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Detection of Biofilm Formation in *Staphylococcus aureus*. Does it have a Role in Treatment of MRSA Infections?

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ABSTRACT

Staphylococcus aureus is a causative agent of many types of diseases throughout the world. Staphylococcal infections are of particular concern because of the causative bacteria offering resistance to a wide range of commonly used antibiotics. The formation of biofilm is the hallmark characteristic of *S. aureus* infection. Biofilms constitute reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections. In this study 262 clinical strains of *S. aureus* were screened by tissue culture plate method, tube method and congo red agar method for biofilm formation. Antimicrobial sensitivity testing of these strains was done by Kirby bauer disc diffusion method. Tissue culture plate method detected 38 (14.51%) isolates as strong biofilm producers, 132 (50.38%) as moderate biofilm producers and 92 (35.11%) strains as nonproducers of biofilm. The congo red agar method had a low sensitivity and specificity of 67.65 and 89.13%. The tube method correlated well with tissue culture plate method with a sensitivity and specificity of 99.40 and 95.78% but the interpretation are observer dependent. Biofilm production was higher in methicillin resistant strains as compared to the methicillin sensitive strains of *S. aureus*. Biofilm producers were found to be more resistant to almost all the groups of antibiotics.

Key words: Antimicrobial resistance, MRSA, *Staphylococcus aureus,* biofilm, tissue culture plate, congo red agar, tube method

INTRODUCTION

Staphylococcus aureus is a causative agent of many types of diseases throughout the world. *Staphylococcal* infections are of particular concern because of the causative bacteria offering resistance to a wide range of commonly used antibiotics (Siddiqi *et al*., 2002). *S. aureus* is consistently one of the top four causes of nosocomial infections (Akhi *et al*., 2008). Patients hospitalized for long periods of time are usually predisposed to infection by methicillin resistant *Staphylococcus aureus* (Saeed and Ahmed, 2009). At present approximately 40% of *Staphylococcus aureus* are resistant to methicillin and the incidence of methicillin resistance increases year by year (Akhi *et al*., 2008). MRSA poses increasingly serious health care problem in many parts of the world. Several studies have reported increased morbidity and mortality associated with MRSA compared to methicillin sensitive *Staphylococcus aureus* infections (Dadgar *et al*., 2006). Methicillin resistant strains of *S.aureus* are more difficult to treat because multidrug resistance is more common

in these isolates as compared to the MSSA isolates. Vancomycin is considered as the treatment of choice for MRSA cases but recently there are reports of emergence of vancomycin resistance in *S. aureus* (Jasmine *et al.*, 2007).

Staphylococcus aureus is an adaptable, pathogenic organism. In the presence of environmental challenges, *S. aureus* can alter its genotype and/or phenotype to adapt to its surroundings. An example of genotypic change is the acquisition of the β-lactamase gene conferring penicillin resistance. The formation of biofilm is an example of phenotypic change. Formation of a biofilm is the hallmark characteristic of *S. aureus* infection which consists of multiple layers of bacteria encased within an exopolysaccharide glycocalyx. Presence of glycocalyx protects the enclosed bacteria from host defences and impedes delivery of antibiotics (Stewart, 2002). Infact biofilms can resist antibiotic concentration 10-10,000 fold higher than those required to inhibit the growth of free floating bacteria (Jefferson *et al.*, 2005). Biofilm formation in *S. aureus* is regulated by expression of Polysaccharide Intracellular Adhesion (PIA) which mediates cell to cell adhesion and is the gene product of ica ABDC (Ammendolia *et al.*, 1999).

Infectious processes in which biofilms have been implicated include common problems such as urinary tract infections, catheter infections, middle ear infections, formation of dental plaque, gingivitis, coating contact lenses and less common but more lethal processes such as infective endocarditis, cystic fibrosis and infections of permanent indwelling devices such as joint prosthesis and heart valves (Lewis, 2001; Parsek and Singh, 2003).

The present study was undertaken to detect the prevalence of biofilm production in *S. aureus* in a tertiary care hospital in North India and to evaluate three different methods, viz., Tissue Culture Plate method (TCP), Tube Method (TM) and Congo Red Agar (CRA) method for detection of biofilms and to see its relation with antimicrobial resistance.

MATERIALS AND METHODS

A total of 262 non-repetitive, clinical strains of *S. aureus* were isolated from various clinical samples of the indoor and outdoor patients of Jawaharlal Nehru Medical College and Hospital, Aligarh, India during one year period from August 2006 to August 2007. Isolates were identified by standard microbiological techniques including gram stain, catalase test, coagulase test, phosphatase test and DNAase test (Baird, 2006). All the isolates were classified into hospital and community acquired *S. aureus*. *S. aureus* was considered as community acquired when it was isolated from the out patient setting or by a culture positive for *S. aureus* within 48 h after admission to the hospital with no history of permanent indwelling catheters or medical devices that pass through the skin into the body, any medical history of MRSA infection or colonization, any history in the past year of hospitalization, admission to a nursing home or skilled nursing facility, dialysis unit or for surgical intervention. Otherwise the patient was considered to have hospital acquired infection.

Oxacillin disc diffusion test: All the isolates were subjected to oxacillin disc diffusion test using oxacillin 1 μg disc. A 0.5 McFarland turbidity standard suspension of the isolate was made and lawn culture was done on Mueller-Hinton Agar (MHA) plates containing 4% NaCl. Plates were incubated at 37°C for 18 h and zone diameters were measured. An inhibition zone diameter of ≤10 mm was reported as methicillin resistant and ≥13 mm was taken as methicillin sensitive.

PCR amplification for mec A and fem B genes: Multiplex PCR (Geha *et al.*, 1994) was carried out on all the *S. aureus* strains found methicillin resistant on MIC determination. All the MRSA strains were screened for the *mec A* and *femB* genes using the following oligonucleotides sequence.*mec* A1-5' GTA GAA ATG ACT GAA CGT CCG ATA A-3', *mec* A2-5' CCA ATT CCA CAT TGT TTC CGT CTA A-3', *fem* B1-5' TTA CAG AGT TAA CTG TTA CC-3', *fem* B2-5' ATA CAA ATC CAG CAC GCT CT-3'. A 50 µL PCR reaction mixture consisted of 45 µL of mastermix containing PCR buffer (1X), dNTP mix (0.2 mM of each), primer (0.5 µM), Taq DNA polymerase (0.25 U) and $MgCl_2$ (1.5 mM) with 5 µL of template DNA. Cycling parameters were set to- hot start 94°C for 4 min followed by 35 cycles of melting at 94°C for 45 sec, annealing at 50°C for 45 sec and extension at 72°C for 1 min. Analysis of amplified products was done by gel electrophoresis. Amplicons of 310 bp were consistent with *mec* A and of 651 bp with *fem* B gene amplification.

Antimicrobial sensitivity testing: Antimicrobial sensitivity testing was done by Kirby bauer disc diffusion method for the following antimicrobial agents: amikacin 30 µg, ciprofloxacin 5 µg, clindamycin 2 µg, cotrimoxazole 25 µg, erythromycin 15 µg, gatifloxacin 5 µg, gentamycin 10 µg, levofloxacin 5 µg, linezolid 30 µg, ofloxacin 5 µg, sparfloxacin 5 µg, tobramycin 10 µg, vancomycin 30 µg.

Detection of biofilm formation was done by the following methods:

- Tissue culture plate method (Christensen *et al.*, 1985)
- Tube method (Christensen *et al.*, 1985; Mathur *et al.*, 2006)
- Congo red agar method (Christensen *et al.*, 1985; Mathur *et al.*, 2006)

Statistical analysis: Statistical analysis was done using Microsoft Excel 2007.

RESULTS

A total of 262 clinical strains of *S. aureus* were isolated from various clinical samples, of which 217 (82.83%) samples were hospital acquired and 45 (17.17%) were community acquired. Out of the total of 262 *S. aureus* isolates tested for methicillin susceptibility 89 (33.97%) were found to be resistant to methicillin (MRSA) whereas 173 (66.03%) were sensitive (MSSA) by oxacillin disc diffusion method. However, 85 (32.44%) strains had both *mec* A (310 bp) and *fem* B (651 bp) and were confirmed to be methicillin resistant. MRSA was found to be more prevalent in hospital acquired isolates 72 (33.17%) of *S. aureus*. Among the OPD patients rate of isolation of MRSA was 28.89% (Table 1).

Biofilm production by *S. aureus*: Out of the 262 isolates tested for biofilm formation by CRA method, 182 isolates produced black colonies. However, only 125 (47.71%) colonies were black in colour with dry crystalline consistency which is indicative of biofilm formation. The 57 (21.76%) isolates were black in colour but were not dry and crystalline and were indeterminate for biofilm formation. These isolates were also taken as negative for biofilm formation. The 80 (30.53%) isolates produced pink colonies which were taken as negative for biofilm formation (Table 2, Fig. 1a).

Out of 262 isolates tested for biofilm by Tube Method, 35 (13.36%) were strongly positive. However, maximum number of isolates 132 (50.38%) were moderately positive and 95 (36.26%) did not show any biofilm formation (Table 3, Fig. 1b).

Table 1: Isolation rate of MRSA in relation to hospital and community acquired strains

Source	Tested No. (%)	No. of MRSA isolates (%)
Hospital	217 (82.83)	72 (33.17)
Community	45 (17.17)	13 (28.89)
Total	262 (100.00)	85 (32.44)

Table 2: Biofilm formations by *S. aureus* (Congo red agar method) (n = 262)

Colony appearance on congo red agar (CRA)	No. of isolates (%)
Pink/red (negative)	80 (30.53)
Black colonies without dry crystalline consistency (indeterminate)	57 (21.76)
Black colonies with dry crystalline consistency (positive)	125 (47.71)
Total	262 (100.00)

Table 3: Biofilm formation by *S. aureus* (Tube method)

Biofilm production	No. of isolates (%)
0/1 (negative/weak)	95 (36.26)
2 +(moderate)	132 (50.38)
3 + (strong)	35 (13.36)

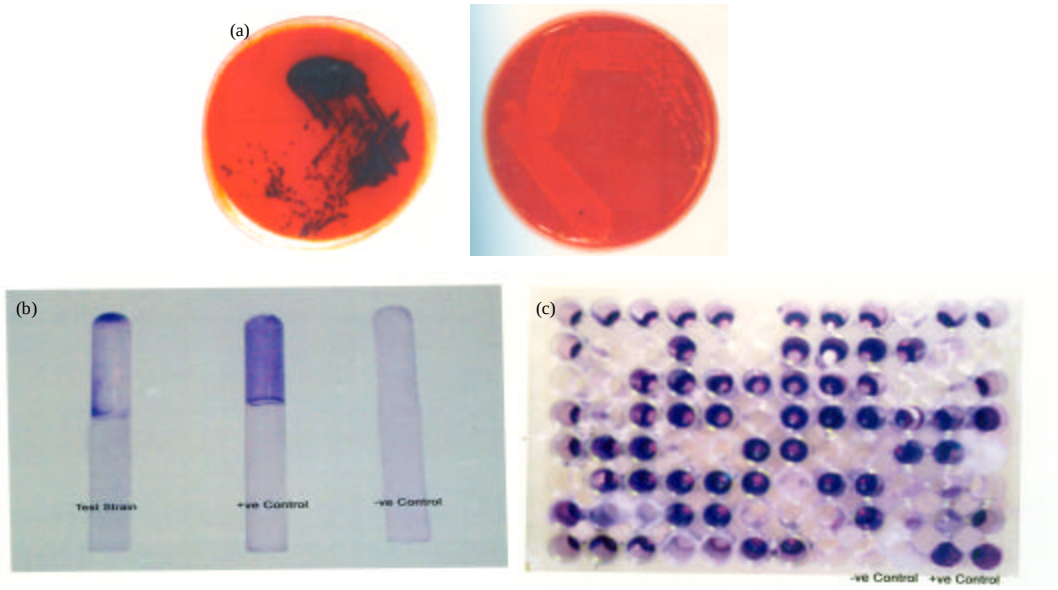

Fig. 1(a-c): (a) Biofilm producer and nonproducer on Congo-red agar, (b) Biofilm production by tube method with positive and negative control and (c) Tissue culture plate method for detection of biofilm production

By tissue culture plate method, 38 (14.51%) isolates were strongly positive for biofilm production, 132 (50.38%) were moderate biofilm producers whereas 92 (35.11%) were negative for biofilm formation (Table 4, Fig. 1c).

When the results of CRA and Tube method for biofilm formation were compared with those of tissue culture plate method, it was found that the specificity and sensitivity of CRA was 89.13% and 67.65% and for Tube method, the specificity and sensitivity were 95.78 and 99.40% (Table 5).

Table 4: Biofilm formation by *S. aureus* (tissue culture plate) method

Biofilm production	No. of isolates (%)
Negative (< 0.120 OD)	92 (35.11)
1+ (0.12- 0.24 OD)	132 (50.38)
2+ (> 0.24 OD)	38 (14.51)

Table 5: Statistical evaluation of TM and CRA for detection of biofilm formation in *S. auerus*

	Test characteristics (%)			
Screening method*	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Tube method	95.78	99.40	99.11	95.29
CRA method	89.13	67.65	91.73	69.83

*Comparative evaluation of Tube method and CRA method with TCP method for detection of biofilm formation in *S. aureus* (n = 262)

Antimicrobial susceptibility profile in relation to biofilm production: It was found that biofilm producing strains were more resistant when compared to the biofilm non producers (Fig. 2). All the strong biofilm producers thirtyeight (by tissue culture plate method) were found to be methiclin resistant. Out of the remaining 47 MRSA strains 40 were moderate biofilm producers and just 7 (8.23%) strains did not produced any biofilm. Amongst the 177 MSSA strains 92 strains (51.98%) were found to be moderate biofilm producers and none was strong producer of biofilm. The resistance pattern of biofilm producing strains when compared to biofilm non producers was for amikacin 73.53/55.43%, chloramphenicol 54.94/34.78%, ciprofloxacin 83.53/76.09%, clindamycin 87.79/78.26%, cotrimoxazole 93.60/79.35%, erythromycin 65.29/53.26%, gatifloxacin 48.23/40.22%, gentamycin 70.00/67.39%, levofloxacin 12.35/6.42%, ofloxacin 24.71/21.74%, sparfloxacin 43.53/33.69%. However, all the strains were sensitive to linezolid and vancomycin.

DISCUSSION

Bioilm formation is an important characteristic of all staphylococcal species, associated with the infection of biomedical devices (Christensen *et al*., 1985; Kloos and Bannerman, 1994; O'Gara and Humphreys, 2001). Polysaccharide Intercellular Adhesion (PIA) plays an important role in the pathogenesis as it mediates the contact of bacterial cells with each other, resulting in the accumulation of a multilayered biofilm (Chaudhary *et al*., 2009). Biofilms constitute reservoir of pathogens and are associated with resistance to antimicrobial agents and chronic infections.

In present study, a total of 262 strains of *S. aureus* were isolated, out of which 85 (32.44%) were methicillin resistant. More than 2/3rd of the isolates were from patients having hospital acquired infections (around 80%). Community acquired isolates accounted for just 17% of the isolates of *S. aureus* (Table 1). Among the hospital acquired isolates 33.17% were found to be methicillin resistant whereas among the community acquired isolates around 29% isolates were resistant to methicillin. Methicillin resistant *S. aureus* is a known cause of nosocomial infections, however, recently there has been emergence of community acquired infections associated with MRSA (Eguia and Chambers, 2003; Oberoi and Verghese, 2006). It was also noted that methicillin resistant strains of *S.aureus* were more prone to biofilm formation as compared to themethicillin sensitive strains of *S. aureus*. Methicillin susceptibility of *S. aureus* has been shown to influence the biofilm formation (O'Neill *et al*., 2007).

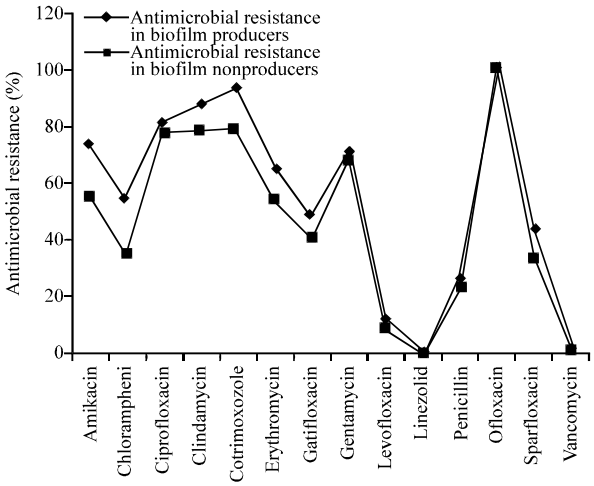

Fig. 2: Antimicrobial resistance of biofilm producers and non-producers

On antimicrobial sensitivity testing by Kirby bauer disc diffusion method it was noted that biofilm forming strains showed higher resistance to almost all the groups of antibiotics as compared to the biofilm non-producers (Fig. 2). The increased resistance of biofilm producing strains to antibiotics may be because the biofilm bacteria exhibit a slow rate of metabolism and divide infrequently resulting in decreased sensitivity to antibiotics targeted at cell wall synthesis (Monroe, 2007). However, even antibiotics targeted at cellular functions such as protein and DNA synthesis which should affect cells at a quiescent state are ineffective against biofilms (Lewis, 2007).

All the 262 isolates of *S. aureus* were subjected to three *in-vitro* screening procedures for their potential to form biofilm. By the tissue culture plate method 170 (64.89%) isolates were found to be biofilm producers and 92 (35.11%) strains did not produced biofilm (Table 4). However, other studies have reported a slightly less number of biofilm production by staphylococcal species (Mathur *et al*., 2006; Bose *et al*., 2009). By tube method, biofilm formation was observed in 167 (63.74%) isolates whereas 95 (36.26%) strains did not show any biofilm formation (Table 3). In our study positivity for biofilm formation by CRA method was 125 (47.79%) (Table 2) which is much higher than reported by Mathur *et al*. (2006) and Bose *et al*. (2009).

Highly accurate methods like PCR for detection of *ica* gene are available to check the capability of *S. aureus* strains to produce biofilm but these are beyond the scope of most of the microbiology laboratories in the developing countries like India. Among the phenotypic methods Tissue culture plate method has been reported as gold standard for biofilm formation (Mathur *et al*., 2006). We compared the results of tube method and CRA method to the tissue culture method for biofilm formation. It was noted that both the sensitivity and specificity of tube method (95.78/99.40%) were higher than those for CRA method (89.13/67.65%).

CRA method is easy to perform and interpret but due to its low specificity and sensitivity we do not recommend it for detection of biofilm formation. Tube method has high sensitivity and sensitivity and the results of the tube test correlate well with the TCP method but the interpretation is observer dependent and there are chances of subjective errors. 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 methicillin resistant *S. aureus* 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.

CONCLUSIONS

Treatment of MRSA is one of the most challenging task for the clinicians and the microbiologists. With the emergence of vancomycin resistance in Staphylococcus aureus role of antimicrobials is becoming limited. Hospital acquired strains of MRSA should be routinely screened for biofilm formation using the tissue culture plate method and treated accordingly.

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