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Original Article

Microbiology Section

Biofilm: Detection Methods and Correlation with Antimicrobial Resistance in *Staphylococcus*

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ABSTRACT

Introduction: *Staphylococcus* is known for its ability to produce biofilm which is considered to be an important virulence factor as well as one of the mechanisms for antimicrobial resistance. Identification of biofilm production and its relation with antimicrobial resistance might help to elucidate the impact of *staphylococci* in diagnosis of various infections.

Material and Methods: In this study, production of biofilm in 96 *Staphylococcus* aureus (Coagulase-positive *Staphylococcus*) isolated from various clinical specimens was investigated with Congo Red Agar (CRA) and Micro titer plate (MTP) methods and the results were compared to each other.

Results: The rate of biofilm production in all *Staphylococcus*, investigated with CRA and MTP were 72.9%, and 47.9%, respectively. The existence of *Staphylococcus* spp. resistance against various antibiotics was also determined by the agar disk diffusion method. The percentage of resistance against Ciprofloxacin, Erythromycin, methicillin (MRSA), and Co-trimoxazole in biofilm producing *Staphylococcus* was 49.0%, 24.5%, 23.6%, and 13.6%, respectively, whereas for non-biofilm producing strains it was 42.9%, 15.7%, 14.2%, and 12.9%, respectively. The comparison of biofilm producer strains with non-biofilm producer strains revealed that biofilm producer strains had more resistance to those antibiotics.

Conclusion: In conclusion, the MTP test could be used for the detection of slime production in *Staphylococcus* spp. because it is reliable and practical.

Key Words: *Staphylococcus*, Biofilm, MRSA

INTRODUCTION

Biofilms may form on a wide variety of surfaces, including living tissues, indwelling medical devices, industrial or potable water system piping or natural aquatic systems. Biofilm is a microbial derived sessile community characterised by cells attached to a substratum or interface or each other, embedded in a matrix of extracellular polymeric substances that they have produced, and exist in an altered phenotype with respect to growth rate and gene transcription [1]. In recent years, *staphylococci* have emerged as important pathogens that can cause substantial economic loss. *Staphylococci* are the most notable pathogens in foreign body-related infections [2,3]. The ability of *staphylococci* to form biofilms depends on their capacity to produce and secrete an extracellular material often termed slime [4,5]. Slime/biofilm production could play an important role in the adherence of these microorganisms to mucous epithelia [6]. The main component of slime is an exopolysaccharide substance, which mediates intercellular adhesion and allows the bacteria to grow and agglomerate in large cell clusters till forming multi-layer-structured biofilms. Its role in the pathogenesis of staphylococcal infections strictly related to its importance in determining bacterial adhesion and accumulation [7,8] has led progressively to consider biofilm formation as a crucial virulence marker. Biofilm can reduce the immune response and phagocytosis, by interfering with host defense mechanisms. The ability of an organism to produce biofilm is significantly associated with its capability to produce diverse illnesses. In light of this a variety of sensitive techniques have been developed to detect biofilm-positive virulent strains. Here we compare the various methods of detection of biofim production and correlated biofilm production with antimicrobial resistance.

MATERIAL AND METHODS

[Table/Fig-1] In this study a total of 96 isolates of *Staphylococci* were obtained from various clinical specimens from patients admitted in different wards from S.S. Hospital, I.M.S. BHU, Varanasi. Most of these (89.5%) were obtained from pus, followed by blood, Cathetertip, Eyedischarge4.2%,4.2%,2.1%. [Table/Fig-2] Sample were directly streaked on to 10% sheep blood agar and incubated aerobically at 37°C for 48 h. After the incubation period, *Staphylococci* were identified on a

predetermined protocol i.e. colony characteristics, Gram staining, catalase production, oxidase test, and Oxidation-Fermentation tests. Gram-positive, cluster forming, catalase positive, oxidase negative, resistant to bacitracin, coagulase and fermentative strains of *Staphylococci* were identified by standard methods. Antimicrobial susceptibility testing was performed for 13 different therapeutically relevant antibiotics by Kirby Bauer disk diffusion method according to norms of Clinical Laboratory Standards Institute (CSLI). Antibiotics tested included Penicillin(10 IU), Cefoxitin (30 µg), Gentamycin (10 µg), Ciprofloxacin (30 µg), Erythromycin (15µg), Cotrimoxazole (1.25/23.75 µg), Levofloxacin (5µg), Clindamycin (2 µg), Vancomycin(30 µg), Linezolid (30 µg), Piperacillin-tazobactam (100/10 µg) and tigecycline (15µg)s. For the susceptibility test, isolates were suspended in TSB and the suspension was adjusted to a turbidity equivalent to a 0.5 McFarland standard. The antibiotic susceptibility test was performed with the agar disk diffusion method [10]. Isolates were categorised as susceptible, moderately susceptible, and resistant, based upon interpretive criteria developed by the Clinical and Laboratory Standards Institute (CLSI) [10].

S. No	Unit	Number	percent
1	ARC	2	2.1
2	burn	2	2.1
3	ENT	4	4.2
4	med	4	4.2
5	nephro	2	2.1
6	neuro	2	2.1
7	nicu	4	4.2
9	ortho	24	25
10	Paediatric ward	2	2.1
11	plastic	2	2.1
12	shalya	2	2.1
13	skin	6	6.3
14	surgery	38	40
15	trauma	2	2.1
16	**Total**	**96**	**100**

[Table/Fig-1]: Clinical specimens obtained from different wards OPDs

S. No.	Specimen	Number	Percent
1	Blood	4	4.2%
2	Catheter tip	2	2.1%
3	Eye discharge	2	2.1%
4	Neck line	2	2.1%
5	Pus	86	89.5%
Total		96	100%

[Table/Fig-2]: Types of clinical specimens obtained for the study

Biofilm production

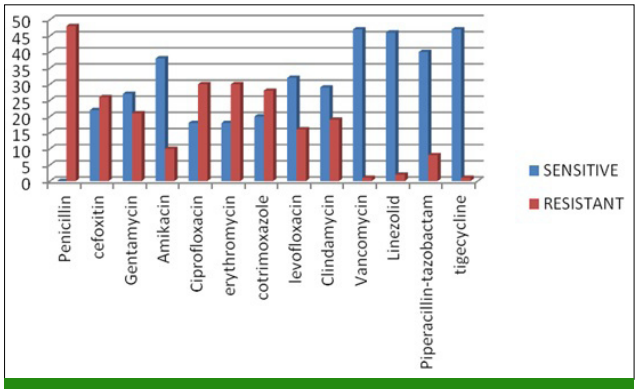
The isolates were simultaneously examined by two methods:

Detection of biofilm production by Congo Red Agar (CRA) method: The method developed by Freeman et al., [11]. was used in this study. The composition of medium (CRA) was brain heart infusion broth (BHIB) 37 g/l, sucrose 50 g/l, agar 10 g/l, and Congo Red 0.8 g/l. The Congo Red stain was prepared as a concentrated aqueous solution and autoclaved separately at 121˚C for 15 min. and was added when the agar had cooled to 55°C. Plates were inoculated and incubated aerobically at 37˚C for 24h. Isolates that produced black colonies with dry crystalline consistency were regarded as biofilm producer, whereas those showing pink colonies were non producer and those with black colonies without dry crystalline consistency were reported as indeterminate producers.

Detection of slime by Micro titer plate (MTP) method: Quantitative determination was carried out with the MTP method proposed by Christensen et al., [12]. using tissue plates with 96 flat-bottomed wells. Each well was filled with 0.2 ml of 105 CFU/ml of a bacterial suspension in Trypticase Soy Broth (TSB). After 48h incubation at 37˚C, the contents were aspirated and the plates were washed twice with phosphate-buffered saline (PBS; pH: 7.2). The wells were stained with 0.25% safranine for 30 s. The plates were read in an enzyme-linked immunosorbent assay (ELISA) reader to 490nm. Sterile TSB was used as a negative control. All the experiments were repeated at least twice, the values of optical density were then averaged. A 3-grade scale was used to evaluate the strain slime producing ability; (-): ODs < 0.500; (+): ODs 0.500-1.500; (++): ODs > 1.500.

RESULTS

Congo red agar methods have high sensitivity showing 73% biofilm positive isolates whereas MTP method is more specific shown only 48% isolates forming biofilm. Comparison of biofilm production with drug resistance has shown in [Table/Fig-3].



[Table/Fig-3]: Antimicrobial susceptibility pattern

		Congo red method				Total
		Negative	Mild	Indeterminate	High	
TCP	Negative	10(10.4%)	6(6.2%)	26(27.1%)	8(8.3%)	50(52.1%)
	Mild	6(6.2%)	4(4.2%)	6(6.2%)	0(%)	16(16.7%)
	Moderate	4(4.2%)	4(4.2%)	0(0%)	2(2.1%)	10(10.4%)
	High	6(6.2%)	6(6.2%)	8(8.3%)	0(0%)	20(20.8%)
Total		**26(27.1%)**	**20(20.8%)**	**40(41.7%)**	**10(10.4%)**	**96(100%)**

[Table/Fig-4]: Congo-red TCP comparison

DISCUSSSION

Biofilm has been described as a barrier, which is produced by microorganisms to survive and protect themselves against various environments. Biofilm production is an important mechanism that allows microbes to escape host defenses and antimicrobial therapy. *Staphylococcus* sp. is a common cause of nosocomial and environmental infection. It has been thought that testing for biofilm formation could be a useful marker for the pathogenicity of *Staphylococci* [13,14]. Baselga et al., [6]. reported that 47.7% *Staphylococcus* isolates from various clinical specimens were biofilm producer. Ammendolia et al., [15] reported that 88.9% of the Coagulase positive strains were biofilm producer. In our study MTP method and CRA methods detected 48% and 73% isolates was biofilm producer respectively. CRA method is more sensitive than MTP for biofilm production. The microtitre plate method (MTP) was compared to the Congo red agar based method for the detection of biofilm production. It was easy to discriminate between strong biofilm producers, moderate and non producers when using the MTP, while the CRA there is subjective variability in result interpretation. There was a good correlation between the two methods, ($p<0.05$), but the MTP methods was the one used for further analysis, because it was found to be more sensitive and easy to read, although, both methods were easy to perform. With the polystyrene microtitre plate, 20 (20.8%) were strong producers, 10 (10.4%) were moderate biofilm producers, 16 (16.7%) were mild biofilm producers while 50 (52.1%) were non-biofilm producers. With the Congo red methods, 10 (10.4%) high biofilm producers, 40 (41.7%) were indeterminate biofilm producers and 20 (20.8%) were mild biofilm producers whereas 26 (27.1%) were non-biofilm producers. The microtitre plate method was more specific as compared to the Congo red agar method, and it was the one used for further analysis in this study [Table/Fig-4].

All isolates were resistant to Penicillin. Cefoxitin resistance (MRSA) among non-biofilm producing and biofilm producing strains were 31% and 22% respectively. 50% of high biofilm producers were MRSA positive whereas 60% of non-biofilm producers were MRSA positive. Fifty-six (56%) biofilm producing strains were MRSA positive compared to 48% among the MSSA isolates. Moreover, when isolates were classified based on biofilm producing capacity, 37.8% of the strong biofilm producers were MRSA positive [16].

Ciprofloxacin resistance was 35% and 29%, and Erythromycin resistance was 35% and 27% respectively among biofilm producers and non producers. All isolates were sensitive to Vancomycin, Linezolid and Tigecyclin.

CONCLUSION

Biofilm production is one of the virulence factor of staphycococcus and should be looked for along with coagulase production and other virulence factors. It is associated resistance of the organism for various antibiotics as this reduces the availability of the drug to the organism. MTP method for the detection of biofilm is a reliable method than CRA method as it is more specific and results are more reproducible. Clinical microbiology labs must look for biofilm producing ability of the *Staphylococcus* along with antibiotic sensitivity as it may be a answer to the clinical resistance in case of microbiology laboratory susceptible antibiotic.

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