Section A-Research paper

Biofilm formation by clinical isolates of *Staphylococcus aureus* and their association with antimicrobial resistance.

Authors : 1. Dr. Nilima Sanjay Naik
Assistant professor, Department of Gen. Pathology and microbiology,
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Sangli. Maharashtra
Mobile: 9423023429
Mail ID - patilnilima@rocketmail.com
2 Dr. Madhuri Sale
Assistant professor, Department of Oral Pathology and microbiology,
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Sangli. Maharashtra
Mail ID - drmadhurisp@gmail.com
3. Dr. Lalita Girish Nanjannwar
Associated professor,Dept. of Orthodontic and Dentofacial Orthopedics
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Sangli. Maharashtra
Mail ID: drlalitagn@gmail.com
4. Dr. Chetan Patil
Assistant professor, public health dentistry,
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Sangli. Maharashtra
Mail ID: chetan.patil@bharatividyapeeth.edu
5. Dr. Shivdas M.Mali
Assistant professor, Department of Gen. Pathology and microbiology,
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Pune. Maharashtra
Mail ID – shivdasmali84@gmail.com
6. Dr. Amol Karagir
Assistant professor, Oral medicine and radiology,
Bharati vidyapeeth deemed to be university, Dental College and hospital,
Sangli. Maharashtra
Mail ID:amol.karagir@bharatividyapeeth.edu

Abstract: - *Staphylococcus* species has the tendency to form biofilms, and causes significant mortality and morbidity in the patients. Aim: Our study was aimed to determine the prevalence of biofilm production in *Staphylococcus aureus* isolates from various clinical samples and its

antibiotic sensitivity pattern in the tertiary care hospital. **Methods:** The study included 150 staphylococcal isolates. Biofilm detection in staphylococci was performed using tissue culture plate (TCP). **Results:** The TCP method detected total positive biofilm production in 96 (64%) staphylococcal isolates. Out of these strong positive was 52 (54.16%), among there 30 were *S. aureus* and 22 were Coagulase negative Staphylococcus and moderate positive were 44 (45.83%) 31 were *S. aureus* and 13 were Coagulase negative Staphylococcus. They were resistant to most antibiotics except vancomycin and linezolid. **Conclusions:** The clinical isolates of *Staphylococcus aureus* exhibit a high degree of biofilm formation. Higher rate of antimicrobial resistance is demonstrated by biofilm producers than non-producers. Therefore, we recommend regular surveillance of biofilm formation in *S. aureus* and their antimicrobial resistance profiles.
Abbreviations: *Staphylococcus aureus*, Biofilm, Coagulase negative Staphylococcus, Tissue culture plate.

Introduction:

Staphylococcal infections occur by two mechanisms including direct invasion to the tissue, e.g. endocarditis and septic arthritis and through the production of exotoxins. Some of these exotoxins have local effects and others have severe systemic effects as they induce release of cytokine from T cells causing disease to all parts of the host body [1]. Their pathogenicity is due not only to the virulence factors that they express, but also to the ability of these bacteria to form biofilms. Staphylococcal infections represents major concern in the medical practice especially when it related to biofilms formation in implanted medical devices like catheters and prosthesis. The production of biofilms by bacteria is an important factor, leading to treatment failures also. These infections are persistent in nature being highly refractory to various problems including antibiotics resistance. Bacterial biofilms create significant obstacles in both medical and industrial settings. In the medical field, it is estimated that biofilms cause over 80% of microbial infections in the body and approximately 65% of nosocomial infections involve biofilms. The ability to form biofilm, an important virulence factor is expressed by many pathogenic bacteria, and the *Staphylococci* are the most common etiological agents of device related infections [2].

Scientists have recently understood that bacteria are not always living as free cells in nature; on the contrary, most of the time, bacteria build a real social life in a resistant community surrounded by a matrix composed of polysaccharides, extracellular DNA, proteins, lipids and other components [3, 4]. Biofilm is defined as a multicellular lifestyle an organized structure built by almost all bacterial species. Even if the term "biofilm" has been used for more than 60 years, the understanding of this structure started but recently. Fossilized biofilms of 3.5 billion years have been discovered and highlight the hypothesis that biofilm is a survival strategy always used by microorganisms since the dawn of time [5]. Biofilms are the aggregation of bacteria embedded in a self-produced extracellular matrix of exopolysaccharides (EPSs), proteins and some micromolecules such as DNA. They can form on both biotic and abiotic surfaces [6].

Biofilm is present on biotic or abiotic surface and bacteria embedded inside are 10–1000 times more resistant to conventional antibiotics than free-floating bacteria according to the strains, the molecule applied and the model of study. Life cycle of biofilm is nowadays well-described. First, bacteria adhere on a surface and they enhance different mechanisms to irreversibly be attached. Then, the program of biofilm starts with a maturation of the multicellular structure. To complete this cycle, dispersion of swimming cells occurs under specific conditions [7]. In the medical domain, numerous difficulties to treat biofilm-associated infections like it is resistance to antibiotics and to immune system, spread of infection, sepsis shock and surgical risks to remove infected implant or tissues [8, 9].

In addition, biofilm also protect the embedded bacterial cells from the host immune cells thus facilitating the survival of pathogens for a prolonged period. The ability of *S. aureus* to form biofilm on biotic and abiotic surfaces is its major virulence property. Biofilm formation of Staphylococcus aureus of primary public and animal health concern [10]. Therefore our study was aimed to determine the prevalence of biofilm production in *Staphylococcus aureus* isolates from various clinical samples and its antibiotic sensitivity pattern in the tertiary care hospital.

Materials and methods:

Sample source: A total 150 *Staphylococcus species* were isolated from randomly selected various clinical specimens like pus, blood and various aspirations received in the department of Microbiology, B.V.D.U.M.C & H. Sangli.

Identification of Staphylococcus species:

All the Staphylococcus were identified by conventional microbiological methods including colony colour, colony morphology, Gram stain, slide coagulation test, tube coagulation test, mannitol fermentation test and catalase test to differentiate it from *Streptococcus* species and DNase test to differentiate *S. aureus* from coagulase- negative staphylococci (CONS) and then subjected to antibiotic susceptibility testing by Kirby – Bauers disc diffusion method on Muller Hinton agar plate using routine antibiotic discs as per CLSI guidelines [11].

Biofilm formation in these isolates was detected by tissue culture plate method. Tissue culture plate (TCP) method is the standard gold method as reported by Mathur et al., 2006 [12]. Hence it was considered a standard method for interpretation of our results. Tests for biofilm production Control strains: *S. aureus* ATCC 35556 and *S. aureus* ATCC 25923 were used as positive and negative controls, respectively, for the biofilm assay were used.

Tissue culture plate method

Tissue culture plate method was done for quantitative assessment of biofilm production. It is gold standard method for biofilm detection was carried out as described by Christensen et al[13]. In this method, isolated colony of *S. aureus* was inoculated in 2 mL of trypticase soya broth. The broth was incubated at 37 °C for 24hrs. The culture was then diluted to 1:100 with fresh medium. A sterile individual plate with 96 flat bottom polystyrene wells was filled with

200 µL of the diluted culture. The control organisms were also processed in a similar manner. The plate was incubated at 37 °C for 24 hours. After incubation, the contents of each well were removed by gentle tapping. The wells were washed with 200 µL of phosphate buffer saline (pH 7.3) to remove free-floating bacteria. Biofilms formed by bacteria adherent to the wells were fixed by 99% methanol and stained with 0.1% crystal violet. Excess stain was washed gently and the plate was kept for drying. The optical density of the stained adherent biofilm was measured using a micro- ELISA auto-reader at a wavelength of 570 nm. Interpretation of biofilm production was performed as per the criteria described by Stepanovic et al, [14] and the bacteria were categorized into biofilm nonproducers, or weak, moderate or strong biofilm producers. The optical density (OD) value of each isolate was interpreted according to the following table to assess the degree of the biofilm (Table 1).

Table 1: Interpretation of results of Tissue Culture Plate method

OD Value	Biofilm Formation
<0.120	Non biofilm producer
0.120 -0.240	Moderate biofilm producer
>0.240	Strong biofilm producer

Abbreviation: OD- Optical density

Antimicrobial susceptibility test:

Kirby – Bauers disc diffusion method was used for antimicrobial susceptibility testing on Muller Hinton agar plate using routine antibiotic discs as per CLSI guidelines. The following antibiotic discs were used. Diameter of zone of inhibition was measured by scale and compared with NCCL zone size interpretation chart. Antibiotics tested were Ampicillin , Co- trimaxazole , Cefoxitin , Ciprofloxacin , Gentamicin , Vancomycin and Linezolid (Hi Media Mumbai). Zone diameters were measured following CLSI criteria **[11]**.

Results

A total 150 *Staphylococcus species were* isolated from various clinical samples. Among these 90 strains were *S. aureus* and 60 were Coagulase negative Staphylococcus (CONS). The percentage of biofilm producing *S. aureus* isolates from different samples is shown in Table 2, which indicates higher incidence of biofilm producers (moderate to high) in pus and blood samples, while in case of fluids and urine samples though the overall biofilm producers are less.

Biofilm detection in staphylococci was performed using Tissue culture plate method. The TCP method detected total positive biofilm production in 96 (64%) staphylococcal isolates.

Out of these strong positive was 52 (54.16%), among there 30 were *S. aureus* and 22 were Coagulase negative Staphylococcus and moderate positive were 44 (45.83%) 31 were *S. aureus* and 13 were Coagulase negative Staphylococcus.

Table 2. Distribution of Biofilm Producers According to Clinical Samples

Specimen	Biofilm Producer		Strongly Positive		Moderate Positive	
	No	%	No	%	No	%
Pus	34	35.41	22	42.30	12	27.2
Blood	32	33.33	26	50	06	13.63
Sputum	16	16.66	07	13.46	09	20.45
Fluids	04	04.16	01	1.92	03	06.81
Urine	14	14.58	09	17.30	05	11.36
Total	96		52		44	

As shown in Table 3, the biofilm-producing *Staphylococcus species* were associated with higher incidence of antimicrobial resistance when compared to the non- biofilm producers. All the isolates were sensitive to vancomycin and linezolid.

Table No 3. Antimicrobial resistance pattern of biofilm producer and non - biofilm producer *Staphylococcus* Species

Antibiotic agent	**Biofilm producer (n=96)**		**Non- biofilm producer (n=54)**	
	No	%	No	%
Ampicillin	79	82.29	28	51.85
Cefoxitin	35	36.45	15	27.77
Cotrimoxazole	58	60.41	25	46.29
Ciprofloxacin	71	73.95	28	51.85
Gentamicin	51	53.12	30	55.55

Vancomycin	0	0	0	0
Linezolid	0	0	0	0

Abbreviations: BP- biofilm producer; BN-biofilm non-producer;

Discussion:

In the present study, we detected the in vitro biofilm-forming ability of *S. aureus* and Coagulase negative *staphylococci* isolated from clinical samples and their association with antimicrobial resistance. Early detection of virulent staphylococci therefore warrants one of the most essential steps for prevention, management, and cure of staphylococcal infections.

The prevalence of staphylococcal biofilm formation in the present study was 64% detected by TCP. Differences in the prevalence of biofilm formation have been reported, with data ranging from <50% to >70% [15–16]. Our findings are correlates with a study done by Hassan et al where the number of isolates showing biofilm formation by TCP method was 64.7%, and non-biofilm producers were 36.3%.[17]. Fatima et al., 2011 [18] also reported a high percentage of *S. aureus* as biofilm producers (64.89%). Study done by Gogoi M et al reported 61.7% biofilm formation by TCP method [19]. Our findings are comparatively more than studies done by Bose et al found that biofilm formation in TCP method was 54.19% and non-biofilm producers were 45.81%.[20] and Manandhar et al reported a prevalence 43% of this bacterium in clinical specimens **[21]**.Our findings are comparatively less than studies done by Rania M 2018 reported (74%) staphylococcal isolates were biofilm producers. the strong positive was 65 (43.3%), and moderate positive were 46 (30.7%)[22]. This might be attributed to the difference in the sources from which their strains were isolated. Biofilm formation depends on many factors such as environment, geographical origin, availability of nutrients, types of specimen, surface adhesion characteristics and genetic makeup of the organism [23]. These factors may have affected the data and contributed to the high prevalence observed in the present study. Biofilms can form on any wound when planktonic bacteria are not eliminated by the host's immune system or by exogenous antimicrobial agents [24].

In the present study the potential for biofilm formation in pus may be similar to that in the blood. Biofilm infections are clinically important because bacteria in biofilms exhibit resistance to antimicrobial compounds [25]. The biofilm-producing *S. aureus* were more resistant to various antimicrobials than the biofilm non-producers [17, 26]. Antimicrobial approach in the control of staphylococcal infections has often become ineffective due to the emergence of multi drug resistance (MDR). Threats of MDR transforming to PAN drug resistance in near future requires a steady research outcome to combat such kind of infections. Biofilm producing strains in our work were resistant to almost all groups of antibiotics. Among

our isolates, Biofilm is one of the major factors in emphasizing antibiotic resistance and hence biofilm detection facilitates the investigation of severity of infection among invasive *S. aureus*.

The higher rate of resistance in biofilm-producing Gram-positive bacteria toward Cotrimoxazole and Ciprofloxacin has been reported earlier [17]. Our result correlate to previous study which reported that resistance toward Co-trimoxazole was increased due to the excessive use of these drugs for the treatment of staphylococcal infections. Therefore, the antimicrobial resistance seen in the present study was higher among biofilm-producing *S. aureus* than among the non-producers. These results indicate that biofilm formation may be one of the crucial factors for increasing resistance toward commonly used antibiotics. Linezolid is a worldwide effective and well tolerated antimicrobial in patients with *S. aureus* infections. In a study done in Nepal, linezolid showed the highest rate of susceptibility (100%) [27].

Conclusion

The clinical isolates of *Staphylococcus aureus* recovered from various clinical specimens of patients exhibit a high degree of biofilm formation. Higher rate of antimicrobial resistance is demonstrated by biofilm producers than non-producers. This may lead to the high risk of impairment in the healing and dissemination of the infections. Therefore, we recommend regular surveillance of biofilm formation in *S. aureus* isolates and their antimicrobial resistance profiles. This may help us to formulate an effective antimicrobial policy for the early treatment.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

The authors are grateful to the laboratory staff and management of B V D U M C & H, Sangli for their help and cooperation during the study.

References:

1. Coates R, Moran J, Horsburgh MJ. Staphylococci: colonizers and pathogens of human skin. Future Microbiol. 2014; 9(1):75–91.

2. Percival SL, Suleman L, Vuotto C, Donelli G (2015) Healthcare-associated infections, medical devices and biofilms: risk, tolerance and control. J Med Microbiol 64: 323-334.

3.Høiby N, Ciofu O, Johansen HK, Song Z, Moser C, Jensen PØ, et al. The clinical impact of bacterial biofilms. Int J Oral Sci. 2011 Apr; 3(2):55–65.

4. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science. 1999 May 21; 284(5418):1318–22.

5 Hall-Stoodley L, Costerton JW, Stoodley P. Bacterial biofilms: from the natural environment to infectious diseases. Nat Rev Microbiol. 2004 Feb; 2(2):95–108.

6 Gowrishankar S, Duncun Mosioma N, Karutha Pandian S. Coral-associated bacteria as a promising antibiofilm agent against methicillin-resistant and susceptible Staphylococcus aureus biofilms. *Evid Based Complement Alternat Med.* 2012; 2012:862374

7 de la Fuente-Núñez C, Reffuveille F, Fernández L, Hancock REW. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. Curr Opin Microbiol. 2013 Oct; 16(5):580–9.

8. Bryers JD. Medical biofilms. Biotechnol Bioeng. 2008 May 1; 100 (1):1–18.

9. Römling U, Balsalobre C. Biofilm infections, their resilience to therapy and innovative treatment strategies. J Intern Med. 2012 Dec; 272 (6):541–61.

10. Tam, K.; Torres, V.J. Staphylococcus aureus secreted toxins and extracellular enzymes. Microbiol. Spectr. 2019, 7

11. Clinical Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; Eighteenth Informational Supplement. Zone Diameter Interpretive Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for Staphylococcus spp., Jan 2008; M100-S18; 28(1): 48.

12. Mathur T, Singhal S, Khan S, et al. Detection of Biofilm Formation among the Clinical Isolates of Staphylococci: An Evaluation of Three Different Screening Methods. Ind J Med Microbiol. 2006; 24(1):25-29.

13. Christensen GD, Simpson WA, Younger JJ, et al. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices. *J Clin Microbiol*. 1985; 22(6):996–1006.

14. Stepanovic S, Vukovic D, Hola V, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*. 2007;115 (8):891–899.

15. Samie A, Shivambu N. Biofilm production and antibiotic susceptibility profiles of Staphylococcus aureus isolated from HIV and AIDS patients in the Limpopo Province, South Africa. *Afr J Biotechnol*. 2011; 10(65):14625–14636.

16. Cha JO, Yoo JI, Yoo JS, et al. Investigation of biofilm formation and its association with the molecular and clinical characteristics of methicillin resistant staphylococcus aureus. *Osong Public Health Res Perspect.*2013;4(5):225–232.

17. Hassan A, Usman J, Kaleem F, Omair M, Khalid A, Iqbal M. Evaluation of different detection methods of biofilm formation in clinical isolates. Braz J Infect Dis. 2011;15 (4):305-11.

18. Fatima K, Indu S, Meher R, et al. Detection of Biofilm formation in Staphylococcus aureus. Does it have a role in t/t of MRSA infections? Trends in Med Res. 2011; 2:116-123.

19 *Gogoi M et al.* Biofilm formation by bacteria isolated from intensive care units of a tertiary care hospital, with special relevance to its risk factors*Int J Res Med Sci. 2021 Oct;9(10):2959-2965*

20. Bose S, Khodke M, Basak S, Mallick SK. Detection of biofilm producing Staphylococci: need of the hour. J Clin Diagn Res. 2009;3 :1915-20.

21. Manandhar S, Singh A, Varma A, Pandey S, Shrivastava N. Evaluation of methods to detect in vitro biofilm formation by staphylococcal clinical Isolates. BMC Res Notes. 2018; 11: 714.

22.Rania M. Abdel Halim*, Nevine N. Kassem, Basma S. Mahmoud, Detection of Biofilm Producing Staphylococci among Different Clinical Isolates and Its Relation to Methicillin Susceptibility, Macedonian Journal of Medical Sciences, 2018 Aug 20; 6(8):1335-1341.

23. Kokare CR, Chakraborty S, Khopade AN, Mahadik KR. Biofilm: importance and applications. *Indian J Biotechnol*. 2009;8: 159–168.

24. Hurlow J, Couch K, Laforet K, Bolton L, Metcalf D, Bowler P. Clinical biofilms: a challenging frontier in wound care. *Adv Wound Care (New Rochelle)*. 2015;4(5):295–301.

25. Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: an evaluation of three different screening methods. *Indian J Med Microbiol*. 2006;24(1):25–29.

26. CharanKaur D, Khare AS. Biofilm formation and antibiotic susceptibility pattern in MRSA strains in a tertiary care rural hospital. *IJAR*. 2013;3 (1): 37–44.

27. Ansari S, Nepal HP, Gautam R, et al. Threat of drug resistant Staphylococcus aureus to health in Nepal. *BMC Infect Dis*. 2014;14: 157.