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Evaluation of Different Phenotypic Methods for Detection of Biofilm Formation among the Clinical Isolates

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A B S T R A C T

Biofilm-producing staphylococci have been identified from various clinical samples such as pus, blood, urine and skin surfaces, in addition to device associated illnesses. In the study included 100 clinical isolates of Staphylococci spp. Biofilm detection was performed on clinical staphylococci spp. isolates using two different phenotypic methods, including Tissue Culture plate (TCP) and Congo red agar (CRA).By the TCP method revealed that, 55 (55%) Staphylococcus isolates were biofilm producers, while 45 (45%) were non- or weak biofilm producers. Among 55(55%) biofilm producers, 31 (56.36%) were strong biofilm producers, while 24 (43.63%) were moderate biofilm producers. By the CRA method, among 100 staphylococcal isolates, 10(10%) were produced biofilm while 90 (90%) were non/weak biofilm producers. Among the 10 biofilm producers, strong biofilm was produced by 3(30%) and moderate biofilm was produced by 7 (70%). TCP is considered a highly significant test, p = 0.001. As for CRA, it detected a very low number of biofilm producers compared to total positive biofilm producers. According to the p = 0.381, CRA method is considered the non-significant test.The two phenotypic methods were evaluated and it was found that CRA method was less sensitive in detecting biofilm formation. TCP can be recommended as a general screening method for detection of biofilm producing bacteria.

Introduction

The genus Staphylococcus is a Gram-positive coccus in the family Micrococcaceae. *Staphylococcus aureus* and *Staphylococcus epidermidis* are well-known pathogens that causes skin and soft tissue infection, bloodstream infection, osteomyelitis, endovascular and respiratory tract infections in humans. Other staphylococci species commonly found in clinical specimens include *S. haemolyticus, S. lugdunensis, S. capitis, S. cohnii, S. warneri, S. saprophyticus, S. saccharolyticus* and *S. hominis.* These are ubiquitous in the environment and are also associated with human infections (Thakkar, 2015; Tong, 2015; Nasr, 2012).

Staphylococcus aureus is a nosocomial opportunistic infection that has developed resistance to various

antibiotic classes. Methicillin Resistant *Staphylococcus aureus* (MRSA) strains cause by changes in the penicillin binding protein (PBP). Oxacillin's minimum inhibitory concentration (MIC) is < 4 mg/L. In the majority of MRSA strains (PBP2a), the mecA gene, which encodes a modified penicillin-binding protein 2a, is found on the staphylococcal cassette chromosome mec (SCCmec). Infections caused by MRSA range from skin and surgical site to infections involving catheters and prosthetic implants, endocarditis and pneumoniae (Siva Sankari, 2003; Lienen, 2021).

In recent years, increased incidences of staphylococci associated with medical device-related infections have also been observed. This may be due to the organisms able to produce biofilms on a variety of surface. Antibiotics and host defense mechanisms are resistant to biofilm-forming bacteria which complicates the treatment. Biofilm are a group of micro-organisms adhered to a surface and protected by an exopolysaccharide matrix. During their transition from planktonic to surface-attached communities, they undergo a variety of modifications. Biofilm acts as a diffusion barrier to slow down the infiltration of antimicrobial agents (Triveni *et al.,* 2018).

Biofilm-producing staphylococci have been identified from various clinical samples such as pus, blood, urine and skin surfaces, in addition to device associated illnesses. In many chronic and refractory conditions, including native valve endocarditis, cystic fibrosis, pneumoniae and periodontitis, biofilm formation among bacteria is an increasing cause of mortality and morbidity. Biofilm associated infections was difficult to cure because the biofilm confers resistant against the host immune system as well as make the bacterial cells impervious to the antibiotics (Bose, 2009; Hou, 2012).

To identify slime production by staphylococci, a variety of tests are available, including quantitative methods like Tissue culture plate (TCP) which is regarded as the gold-standard method for biofilm identification and qualitative methods like Congo red agar (CRA) (Halim *et al.,* 2018). Hence, the current study is aimed to evaluate the biofilm forming capacity of clinical isolates of Staphylococcus species in tertiary care hospital.

Materials and Methods

A prospective Hospital based study was conducted in Department of Microbiology JSS Medical College & Hospital (JSSMC), for the duration of 1 year.

Sample collection and Microbiological processing

The study included 100 clinical isolates of Staphylococci spp. The various clinical samples such as pus, blood, ear swabs, bile, endotracheal aspirates which were received in the laboratory for culture and sensitivity were processed according to the standard protocol.

Identification of *Staphylococcus* spp.

Bacterial identification was performed using conventional bacteriological techniques including colony morphology on blood agar which showed either β-hemolytic (golden yellow) and non-hemolytic (grey-white) colonies, Gram staining showed Gram positive cocci in clusters. After Gram staining, isolates were tested for catalase to distinguish them from Streptococcus species, and then coagulase (slide and tube coagulase) to distinguish *S.aureus* from Coagulase negative Staphylococci (CONS).

All clinical and environmental isolates were identified and an Antimicrobial Susceptibility Testing (AST) was performed using an automated identification system (Vitek-2 method) and Kirby-Bauer's Disk Diffusion Method accordance to the CLSI guidelines.

Detection of MRSA

Most Methicillin resistance is mediated by mecA, encoding PBP2a. Detection of mecA and PBP2a are

the foremost definitive tests for detection of Methicillin Resistant Staphylococci species. Isolates that test positive for mecA or PBP2a or are resistant by any of the recommended phenotypic methods should be reported as Methicillin resistant.

Cefoxitin was used as a surrogate test for diagnosing methicillin resistant mediated by mecA. Results were recorded and interpreted as per the CLSI guidelines as shown in the (Table 1).

Detection of Biofilm production

Biofilm detection was performed on clinical staphylococci spp. isolates using two different phenotypic methods, including Tissue Culture plate (TCP) and Congo red agar (CRA).

Tissue culture plate method

This method was taken as the gold standard method for detecting the formation of biofilms. Christensen *et al.,* (1985) described TCP method.

A loopful of culture isolate was inoculated from fresh blood agar plate into BHI broth with 2% sucrose and kept for incubation at 37 ℃ for 24 hours in stationary condition and diluted 1 µl in 100 µl with fresh medium. 200 µl of prepared bacterial suspension was aseptically transferred in the wells of a flat-bottomed micro titer plates. The inoculated Tissue culture plate was incubated at 37℃ for 24 hours.

The contents of the wells were carefully tapped out of the plate after incubation. After that, 200 µl of phosphate buffer saline was used to wash the contents (pH 7.2) which removed the free floating bacteria from the wells. The washing process was repeated four times. The plates were air dry at room temperature. Biofilm produced by bacteria adhering to the wells was fixed for 30 minutes with 2% sodium acetate and stained with 0.1% crystal violet for 30 minutes. After 30 minutes, the wells were completely rinsed with deionized water to remove any remaining stain and the plates were retained for drying. After drying, the Optical density (OD) of stained adhering biofilm was measured by microELISA auto reader at 570 nm wavelength. Biofilm formation used the OD values as an indicator of bacterial adhesion to the wells. The procedure was repeated three times and three average OD values were taken. The OD readings from the sterile medium, fixative and dye were averaged and subtracted from all the test values to account for background absorbance. All test OD values were deducted from the mean OD values obtained from the media control wells. Biofilm production was graded as Strong ((> 0.240), Moderate (0.120-0.240) and Non/Weak (< 0.120) based on the OD values.

Congo red agar method

The Congo red agar method was performed as described by Freeman *et al.,* 1989 for qualitative assessment for detection of biofilm formation. The Congo red agar plates were quadrant streak method inoculated with the test organisms and incubated aerobically at 37℃ for 24-48 hours. The interpretation of result such as Black coloured colonies with a dry crystalline consistency indicated as strong result. Darkening of the colonies with absence of dry crystalline consistency indicated as moderate result. Weak or non-slime producers showed pink colonies and occasionally darkening were observed at the center of colonies.

Statistical analysis

Statistical analysis of biofilm formation was performed using SPSS software version 26 for Windows. P values were calculated using the chi-square test. A p value of < 0.05 was considered significant.

Results and Discussion

Patients Characteristics

In the present study, a total 100 Staphylococci isolates were collected from Jan 2020 to Dec 2020

for a period of 1 year. Figure 1 represented that maximum number of staphylococci were isolated from patients aged 41-50 years accounting for 20 (20%) followed by 51-60 years of age group, accounting to 19 (19%),21-30 years accounting to16(16%), 0-10 years 14 (14%), 61-70 years accounting to 11 (11%), 31-40 years accounting to 8 (8%), 71-80 years accounting to 6 (6%), 11-20 years accounting to 5(5%) and age group ≥ 80 years accounted for 1(1%). In the present study, 67 (67%) staphylococci were isolated from male patients and 37(37%) staphylococci were isolated from female patients, this account to a male to female ratio of 2:1.

Methicillin Sensitive and Resistant *Staphylococcus* spp.

In this study, among 100 clinical isolates of Staphylococcus spp. 73 (73%) isolates were *S. aureus* and 27 (27%) isolates were Coagulase Negative Staphylococci (CONS). Among 73 clinical isolates of S. aureus, 51 (69.86%) were Methicillin Resistant *S. aureus* (MRSA) and 22 (30.13 %) were Methicillin Sensitive *S. aureus* (MSSA). Among 27 CONS isolates, 23 (85.18%) were Methicillin Resistant - Coagulase Negative Staphylococci (MR-CONS) and only 4 (14.81%) were Methicillin Sensitive - Coagulase Negative Staphylococci (MS-CONS).

The vitek-2 method was used to identify all the clinical isolates of CONS 12 (44.44%), these included, S. epidermidis, 8 (29.62%) were S. hemolyticus, 3 (11.11%) were *S. hominis*, 2 (7.40%) were *S. warneri* and one isolate each i.e. 3.70% of *S. lugdunensis* and *S. lentus* which were represented in figure 02.

Antimicrobial Susceptibility Testing (AST) Pattern in Clinical Isolates of Staphylococcus aureus and CONS by Vitek -2 Method

AST pattern of 73 clinical isolates of *Staphylococcus aureus* revealed that all the isolates were (100%) sensitive to Vancomycin, Linezolid, Daptomycin and Teicoplanin followed by 65 (89%) Tetracyclin, 62 (84.93%) Gentamycin, 61 (83.56%) Clindamycin, 58 (79.45%) Trimethoprim in combination with sulphamethoxazole and 51 (69.86%) isolates were resistant to Oxacillin/ Cefoxitin, followed by resistance to Ciprofloxacin and Levofolxacin 60 (82.79%) and Erythromycin 35 (47.94%).

AST pattern of 27 clinical isolates of CONS revealed that all isolates were (100%) sensitive to Linezolid, vancomycin and Daptomycin followed by Teicoplanin 26 (96.29%) Genyamycin 24 (88.88%), Tetracyclin 19 (70.37%), Trimethoprim in combination with sulphamethoxazole 19 (70.37%) and 23 (85.18%) isolates were resistant to Oxacillin/Cefoxitin followed by Clindamycin 20 (74%), Erythromycin 14 (51.85%).

Detection of Biofilm production

Two phenotypic methods, TCP and CRA were used to detect the biofilm forming capacityof100 clinical isolates of Staphylococcus spp. and CONS.

TCP Method

By the TCP method revealed that, 55 (55%) Staphylococcus isolates were biofilm producers, while 45 (45%) were non- or weak biofilm producers. Among 55(55%) biofilm producers, 31 (56.36%) were strong biofilm producers, while 24 (43.63%) were moderate biofilm producers. Out of the 55 biofilm-producing isolates, 41 (74.54%) accounted for *S. aureus*, while 14 (25.45%) accounted for CONS. Out of 73 S. aureus, 41 (56.16%) were produced biofilms, among them 25 (34.24%) were strong biofilms producers and 16 (21.91%) were moderate biofilm producers. Out of 27 CONS, 14(51.85%) were biofilm producers, among them 6 (22.22%) were produced strong biofilm and 8 (29.62%) were produced moderate biofilm. Out of 51 MRSA, 19 (37.25%) were produced strong biofilms and 9 (17.64%) were produced moderate biofilm. Out of 22 MSSA, 6 (27.27%) were produced strong biofilm while7

(31.81%) were produced moderate biofilm. Out of 23 MR-CONS, 5 (21.71%) were produced strong biofilm while 6 (26.08%) were produced moderate biofilm. Out of 4 MS-CONS, 1 (25%) were produced strong biofilm and 2 (50%) were produced moderate biofilm.

Production of biofilm was found to be highest among the CoNS isolates in *S. epidermidis* (8), with 3 (25%) were produced strong biofilm and 5 (41.66%) were produced moderate biofilm. Out of 8 *S. hemolyticus* 1 (12.55%) were produced strong biofilm, while the other 3 (37.5%) were moderate biofilm producers. Out of 2 *S. warneri*, 1 (50%) were strong biofilm producer, and out of 3 *S. hominis* 1 (33.33%) were strong biofilm producer.

CRA Method

By the CRA method, among 100 staphylococcal isolates, 10(10%) were produced biofilm while 90 (90%) were non/weak biofilm producers. Among the 10 biofilm producers, strong biofilm was produced by 3(30%) and moderate biofilm was produced by 7 (70%). Out of 10 biofilm producers, 5 (50%) were S. aureus and 5(50%) were CONS. Out of 73 S. aureus isolates, 5 (6.84%) were biofilm producers and among them 2 (2.7%) were strong biofilm producers while 3 (4.1%) were moderate biofilm producers. Out of 27 CONS isolates, 5 (18.5%) were biofilm producers, among them 1(3.7%) was strong biofilm producer and 4 (14.8%) were moderate biofilm producers. Out of 51 MRSA, 2 (3.9%) were strong biofilm producers while 2 (3.9%) were moderate biofilm producers. Out of 22 MSSA only 1 (4.5%) was moderate biofilm producer. Out of 23 MR-CONS, 1 (4.3%) was strong biofilm producer and 3 (13.4%) were moderate biofilm producers. Out of 4 MS-CONS only 1 (25%) isolate was moderate biofilm producer. Out of 12 *S. epidermidis*, 1 (25%) were strong biofilm producer and 2 (41.66%) were moderate biofilm producers. Out of 8 *S. hemolyticus*, 1 (37.5%) were moderate biofilm producer, out of 3 *S. hominis*, 1 (33.33%) were moderate biofilm producer.

Comparison between TCP and CRA

Table 2 shows a comparison between the results of biofilm production by TCP and CRA, using the Chi-square test.

TCP is considered a highly significant test, $p = 0.001$. As for CRA, it detected a very low number of biofilm producers compared to total positive biofilm producers. According to the $p = 0.381$, CRA method is considered the non-significant test.

Development of biofilm is an important virulence component for all staphylococcal species associated to infections caused by bio-medical device. Biofilm-producing staphylococcal isolates from different clinical samples have a great clinical importance because it serves as a reservoir for pathogens and is linked to antibiotic resistance and persistent infections.

In the present study, most of the samples were from patients age group ranging from 41-50 years which accounts to 20%. Whereas, in another study, conducted by Das *et al.*, (2020), a higher percentage of samples from patients between the age range of 41-50 (29%) was reported.

In the present study, the male to female ratio was 2:1 i.e. about 67% were males and 37% were females. The reported by Lienen *et al.*, (2021). 62% of samples were from males and 38% of samples were from females. Majority of the isolates were pus samples (89%) followed by blood (8%). Siva Sankari *et al.*, (2003), have reported 57% of samples from pus samples and 15.6% from blood samples.

Table.1 Detection of MRSA in accordance with CLSI guidelines

Drugs	*Staphylococcus* spp.	Disk content	Incubation period	Results		
				S	I	R
Cefoxitin	*S.aureus, S.lugdunensis*	30 µgCefoxitin	16-18 hours	≥ 22mm	-	≤ 21mm
	S.epidermidis S.pseudintermedius S.schleiferi	30 µgCefoxitin	16-18 hours	≥ 18mm	-	≤ 17mm
	Staphylococcus spp. except *S.aureus, S.lugdunensis, S.pseudintermedius* (not recommended) *S.schleiferi* (not recommended)	30 µgCefoxitin	24 hours (Reported after 18 hours, if resistant)	≥ 25mm	-	≤ 24mm

Table.2 Comparison between TCP and CRA as regards biofilm production

Methods / Biofilm producer	Non producers	Moderate producers	Strong producers	Total Staph. isolates	P value	Chi-square
TCP	13	8	6	27	0.001	19.6744
	13%	8%	6%			
CRA	90	7	3	100		
	90%	7%	3%			
Total Staph. isolates	103	15	9	127		

Fig.1 Age and Gender-wise distribution of patients

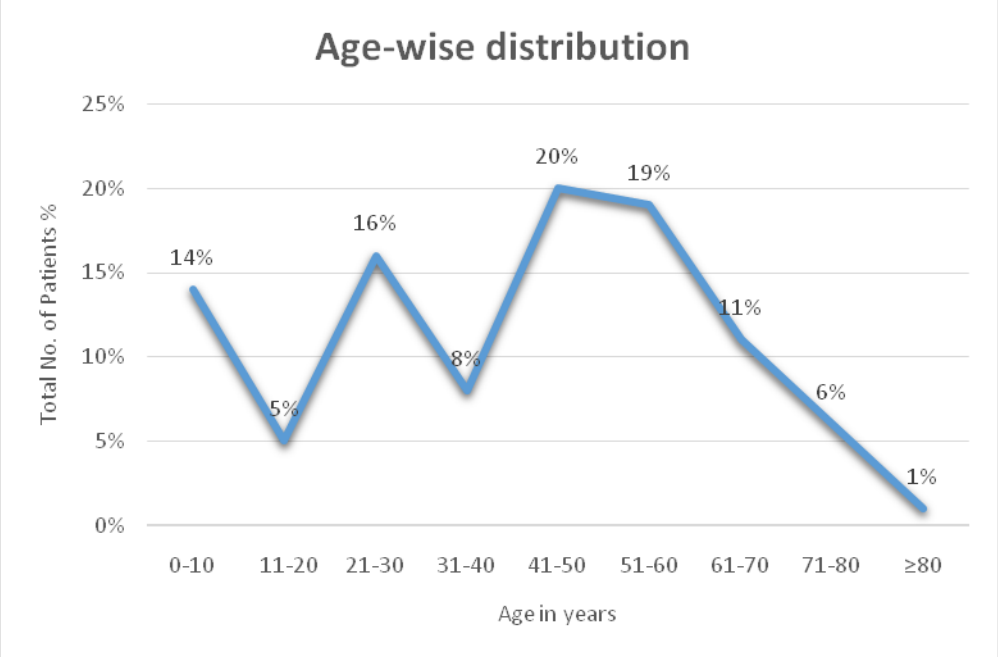
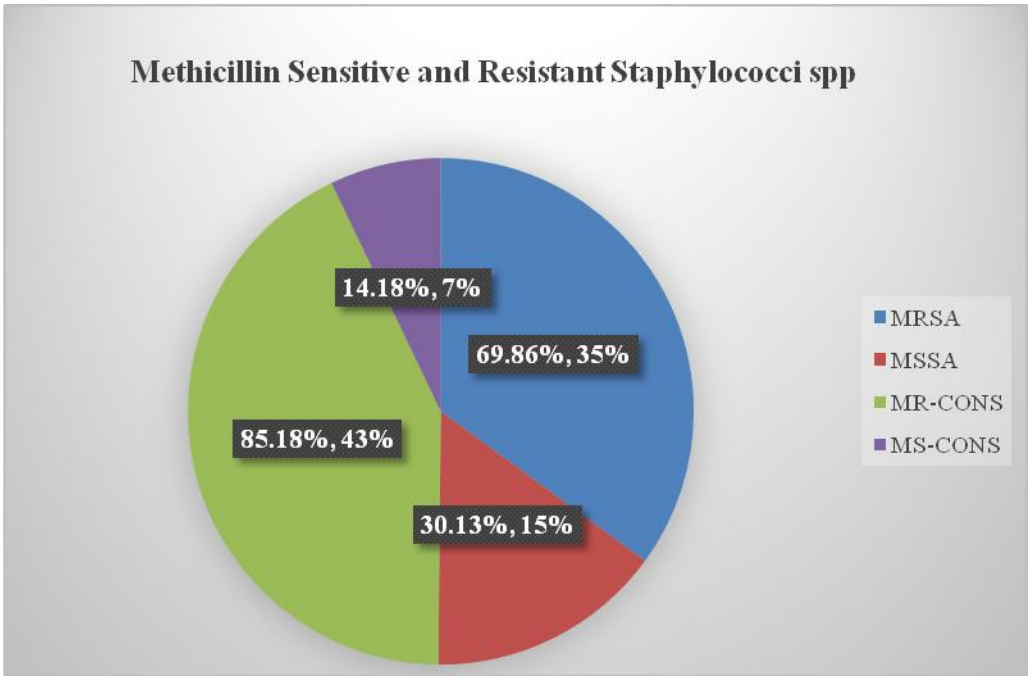


Fig.2 Methicillin sensitivity and resistant Staphylococci spp.



In the present study, a total 100 clinical isolates of Staphylococci, 73 (73%) were *S. aureus* and 27 (27%) were CONS. Whereas in another study, conducted by Sarita Manandhar *et al.,* (2018) 57% were *S. aureus* and 43% were CONS. In our study, majority of the CONS isolates were *S. epidermidis* 12 (44.44%), a similar result was reported in another study conducted by De Paulis *et al., S. epidermidis* 50.5%. TCP method is regarded as the gold standard method for detecting biofilm which was reported by Mathur *et al.,* (2006). Study entitled "Detection of biofilm formation among the clinical isolates of staphylococci: an evaluation of three different screening methods" hence it was considered a standard method for interpreting our findings.

In our study, by the TCP method 55/100 (55%) clinical isolates of staphylococci were biofilm producing, among 55 biofilm producers, 56.36% were produced strong biofilm and 43.63 % were produced moderate biofilm. Whereas in a study conducted by Halim *et al.*, (2018).

A higher percentage of staphylococci 74% were biofilm producing, however a lower percentage 43.3% of these isolates were strong biofilm producers and a lower percentage (30.7%) were moderate biofilm producers. Among 73 *S. aureus* isolates, 41 (56.16%) were produced biofilm and among 27 CONS isolates 14 (51.85%) were biofilm producers. According to Akinkunmi *et al.,* (2012), Biofilm producers account for 36% of *S. aureus* isolates and 32.9% of CONS isolates. These findings are lower than what is reported in this study. However, Ramakrishna *et al.,* (2014) have reported a lower incidence of biofilm production among *S. aureus* (38%) and a higher percentage (84%) of CONS as biofilm producers. This may be due to their strains were collected from different sources.

In the present study 13 /22 (59.09%) of biofilm producers were MSSA and 28/51(54.9%) of biofilm producers were MRSA. However, Rewatkar *et al.*, (2013), have reported lower incidence of biofilm production among MSSA (15%) and a higher incidence among MRSA (85%) whereas O Neill *et al.*, (2007), have reported a high incidence of biofilm production among MSSA (84%) and MRSA

(74%). In the present study 75% of biofilm producing CONS were MS-CONS and 47.8% were MR- CONS. Whereas, the study conducted by Halim *et al.,* (2018), have found a higher incidence of biofilm production 77.8% by MS-CONS and MR-CONS (71,4%). By CRA method only10% of Staphylococcal isolates were found to be biofilm-producers. In a study conducted by Mathur *et al.,* (2006), similarly found a lower percentage of biofilm detection using the CRA method (3.8% and 5.3% respectively). Whereas Kaiser *et al.,* (2013), in their study entitled "Modification of the Congo red agar method to detect biofilm production by Staphylococcus epidermidis" have reported in higher percentage (25.3%) of biofilm detection by CRA method. This might be due to the used of modified CRA which contained (BHIA with 5%sucrose, 1.5%NaCl, 2% Glucose, 0.08% Congo red and 0.5mg/mL Vancomycin) which was not used in the present study. Probably this might be the caused for the higher incidence of biofilm detection in their method.

Biofilm-producing *S. aureus* was found to be more resistant to almost all the antibiotics in the present study. It was observed that 70.73%were resistant to Oxacillin/Cefoxitin followed by 85.36% resistant to Ciprafloxacin, 85.36%, to Levofloxacin, 48.44% to Erythromycin, 36.58% to Clindamycin, 21.95% to Trimethoprim/sulfamethoxazole and 14.63% to Gentamycin. This result is accordance to the finding by Singh *et al.,* who in their study entitled "Standardization and classification of in vitro biofilm formation by clinical isolates of *Staphylococcus aureus*.

Similarly, Akinkunmi *et al.,* (2012) studied by all the CONS isolates which are biofilm producers were also resistant to almost all the antibiotics. It was observed that85.71% were resistant to Oxacillin /Cefoxitin followed by 42.85% resistant to Ciprafloxacin, 50% to Levofloxacin, 57.14% to Erythromycin, 28.57% to Clindamycin, 35.71% to Trimethoprim/sulfamethoxazole and 14.2% to Gentamycin.

Biofilm formation is a virulence marker in clinical isolates of staphylococci spp. Detection of this virulence factor is important as biofilm producing isolates are more resistant to antibiotics and thus result in chronic infections. Detection of biofilm forming capacity will also help in adjusting the dose of antibiotic to be administered. In the present study, two phenotypic methods were evaluated and it was found that CRA method was less sensitive in detecting biofilm formation. TCP can be recommended as a general screening method for detection of biofilm producing bacteria.

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