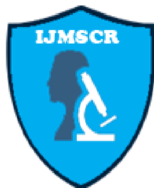
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Study Of Antimicrobial Resistance And Biofilm Detection Among Various Clinical Isolates Of Staphylococcus aureus Using Three Different Methods

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Abstract

Background: *Staphylococcus aureus* is Gram-positive bacteria commonly associated with nosocomial infections. The development of biofilm exhibiting drug resistance especially in foreign body associated infections has enabled the bacterium to draw considerable attention. The purpose of this study was to evaluate three methods for detection of biofilm formation in Staphylococcus aureus.

Methods: A total of 200 S. aureus were isolated from the clinical samples. MRSA detection was done by cefoxitin disk diffusion test. Antibiotic susceptibility testing was done according to Kirby-Bauer disk diffusion method and the results were interpreted according to CLSI 2016 guidelines. Biofilm detection was done by tissue culture plate (TCP), tube method (TM) and Congo red agar (CRA).

Results: Out of the 200 S. aureus isolates,47.5% were MRSA and 52.5% were MSSA. All of them were sensitive to Vancomycin and Linezolid. Most of the isolates were having MIC of 2 mcg/ml to Vancomycin. 26(13%) isolates were constitutive Clindamycin resistant (cMLSB) and 36 (18%) isolates had inducible clindamycin resistance(iMLSB). 87% were resistant to penicillin, high level of resistance was seen to ciprofloxacin.

Maximum strong biofilm producers were detected by TCP 43(21.5%) as compared to TM 36(18%) and CRA 14(7%) respectively. TM showed good correlation with the TCP assay for strong biofilm forming isolates and total 36 were picked up as strong and 57 as moderate and 21 as weak and 86 as negative respectively.

Conclusion: Our data indicates that the TCP method is an accurate and reproducible method for screening and this technique can serve as a reliable quantitative tool for determining biofilm formation by clinical isolates of staphylococci. The presence of biofilm is probably underestimated because of lack of in vitro diagnostics. Thus, research activity is required in this field as these biofilms may be associated with human infectious diseases.

Keywords: Biofilms, S.aureus, MRSA

Introduction

Staphylococcus aureus is a gram positive cocci, most frequently isolated from clinical specimens apart from Enterobacteriaceae. It is both a commensal bacterium and a human pathogen. Approximately 30% of the human population is colonized with *S. aureus* [1]. They colonize healthy individuals & cause severe infections in hospitalized patients especially in ICUs and burns unit. Furthermore, patient colonization with *S. aureus* is associated with a 2-9 fold increased risk of infection [2, 3]. It is the most common cause of surgical site infections and second

common cause of nosocomial bacteremia[4]. It is also a leading cause of infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections.

The study of biofilms has become important due to their impact on many microbiology areas. In the health care system, biofilms have been of great significance due to many pathogenic and non-pathogenic bacteria that can produce biofilm as a part of their virulence mechanism and protection against the host defence. A biofilm is considered a complex microbial community which is attached to a defined surface and embedded within a cell matrix [5].

For a long time, the research on biofilm formation was focused on gram-negative pathogens, especially *Pseudomonas aeruginosa*. Recent advances in Staphylococcal molecular biology have allowed researchers to determine the molecular basis of biofilm formation in Staphylococci [6]. A number of tests are available to detect biofilms.These include visual assesment by electron microscopy, molecular techniques like polymerase chain reaction. The qualitative methods are tube method, congo red agar method and quantitative method such as tissue culture plate method.

Penicillin was the drug of choice for Staphylococcal infections previously, but later penicillin resistance developed. In the 1950s, Penicillin resistance developed due to penicillinase production. Antibiotic resistant strains, such as methicillin-resistant *S. aureus* (MRSA), have emerged as a significant threat in both the hospital and community. Penicillinase resistant penicillins, methicillin and oxacillin were introduced in 1959 & 1960.

The first case of MRSA was isolated way back in 1961 followed by the first major nosocomial epidemic in 1963 [4,7]. This was due to an altered penicillin binding protein called PBP2a, the product of mecA gene which has low affinity towards beta lactam antibiotics [8,9,10]. MRSA has been recognized as one of the major pathogens in hospital & community settings with infected or colonized patients being the reservoir and mode of transmission being through contaminated hands of healthcare workers[11, 12, 13]. Interestingly, surgical patients have been shown to be at a greater risk of developing HAI and surgeons themselves have even been shown, in small-scale studies, to be at an elevated risk of nasal carriage of MRSA in comparison with non-surgical medical doctors[14, 15]. Failure to detect patients and carriers of MRSA leads to inappropriate therapy, treatment failure, increased mortality and health care costs. Hence detection of MRSA rate helps in formulating a proper infection control policy. Vancomycin has been regarded as the first line drug for MRSA[4, 8]. But because of its increased usage for treating MRSA, *Clostridium difficle* and enterococcal infections, there has been development of reduced susceptibility to the drug[16]. The first strain of vancomycin intermediate *S.aureus* (VISA) was isolated in May 1996 and reported in 1997 from Japan [17]. The first vancomycin resistant strain (VRSA) was reported in 2002 from the United States [8, 18, 19]. Since then resistance to vancomycin is on a constant rise. Due to the widespread use of macrolide - lincosamide - streptogramin B (MLSB) group of antibiotics as alternatives, an increase in MLSB resistance is seen in Staphylococci. This resistance can be constitutive or inducible [20].

Hence this study was done to isolate *S.aureus* from clinical samples, study their antibiogram and to detect biofilm which is one of the virulence factors.

Materials and Methods

The study of "**STUDY OF ANTIMICROBIAL RESISTANCE AND BIOFILM DETECTION AMONG VARIOUS CLINICAL ISOLATES OF STAPHYLOCOCCUS AUREUS USING THREE DIFFERENT METHODS**" was carried out in the Department of Microbiology, Bangalore Medical College & Research Institute, Bangalore, from November 2015 to May 2017.

Two hundred non-repetitive clinical isolates of *S.aureus* from different clinical samples like urine, sputum, pus, blood and other samples collected from out-patients and in-patients admitted in the hospital were studied prospectively.

Inclusion Criteria:

All clinical isolates of Staphylococcus aureus were included for the study.

Exclusion Criteria:

All clinical isolates of Coagulase negative staphylococcus,other gram positive organisms,gram negative organisms,fungi were excluded from this study.

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Laboratory Procedures :

Various clinical samples like blood, urine, pus, skin surface, infected devices, etc. were collected under sterile conditions and immediately transferred to the microbiology lab. Smears were made from all samples except urine and blood, heat-fixed and stained by Gram-stain. Smears were examined for the presence of pus cells and Gram positive cocci. The specimens were processed immediately and inoculated onto following media. Urine sample was inoculated with standard loop on MacConkey agar (MA) and blood agar. After 24hrs of incubation at 37°C, cultures with significant bacteriuria were further processed. Pus and other samples were inoculated onto MacConkey agar and 5% sheep Blood agar (SBA) and sputum samples were inoculated into MacConkey agar, 5% SBA and Chocolate agar. The media were incubated aerobically overnight at 37°C and observed for growth on the next day. Blood samples were inoculated into the Brain-heart infusion broth and incubated for 24hrs. On the next day, it was subcultured onto MacConkey agar and 5% SBA. Cultures were reported negative after three subcultures over a period of 10 days. Additionally samples with mixture of organisms like nasal swabs were inoculated on to Mannitol Salt Agar (MSA) as it is selective for *S.aureus*. Depending on the morphology of colonies, the presumptive identification of the organism was made and confirmed by Gram's stain and a battery of biochemical tests..

All 200 isolates were then subjected to antimicrobial susceptibility testing as per CLSI guidelines 2016, and were tested for different phenotypic resistant pattern .Methicillin resistance was identified using Cefoxitin(30mcg) discs on Mueller Hinton agar and a zone size of <22 mm was considered as MRSA as per CLSI guidelines. Antibiotic susceptibility testing on Mueller Hinton Agar (MHA) by Modified Kirby-Bauer disk diffusion method: As per CLSI guidelines, HiMedia discs of the following antibiotics were used: Penicillin (10 units), Erythromycin (15µg), Clindamycin (2 µg), Ciprofloxacin (5µg), Cotrimoxazole (1.25/23.75 µg), Linezolid (30 µg), Chloramphenicol(30 µg), Tetracycline (30 µg), Doxycycline (30µg), Azithromycin (15µg) and Cefoxitin (30 µg). To assess Vancomycin susceptibility, E-strips were used. isolate with MIC ≤ 2µg/mL was considered susceptible, 4-8µg/mL was taken as intermediate (VISA) and ≥ 16µg/mL was reported resistant (VRSA). ATCC® MSSA strain 25923 was used as a reference control.

Test for inducible Clindamycin resistance: Standard disc diffusion procedure was performed as per CLSI guidelines using 15µg Erythromycin and 2µg Clindamycin discs placed 15-26mm apart on MHA.

Detection of biofilm production: Biofilm formation was detected by Tissue culture plate method (TCP) , Tube method (TM) and Congo red agar method(CRA)

A known positive biofilm producer and non producer Staphylococcus aureus, isolated from previous tests was used as positive and negative control respectively.

TCP method:- Overnight culture of isolate was inoculated into Trypticase soy broth [TSB] with 1% glucose. Broth was incubated at 37^0 C for 24 hours. The next day, the broth culture was then diluted 1:100 with fresh TSB medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture plates were filled with 0.2mL of the diluted cultures and incubated for 24 hours at 37°C. After incubation content of each well was gently removed by tapping the plates and washed 4 times with phosphate buffer saline (ph7.2). Biofilm formed by bacteria adherent to the wells was fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent bacteria was determined with an ELISA reader at wavelength of 570nm. Average of OD values of sterile TSB broth(ODc) were calculated and subtracted from all test values. Experiment was performed in triplicates and average OD value was taken for each sample and read as absent, weak, moderate and strong. These OD values were considered as an index of bacterial adherence and biofilm formation.

OD ≤ ODc --- non-biofilm producer

ODc < **OD** ≤ 2 × ODc --- weak biofilm producer

2 × ODc < **OD** ≤ 4 × ODc --- moderate biofilm producer

4 × ODc < **OD** --- strong biofilm producer

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Tube Method:- 10ml Trypticase soy broth with 1%glucose was inoculated with loopful of test organism from overnight culture. Broth was incubated at 37^0C for 24hrs.Tubes were decanted and washed with phosphate buffer saline(pH 7.3).Tubes were dried and stained with 0.1% crystal violet. Tubes were washed and dried in inverted position. A visible stained film lining wall and bottom of tube indicated biofilm formation. Ring formation at the liquid interface was not indicative of biofilm formation. Tubes were examined and the amount of biofilm formation was scored as negative, weak, moderate and strong.

Congo red agar method:- Congo red agar was prepared from brain infusion broth, sucrose, agar no.1 .Congo red stain was prepared separately ,autoclaved and added to above ingrediants.CRA plates were inoculated and then incubated at 37^0C for 24hrs. Black colonies with dry crystalline consistency indicated biofilm production.

Statistical Analysis: Statistical analysis was done by using descriptive analysis and suitable analytical software.

Results

TABLE 1: Antibiotic sensitivity pattern of *Staphylococcus aureus* isolates

Sample	P	TE	CIP	DO	CD	E	LZ	COT	C	AZM	CX	VA
Blood	4 (19%)	17 (80.9%)	4 (19%)	18 (85.7%)	13 (61..9%)	10 (47.6%)	21 (100%)	7 (14%)	17 (81%)	12 (57%)	7 (33%)	21 (100%)
Pus	14 (11%)	110 (87.3%)	29 (23%)	112 (88.9%)	84 (66.6%)	49 (39%)	126 (100%)	50 (39.7%)	114 (90.5%)	74 (58.7%)	72 (57%)	126 (100%)
Urine	1 (7%)	12 (85.7%)	6 (43%)	12 (85.7%)	9 (64.3%)	5 (35.8%)	14 (100%)	5 (35.7%)	13 (93%)	9 (64.3%)	6 (43%)	14 (100%)
Sputum	3 (30%)	9 (90%)	6 (60%)	7 (70%)	7 (70%)	2 (20%)	10 (100%)	2 (20%)	10 (100%)	8 (80%)	4 (40%)	10 (100%)
Fluids	1 (14%)	7 (100%)	2 (28.6%)	7 (100%)	6 (85.8%)	3 (43%)	7 (100%)	2 (28.6%)	7 (100%)	4 (57%)	3 (43%)	7 (100%)
Others	3 (13.6%)	20 (91%)	7 (31.8%)	20 (91%)	19 (86.4%)	11 (50%)	22 (100%)	6 (27%)	20 (91%)	13 (59%)	13 (59%)	22 (100%)
Total	**26 (13%)**	**175 (87.5%)**	**54 (27%)**	**176 (88%)**	**138 (69%)**	**80 (40%)**	**200 (100%)**	**72 (36%)**	**181 (90.5%)**	**120 (60%)**	**105 (52.5%)**	**200 (100%)**

All the 200 isolates were susceptible to Vancomycin (100%) and Linezolid (100%), followed by Chloramphenicol (90.5%), Doxycycline (88%), Tetracycline (87.5%) and Clindamycin (69%).

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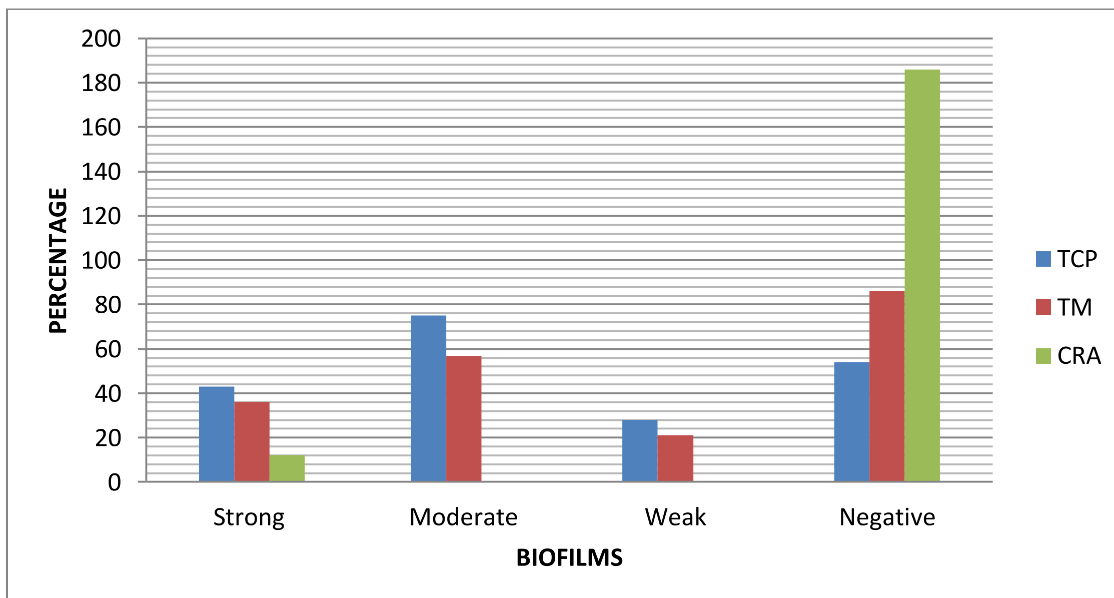
Susceptibility to Penicillin was 13%. Resistance of isolates to Ciprofloxacin was (73%), followed by Cotrimoxazole (64%), Erythromycin (60%), Cefoxitin 47.5% and Azithromycin (40%).

105 *S.aureus* strains were identified as methicillin sensitive i.e sensitive to cefoxitin (52.5%) whereas 95 were found to be methicillin resistant i.e resistant to cefoxitin (47.5%) out of the 200 total samples.

All isolates tested were susceptible to Vancomycin as per CLSI guidelines. Majority of them (39%) showed an MIC of 1.5µg/mL, 32 (41%) being MSSA and 46 (59%) being MRSA strains. 21% strains had an MIC of 1µg/mL and 12.5% had an MIC of 2µg/mL. Of the strains with an MIC of 2µg/mL which is the higher limit of the susceptible range, 72% were MRSA strains.

Figure 1: Comparison of biofilms by TCP, TM, CRA methods



Maximum strong biofilm producers were detected by TCP 43(21.5%) as compared to TM 36(18%) and CRA 14(7%) respectively. TM showed good correlation with the TCP assay for strong biofilm forming isolates and total 36 were picked up as strong and 57 as moderate and 21 as weak and 86 as negative respectively. However, it was difficult to discriminate between moderate and weak biofilm producing isolates.

By CRA method, most strains displayed pink to orange colonies. Only 14/200 (7%) isolates displayed black colonies with dry crystalline morphology.

Table 2: Biofilm producers

Bacterial isolates	Detection methods					
	TCP		TM		CRA	
	n	%	n	%	n	%
n = 200	118	59%	93	46.5%	14	7

TCP method was considered the gold-standard for this study and compared with data from TM and CRA methods. Parameters like sensitivity, specificity, negative predictive value, positive predictive value and accuracy were calculated. True positives were biofilm producers by TCP, TM and CRA method. False positive were biofilm producers by TM(1 isolate) and CRA method(2 isolates) and not by TCP method. False negative were the isolates which were non-biofilm producers by TM and CRA but were producing biofilm by TCP method. True

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negatives are those which were non biofilm producers by all the three methods.

Sensitivity and specificity of TM was 78.60% and 98.8% respectively. For CRA method, sensitivity and specificity remained low and were 10.3% and 97.6% respectively.

Methicillin resistance status in relation to biofilm production: It was found that biofilm producing strains were more resistant when compared to the biofilm non producers. All the strong biofilm producers 43 (by tissue culture plate method) were found to be methicllin resistant. Out of the remaining 52 MRSA strains, 42 were moderate biofilm producers, 7 were weak producers and just 3 strains did not produce any biofilm. Amongst the 105 MSSA strains, 33 strains were found to be moderate biofilm producers, 21 were weak producers and none was strong producer of biofilm. 51 MSSA strains did not produce biofilm .

Table 3: Biofilm production by TCP method in MSSA and MRSA

Organism	STRONG	MODERATE	WEAK	NEGATIVE
MRSA(95)	43(45.3%)	42(44.2%)	7(7.4%)	3(3.2%)
MSSA(105)	0	33(31.4%)	21(20%)	51(48.6)

Table 4 : Comparison of biofilm detection methods in various studies

Year	Author	Biofilm detection methods		
		TCP	TM	CRA
2006	**Turkyilmaz et al[21]**	**50.50%**	**55.55%**	**61.10%**
2006	**Mathur T et al [22]**	**53.9%**	**41.4%**	**5.3%**
2011	**Hassan et al[23]**	**63.63%**	**54%**	**11%**
2011	**Fatima khan et al[24]**	**64.9%**	**63.7%**	**47.7%**
2014	**Nabajit Deka [25]**	**83%**	**57%**	**20%**
2015	**Tayal R et al[26]**	**27%**	**37.96%**	**40.88%**
2015	**Mohamad E et al [27]**	**45.6%**	**38.11%**	**36.9%**
2016	**Pallavi Sayal et al[28]**	**69.9%**	**53.1%**	**9.7%**
2017	**Present study**	**59%**	**46.5%**	**7%**

Discussion

Staphylococcus aureus is a leading cause of morbidity and mortality in nosocomial and community-based infections. It is associated with a number of infections ranging from dental caries, periodontitis, stye, carbuncle, impetigo, and pyoderma to persistent tissue infections such as wound infection, otitis media, osteomyelitis, rhinosinusitis, recurrent urinary tract infection, and endocarditis .It is also one of the most important pathogens in implant-related infections.

In this study 200 *Staphylococcus aureus* isolates were collected during November 2015 – May 2017 among them 95 (47.5%) were MRSA. The incidence of MRSA varies widely in India because of varied population and hospital practices. Various studies have shown incidence of MRSA varies between 25% up to even 50% or

Biofilms : TCP method detected 118/200(59%) as biofilm producers. Out of which, 43 were strong, 75 were moderate and 82 were non biofilm producers. Tube method detected 93/200(46.5%) as biofilm

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producers. Among them, 36 were strong and 57 were moderate and 107 were non biofilm producers. With CRA method most strains displayed red (pink to orange) colonies and only 14(7%) showed black colonies with dry crystalline morphology. Our results were in concordance with studies of Mathur T et al[22], Hassan et al[23], Nabajit Deka et al[25] ,Pallavi Sayal et [28] . however in study by Turkyilmaz et al [21],Fatima Khan et al [24], Tayal et al [26], Mohamad Et al[27] detection of biofilms by CRA was higher than our study as shown in the Table 4 .

Our data indicates that TM is 78.60% sensitive, 98.8% specific and 87% accurate method. Based on our observations we don't recommend CRA method as suitable method for biofilm screening. But due to subjective variations in interpretation and lack of reproducibility among test results TM cannot be suggested as general screening test to identify biofilm producing isolates.

When *S. aureus* assumes the biofilm phenotype, these infections are often extremely difficult to treat. The infection may fail to respond to antibiotic therapy or it may initially respond only to relapse weeks or months later. In such cases, invasive treatments, such as surgical removal and replacement of the infected tissue or device, may be required. So for proper treatment of *S. aureus* infection screening for biofilm production is necessary. However, this might not be feasible in every case, so we recommend that in all cases of *S. aureus* especially MRSA infections and in patients with hospital acquired staphylococcal infection screening for biofilm should be done routinely by tissue culture plate method as this is a cheap method with no subjective errors and requires less expertise.

Conclusion

Staphylococcus aureus infections are becoming difficult to treat owing to emergence of resistant strains to individual drugs as well as multiple drugs. As the prevalence of such resistant strains especially MRSA, has increased in the recent times, such strains need to be identified at the earliest to avoid further spread.

In our study the cefoxitin disc diffusion method was considered the standard method for detection of MRSA (according to CLSI guidelines) in the absence of molecular methods, Vancomycin E-strip testing was found to be highly efficient, accurate and less labour intensive for detecting MIC levels.

Any resistant strain needs to be sent to a reference laboratory for confirmation before notifying the same. Resistance to other agents is also emerging with many strains being multidrug resistant.

Detection of erythromycin and clindamycin resistance phenotypes is of utmost importance as it can guide modifications in therapy. Clindamycin is preferably avoided for strains that are erythromycin resistant as in vivo resistance may develop sooner or later. The D test precisely detected the strains with inducible clindamycin resistance.

Evaluation of effective antibiotic options and rigorous infection control measures will help in the fight against resistant *S.aureus* strains. Antimicrobial stewardship should be implemented in the institution as an important part of efforts to control multidrug resistant organisms. This will help in reducing the prevalence of multidrug resistant *S.aureus*.

Staphylococcus aureus is a clinically relevant pathogen due to its antimicrobial resistance and evasion of the host immune system. In conjunction with the multitude and redundancy of its virulence factors in avoiding host responses and influencing disease, S. aureus is able to form intricate micro-colonies termed biofilms. Although neutrophils are capable of invading the biofilm, the bacterial community is able to thwart this attack and may also skew the immune response to survive attack. Staphylococcus aureus is the etiological agent to a myriad of human acute infections, however, its ability to form biofilm in host emanates into chronic and recalcitrant disease. Current therapies for treating and preventing chronic biofilm-mediated infections are limited to surgical intervention and prolonged antibiotic regiments or addition of antimicrobial compounds to indwelling-medical devices.

We conclude from our study that TCP is a quantitative and reliable method to detect biofilm forming microorganisms. When compared to TM and CRA methods, TCP can be recommended as a general screening method for detection of biofilm producing bacteria in laboratories.

Unique and changing properties of biofilm-positive microbes made them responsible for recalcitrant infection, which are difficult to eradicate. The

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presence of biofilm is probably underestimated because of lack of in vitro diagnostics. Thus, dynamic research activity is required in this field as this bacterial lifestyle may be associated with human infectious diseases. With the emergence of vancomycin resistance in S.aureus role of antimicrobials is becoming limited. Hospital acquired strains of S. aureus should be routinely screened for biofilm formation using the tissue culture plate method and treated accordingly.

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