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Evaluation of two different screening methods for detection of biofilm formation among the clinical isolates of *Staphylococcus aureus*

S.Umadevi [1], *M Sailaja* [2]

1- Assistant Professor, Dept of Microbiology, Govt Medical College, Anantapur, A.P.
2- Assistant Professor, Dept of Microbiology, Kurnool Medical College, Kurnool, A.P.

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Corresponding Author:
Dr.S.Umadevi, Assistant Professor, Dept of Microbiology, Govt Medical College, Anantapur, A.P.
Email: umadevi20378@gmail.com

Abstract:

Staphylococci are most often associated with chronic infections of implanted medical devices. The use of indwelling medical devices is important in the treatment of critically and chronically ill patients, however bacterial colonization of implanted foreign material can cause major medical and economic sequel. The increased use of indwelling medical devices has had considerable impact on the role of *Staphylococci* in clinical medicine.

Key words: Bio film; *Staphylococcus aureus*, intensive care unit.

Introduction

Staphylococci are most often associated with chronic infections of implanted medical devices [1]. The use of indwelling medical devices is important in the treatment of critically and chronically ill patients, however bacterial colonization of implanted foreign material can cause major medical and economic sequel. The increased use of indwelling medical devices has had considerable impact on the role of S*taphylococci* in clinical medicine. The predominant species isolated in these infections are *Staphylococcus epidermidis* and *Staphylococcus aureus*, their major pathogenic factor being ability to form biofilm on polymeric surfaces [2]. Biofilm consists of multilayered cell clusters embedded in a matrix of extracellular polysaccharide (slime), which facilitates the adherence of these microorganisms to biomedical surfaces and protect them from host immune system and antimicrobial therapy [3].

Understanding the interaction between antimicrobial agents and the biofilm in realistic environments presents a challenging problem since the biofilm forming rate and susceptibility depend on surface physico – chemical properties such as hydrophilicity, surface free energy and surface topography. A direct correlation between surface roughness and biofilm development on enamel surface and ortho appliances, catheters and medical implants has been demonstrated [4].

Biofilm formation is regulated by expression of polysaccharide intracellular adhesin, which mediates cell to cell adhesion and is the gene product of *ica*ADBC [5]. Various reports attest to the presence of *ica*ADBC gene in staphylococcus aureus and staphylococcus epidermidis isolated from infections associated with indwelling medical devices [6,7].

A number of tests are available to detect slime production by *Staphylococci*; methods include tissue culture plate (TCP) [8], tube method (TM) [9], congo red agar (CRA) [10,11**]**, bioluminescent assay [12] and light or fluorescence microscopic examination [13]. These methods are often subject to severe analytical limitations and are unable to detect bacterial adherence accurately.

In this study simultaneously screened 100 clinical isolates of *Staphylococcus* spp by TM and CRA methods for determining there ability to form biofilm and also evaluated the reliability of these methods in order to determine most suitable screening method.

Materials and Methods:

264 patients admitted in the intensive care unit (ICU) with any artificial implant, IV canula or urinary catheter were selected as study group from clinical and surgical departments of Narayana General and superspeciality hospital, Nellore. Study period extended for 1year from April 2010 to April 2011. Samples were collected from catheter tips, blood, pus, wound swabs, urine, swabs from orthopaedic implants. Sample collection:- All specimens for microbiological processing were collected in appropriate sterile containers

Microbial methods:- Maki's Semi Quantitative

Method:

The aseptically collected 5cm catheter tip was rolled 4-5 times on the surface of blood agar and incubated for 18-24 hrs at 370c. The culture medium were examined for characters of bacterial growth .keeping the criteria for positive (>15 CFU). The colony morphology and Gram's stain morphology were studied. After MAKI'S method the catheter tip was incubated in brain heart infusion broth for 24-48 hrs and loop full of broth was inoculated on nutrient agar plates and incubated at 370c for 24-48 hours. The plates were examined for growth. If growth was observed, colonial morphology and gram's stain morphology were studied. If no growth was observed, plates were further incubated for the next 24 hours.

The growth on culture plates was examined by gram's stain.

Gram staining:- Smear prepared on microscopic slide and stained with grams stain. Isolation and identification of Staphylococcus aureus

Catalase Test:-

Slide method: Small portion of the suspect colony was spotted onto the centre of a microscopic slide. One drop of hydrogen peroxide solution was placed on a slide. Look for vigorous bubbling occurring within 10 seconds, use a hand lens if necessary to see very slight Catalase production. Observation:- Bubbling seen within 10 seconds. Inference: - Catalase positive.

Tube method: Approximately 0.2 ml of hydrogen peroxide solution taken in a sterile test tube. Colony to be tested was added to H2o2 solution in tube with glass rod. Observation:- vigorous bubbling occurring within 10 seconds.

For all methods:

Positive result: Vigorous bubbling indicates the presence of catalase enzyme. Negative result: No bubbling

Coagulase Test:-

MEDIA & REAGENTS:-

1. Pooled plasma.
2. Quality controls

Coagulability of plasma is tested by adding a drop of 5% calcium chloride to 0.5 ml of plasma. A clot should form with in 10 to 15 seconds. S. aureus - positive, S. epidermidis - negative

Interpretation:-

Positive -------- coagulum formation.

Negative--------plasma remains wholly liquid or shows only a flocculent or ropy

Demonstration of biofilms from same isolate by tube method and Congo red agar method.

1. Tube Method [TM]
2. Congo Red Agar Method [CRA]

Antibiotic Sensitivity Test:--

The Antibiotic sensitivity test was done on mueller-hinton agar [MHA] using Kirby-Bauer disc diffusion method as recommended by NCCLS .

Requirements:-

1.Muller –Hinton agar

2.Bacterial inoculum adjusted to 0.5 Mac –Farland Standard.

Incubation time and temperature:- 37^{o}c for 16 -18 hours.

Lawn culture of the organism was made over the Mueller –Hinton agar with the suspension of organism cultured in peptone water which is standardized with 0.5 Mac –farland standard. After inoculum dried specific antibiotic discs were placed 2 cm apart from each other with sterile forceps and was incubated for 18 -24 hours at 370c aerobically. The zone size was interpreted according to the reference chart provided by the manufacturer according to NCCLS standards for each organism following antibiotic discs.

1. Pencillin (10 units)
2. Ampicillin (10 µg)
3. Ofloxacin (5 µg)
4. Ciproflaxacin (5 µg)
5. Cefotoxime (30 µg)
6. Erythromycin (15 µg)
7. Co-trimoxazole (25 µg)
8. Amikacin (30 µg)
9. Gentamicin (10 µg)
10. Netillmicin (30 µg)
11. Vancomycin (30 µg)

Results

Table 1: Analysis of total samples

	No. of samples	**Percentage**
No. of culture positives	222	84.09
No. of culture negatives	42	16.66
Total	264	100

Table 2: Distribution of different types of samples in the study

Type of sample	**No. of samples**	**Percentage**
Catheters	88	33.33
Pus	55	20.83
Blood	46	17.42
Wound swab	52	19.69
Orthopedic implants	5	1.89
Urine	18	6.81
Total	264	100

Table 3: Prevalence of *Staphylococcus aureus* and other isolates in the total no. of positive samples

Isolates	**No. of isolates**	**Percentage**
Pseudomonas spp	43	19.36
Escherichia coli	31	13.96
Klebsiella spp	21	9.45
Staphylococcus aureus	**52**	**23.42**
CONS	61	27.47
Acinetobacter spp	06	2.70
Candida	08	3.60
Total	222	100

Table 4: Prevalence of *Staphylococcus aureus* in different samples

Sample	**No. of isolates**	**Percentage**
Catheters (88)	**16**	**6.06**
Pus (55)	11	4.16
Blood (46)	9	3.43
Urine (18)	3	1.13
Wound swabs (52)	10	3.78
Orthopedic implants (5)	3	1.13
Total (264)	52	19.69

Table 5: Antimicrobial resistance profiles of *Staphylococcus aureus* isolates by disk-diffusion method (n=52)

Antibiotics	Resistance	Intermediate sensitive	sensitive
Penicillin	50	-	2
Ampicillin	**43**	**7**	**2**
Oflaxacin	3	20	29
Ciprofloxacin	**12**	**23**	**17**
Cefotaxime	5	7	40
Erythromycin	3	16	33
Co-trimoxazole	18	21	13
Amikacin	4	16	32
Gentamicin	5	19	28
Netilmicin	5	2	45
Vancomycin	-	-	**52**

Table 6: Tube method biofilm formation

S.aureus (n=52)	Catheter	blood	pus	urine	Wound swab	Orthopedic implants
Strong	5	2	-	-	1	2
Moderate	2	3	1	1	4	-
Weak	6	2	8	1	5	-
Absent	4	3	1	-	1	-

Table 7: CRA method biofilm results

S.aureus n=52	catheters	blood	pus	urine	Wound swab	Orthopedic implants
Strong	4	1	-	-	1	1
Intermediate	2	2	1	-	2	-
Weak	4	2	3	2	8	-
Absent	6	5	5	1	2	-

Table 8: Screening of *Staphylococcus aureus* isolates for biofilm formation by TM and CRA methods

Clinical isolates n=52	Biofilm formation	TM	CRA
	High	10(19.23%)	7(13.46%)
	Moderate	11 (21.15%)	7 (13.46%)
	Weak	22 (42.30%)	19(36.53%)
	Absent	9 (17.30%)	19(36.53%)

Discussion

Staphylococcal infections are a major source of patient morbidity and implant failure. *Staphylococcal aureus* is the most important pathogen in the genus and is also the most important nosocomial pathogen of surgical wounds [14]. The virulence of *Staphylococcus aureus* is associated with its ability to produce toxins and other extracellular factors, ability to adhere and form biofilm on host surfaces, finally resistance to phagocytosis [15,16].

Bacterial biofilm has long been considered as a virulence factor contributing to infection associated with various medical devices and causing nosocomial infection [17,18].

We isolated 113 *Staphylococcal* Spp from clinical samples namely blood, pus, urine, catheters, swabs from orthopedic implants, wound swabs etc. all isolates were isolated by standard procedure [19] and tested by two in vitro screening tests for biofilm production namely TM and CRA methods. Out of 113 *Staphylococcal* spp 61(53.98%) were *S.epidermidis* and 52(46.01%) were *S.aureus*.

In present study among 52 isolates of *S.aureus* 10 (19.23%) isolates produced strong biofilm, 11 (21.15%) isolates produced moderate biofilm, 31 (59.60%) isolates produced weak/non biofilm by TM method.

T.Mathur et al isolated a total of 152 clinical isolates of *Staphylococcal* spp. from various samples and evaluated biofilm formation by TCP, TM, and CRA methods. The results by TM method were 11.84% strong biofilm producers, 29.60% moderate biofilm producers, 58.55% weak/ non biofilm producers.

Bose S et al (2009) [20] among total of 179 clinical isolates of *Staphylococci* spp 68 isolates were *S.aureus*, biofilm production of *Staphylococci* evaluated by TCP,TM and CRA methods.

The results by TM method were 12.30% strong biofilm producers, 30.16% moderate biofilm producers, 57.54% weak/non biofilm producers.

The positivity rate of TM method (40.38%) results was lower than observed by other workers example T.Mathur et al who has reported 41.44%.

In present study by congo red agar method out of 52 isolates 7(13.46%) isolates produced strong biofilm, 7(13.46%) isolates produced moderate biofilm, 38(73.07%) isolates produced weak/non biofilm.

In our study positivity rate (26.92%) of CRA method was higher than observed by other workers example Mathur et al who has reported 5.26% biofilm producers by CRA method. By these results TM method was considered as best method in this study.

Freeman et al (1989), *S.aureus* isolates examined in CRA test, slime production was established in 8 (11.42%) isolates 10, Baselga et al. (1993) detected 12% positive strains [21] Ammendolia et al. (1999) and Bose et al. (2009) also reported biofilm producers were isolated from catheter tips (intravenous and urinary, 29.6% and 23.4% respectively) followed by urine and pus specimens [20]. Vasudevan et al. (2003) revealed that out of 35 *S.aureus* mastitis isolates, 32 produced typical black colonies within 24-48 hrs, [22] Oliveira et al. (2006) reported 37.5% slime producing *S.aureus* in mastitis isolates [23]. Krukowski et al. (2008) reported 42.37% slime producing S.aureus in mastitis isolates [24]. Baqai et al. (2008) also reported high occurrence of biofilm producing bacteria (75%) among the uropathogens, mainly from *S. aureus* (75%), *E. faecalis* (75%) and *E.coli* (40%). *S. epidermidis* was the major isolate from clinical samples that formed biofilm. They found that after *S.epidermidis*, *S. aureus* (18 out of 51*)* are involved in large number in production of biofilm [25].

In this study antibiotic sensitivity pattern of various samples as obtained. The significant and clinically relevant observation was that the high resistance shown by biofilm producers to conventional antibiotics than non biofilm producers. This observation was supported by other studys also. All strains were sensitive to vancomycin. The results correlated with Zhang Liping et al [26].

The clinically relevant observation was high resistance of biofilm producers to commonly used antibiotics. They have seen that Gram positive biofilm producers showed 100% sensitivity to vancomycin [26].

Conclusion:

1. Staphylococci are most often associated with chronic infections of implanted medical devices
2. The major pathogenic factor being ability to form biofilm on polymeric surfaces
3. TCP, TM, CRA, Bioluminescent assay are different methods available to detect biofilm formation.
4. Among all those TCP and TM are reliable methods to detect biofilm formation.
5. Biofilm formation by staphylococci should be detected because the biofilm producing strains are mostly resistant to commonly used antibiotics and vancomycin might be the drug of choice to treat prosthesis associated infections.

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