi drugs resistance (MDR) was shown by biofilm producers. The highest resistance was shown towards Penicillin, Ampicillin, Methicillin and Erythromycin while Vancomycin and Tetracycline were found to be the most effective agent against biofilm producers. The cholic acid found to exert good antibacterial activity against drug resistant biofilm producers.

Introduction:

Staphylococcus aureus has emerged as one of the most important human pathogens and has over the past several decades, been a leading cause of hospital and a community acquired infections¹. It is associated with a variety of clinical infections including septicemia, pneumonia, wound sepsis, septic arthritis, osteomyelitis and post surgical toxic shock syndrome with substantial rates of morbidity and mortality^{2,3}. Staphylococcus aureus is a virulent organism showing resistance to most of the conventionally prescribed antibiotics. It is difficult to treat long term Staphylococcal infections. One reason these organisms are capable of defending themselves from host immune system is their capability to form biofilm^{4,5}. The interior of the bacterial biofilms present greater resistance to the opsonization by antibodies and to phygocytosis, which explains the chronic character of this infection ⁶.

As many as 60% of bacterial infections treated by physician are related to biofilm formation and are not easily eradicated by conventional antibiotic therapy⁷. In vitro surface – associated in- vivo device associated bacterial biofilms are generally quite resistant to antibiotics. Despite decades of research in this area, treatment options are limited. A factor contributing to this unmet need is the lack of standardized method for determining the drug susceptibility of bacterial biofilms ⁸.

Infections due to the multiple drug resistant strains are becoming more critical due to their capacities to produce biofilm. The role of staphylococci in biofilm-associated human diseases, have allowed researchers to determine the mechanism of biofilm development and molecular basis of virulence in biofilm – forming S. epidermis and S. aureus.

Intervention strategies are of great economical relevance because traditional antibiotic therapy is usually not sufficient to eradicate the biofilm based infections. One major reason for persistence seems to be the capability of the bacteria to grow within the biofilms that protects them from adverse environmental factors. Eradication of slime can achieved by means of minimization of the initial contamination of medical devices or inhibition of quorum sensing or by degradation of matrix. Combination therapy of antibiotics was used to combat the resistance disaster. But considering the ill effect of high dosage of antibiotics and their cost, the concept did not proved satisfactory⁹.

Several investigators analyze the utility of clavulanic acid in

combination with antibiotics which proved effective but clavulanic acid is effective only in case of Penicillin resistance so attention was switched onto analyzed the potentiating ability of cholic acid in combination with antibiotics. In nature, cholic acid is produced in the liver from cholesterol. The liver converts cholesterol in to the conjugated salts of glycocholic and taurocholic acid which are excreted into the bile¹⁰. With the emergence of many strains of multidrug-resistant bacteria has come a renewed interest in new antimicrobial agent. Cationic peptide antibiotics, which have been isolated in organism ranging from bacteria to animals, have received considerable attention in part because of their broad spectrum of activity. Generally, target of these antibiotics are bacterial membrane. Examples of this compound were shown to be bactericidal to broad spectrum of Gram- negative and Gram positive organisms¹¹. However little information is available on potentiating effect of cholic acid on antibacterial activity of antibiotics against multi-drug resistant biofilm producers. Therefore in present investigation the efficacy of antibiotic in combination with cholic acid on the antibacterial activity is proposed to analyze.

Considering the importance of S. aureus as a major human pathogen causing large variety of infections. The present work aimed to study the antibiotic susceptibility pattern among clinical isolates of S. aureus, prevalence of biofilm producing S. aureus and effect of cholic acid along with antibiotics against these isolates.

Material and Methods:

A total 60 clinical samples viz. blood, pus, urine burn wound were collected for the isolation of test organism. The clinical samples were collected from various hospitals and pathology laboratories of Akola city. All the samples were enriched in trypticase soya broth and transferred to selective media. All plates were incubated at 37°C for 24 hrs. Typical colonies from selective media were selected and then subjected for the conventional biochemical analysis for the organism, identified and compared with the standard literature for identification of genus as per Bergy's manual of systemic bacteriology ¹².

Screening of biofilm producing isolates can be done by two phenotypic methods, Congo red agar (CRA) method¹³ and Standard tube (ST) method¹⁴. In CRA method, Congo red agar medium was used which was prepared with Brain Heart Infusion (BHI) broth (Oxide UK) 37 g/L, Sucrose 50 g/L, agar no 1

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10 g/L and Congo red indicator 8 g/L. CRA plates were inoculated with the test organism and incubated at 37°C for 24 to 48 hrs aerobically. Black colonies with a dry crystalline consistency indicate strong biofilm production. Brownish or reddish growth was considered as negative biofilm formation.

In Standard tube method a loopful of test organism was inoculated in 10ml of Trypticase soya-broth with 1% glucose in test tubes. The tubes were incubated at 37°C for 24 hrs. After incubation, tubes were decanted and washed with phosphate buffer saline pH 7.3 and dried. Tubes were then stained with safranine or 0.1% crystal violet. Excess stain was washed with deionised water. Tubes were dried in inverted position. In positive biofilm formation, a visible stained film was seen along the walls and bottom of the tube.

All the isolates of S. aureus then subjected to in-vitro antibiotic susceptibility on Muller-Hinton agar and HI- MEDIA antibiotics disc as per the method described by Kirby and Bauer¹⁵. The zones of inhibition around the disc were measured and drug susceptible/resistance pattern was studied by using the interpretation chart supplied by the antibiotic disc Manufactures (HI-MEDIA, Mumbai, India) according to the Guidelines of CLSI¹⁶. The Effect of 0.05% of sterile Cholic acid on antibacterial activity of antibiotic against biofilm producers has also been studied. Comparative studies were conducted amongst the zone of inhibition developed by antibiotic alone and in combination with cholic acid

Results and Discussion:

Among 38 isolates of Staphylococcus aureus isolated from different clinical samples, 52.63% were found to be positive for biofilm production and 47.37% were found to be negative for biofilm production. The biofilm production of S. aureus strains with regard to source of isolation is depicted in Table No. 1. It was found that highest no. of biofilm producing S. aureus were from urine sample (83.33%) followed by blood (62.5%), pus (30.00%) and burn wound (25.00%) samples.

The biofilm detection among S. aureus isolates has been done by two methods. Out of 38 isolates of S. aureus 52.63% isolates were found to be positive for biofilm production by standard tube (ST) method while 28.94% were found to be positive biofilm producers by congo red agar (CRA) method (Table 2).

After screening the cultures for biofilm production, antibiogram studies were done by Kirby Bauer Disc Diffusion technique¹⁵. The antibiotic resistance exhibited by biofilm producers and non-producers against respective antibiotics was analysed and recorded in Table No. 3. It was observed that the sensitivity of isolates which were biofilm producers was less than those isolates which were non-producers towards different antibiotics.

Highest resistance was recorded against Penicillin, Ampicillin, Methicillin and Erythromycin as only 20%, 25%, 30% and 40% of the isolates found to be sensitive towards the respective antibiotics while sensitivity of isolates towards Kanamycin, Ciprofloxacin and Gentamycin ranges from 51 to 57%. While resistance to Tetracycline and Vancomycine recorded was less as only 10% and 5% isolates showed resistance respectively (Figure 3).

Growth response exhibited by biofilm producers in presence of cholic acid (0.05%) in combination with prescribed dosage of antibiotics was analysed. Change in susceptibility pattern exhibited by isolates was analysed and recorded in Table No. 4.

Table 1: Biofilm production of S. aureus strains with regard to source of isolation

	S. aure				
Source	Biofilm +ve		Biofilm –ve		Total
	No.	%	No.	%	
Urine	10	83.33	02	16.67	12

		-	-	-	
Blood	05	62.50	03	37.50	08
Pus	03	30.00	07	70.00	10
Burn wound	02	25.00	06	75.00	08
Total (No.)	20		18		38
					-

Table 2: Comparison of production of biofilm by clinical isolates of S. aureus by two conventional methods

Method	Biofilm +ve S. aureus (n = 38)			
	No.	%		
Standard Tube Method	20	52.63		
Congo Red Agar Method	11	28.94		

Table 3: Antibiotic susceptibility pattern (%) of biofilm producing and non producing S. aureus isolates.

Antimicrobial Agent	Biofilm Producer (%)	Biofilm non producer (%)		
Penicillin	20	22.22		
Ampicillin	25	55.55		
Methicillin	30	66.66		
Erythromycin	40	72.22		
Kanamycin	50	83.33		
Cirpofloxacin	55	88.88		
Gentamycin	55	94.44		
Nalidixic acid	60	94.44		
Amoxyclave	70	70.12		
Tetracycline	90	91.22		
Vancomycin	95	96.11		

Table	4:	Ant	tibiotic	susceptibil	ity	pattern	(%)	of	biofilm
produ	cin	g S.	aureus	in presenc	e of	f cholic a	cid.		

Name of Antibiotic	Susceptibility of biofilm producing S. aureus (%)
Penicillin	25
Ampicillin	30
Methicillin	35
Erythromycin	45
Kanamycin	60
Cirpofloxacin	60
Gentamycin	65
Nalidixic acid	70
Amoxyclave	75
Tetracycline	100
Vancomycin	100





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Figure 3: Screening of Biofilm producing S. aureus



a) Congo Red Agar method b) Standard tube method

Figure 4: Antibiotic Sensitivity Pattern exhibited by biofilm producing S. aureus



a) In absence of Cholic acid b) In presence of Cholic acid

Discussion:

Biofilm and multidrug resistance have been identified as virulence factors of great magnitude in Staphylococcus aureus infections in clinical settings. S. aureus is a medically important organism associated with a vast variety of diseases, gain increased resistance to antimicrobial agents through biofilm formation¹⁷. Researchers have investigated the strategies employed by micro organism to produce biofilms and to understand the pathogenesis. They discovered that biofilmproducing bacteria secrete certain chemicals that protect them from disinfectant and antimicrobials and phagocytic host immune system¹⁸.

A total of 38 clinical isolates of staphylococci isolated from various clinical samples. They we're further identified by standard conventional methods. We tested 38 clinical isolates of S. aureus by two in vitro screening procedures for their ability to form biofilm. Standard tube method could detect 52.63% of S. aureus isolates to be biofilm producers. Similar results have been reported by Mathur et al., $^{\rm 19}$ that 53.9% of Staphylococci were biofilm producers in their study whereas Bose et al., ²⁰ reported that 54.19% staphylococci were biofilm producers. By CRA method only 28.94% of biofilm producers have been detected. Other studies also detected less biofilm producers using CRA method ²¹. The reason behind this is that the CRA method was found to be easier and faster to perform than other phenotypic methods but could probably identify only strong biofilm producers. It is imprecise in the identification of moderately biofilm producing strains. Hence, it could detect the least number of biofilm producers²¹.

Bacterial identification, screening of biofilm producers followed by susceptibility tests are important for selection of appropriate antimicrobial agent for treatment. For this purpose susceptibility pattern of biofilm producers as well as biofilm non-producers was recorded. Results revealed that high resistance was found against Penicillin, Ampicillin, Methicillin, Erythromycin and Gentamycin among biofilm producers as only 20%, 25%, 30%, 40% and 55% of isolates found to be sensitive. Our results are in accordance with the study of Kandel et al., ²² from South Maharashtra who reported that more than 90% isolates have been found resistant to Penicillin, Ampicillin, Erythromycin and Gentamycin. The Vancomycin was found to be the best antistaphylococcal drug during the study. This result was in consonance with the study of Kesah et al.,²³ who recommended Vancomycin for empiric therapy in the light threatening Staphylococcal infection. But higher price of Vancomycin, its unavailability in many parts of the country, and also the possibility of emergence of resistance to the drug are some of limitations regarding use of Vancomycin which emerges the need for clinicians to look into the alternatives. In the study it was revealed that multi drug resistance was prevalent in biofilm producers as slime producers were remarkably resistant to antibacterial agents i.e. antibiotics.

It was noticed that slime production and multi drug resistance were associated with each other. Reason behind this may be delayed or blocked penetration of antibiotic inside the bacterial cell which automatically makes it resistant to several antibiotics or the altered growth rate in biofilm associated cells making them less susceptible as compared to their planktonic counter parts. Other physiological changes lead to repression or induction of gene during slime production can also lead to multi drug resistant ability. Suci et al., ²⁴ also support the existence of link between multi-drug resistance and slime production.

Considering this it becomes essential to develop some intervention strategy to keep control on this. One of the strategies may be to penetrate the antibiotics through biofilm matrix and kill the biofilm associated cells. Keeping this in mind the potentiating effect induced by cholic acid on the antibacterial activity of antibiotics was analysed and recorded against biofilm producer isolates (Figure 2). It was found that remarkable change recorded in susceptibility of isolates toward antibiotics which were showing less susceptibility alone with antibiotics. Most prominent activity was recorded against Tetracycline and Vancomycin, in presence of Cholic acid as 100% susceptibility were attain by biofilm producers which were showing some resistance towards these antibiotics. This is in agreement with the study of Sheikh et al., 25. Thus in present study it has been observed that cholic acid is effective in potentiating antibacterial activity of antibiotics. The underlying mechanism of this in vitro potentiation still remains undefined but it may be due to enhanced potentiation of antibiotics into the bacterial cell. This also embarks the possibility of utilization of cholic acid in combination therapy with antibiotics to eradicate Staphylococcal infections associated with biofilm production.

Conclusions:

There is an association between biofilm production with persistent infection and antibiotic therapy failure. Hence identification of infection caused by biofilm producing S. aureus might help to modify the antibiotic therapy and prevent infection. Standard tube method is found to be the most reliable method according to our findings for detection of biofilm producing S. aureus. Biofilm producing strains were found to be more resistant to almost all the groups of antibiotics as compared to biofilm non-producing strains. This finding is important because treatment of patients with Staphylococcal infections becomes further more difficult when the strain is biofilm producer as biofilm is known to impede the delivery of antibiotics. Cholic acid found to induce a potentiating effect on antibacterial activity of antibiotics as it may lead to enhancement in penetration of antibiotics across the slime layer which results in increase susceptibility of isolates toward antibiotics. Further studies on some highly accurate methods like PCR analysis to detect ica gene as virulence marker of biofilm and effect of different surface active compounds like cholic acid with antibiotic used in chemotherapy in detail may enlight the good scope in the management of slime producing organisms.

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