Ultrastructural analysis of slime positive & slime negative Staphylococcus epidermidis isolates in infectious keratitis

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Background & objectives: Slime is a major determinant of Staphylococcus epidermidis adherence. The established methods of laboratory detection of slime production by this organism i.e., Christensen’s tube method and congo red agar plate method, can both yield inconclusive and/or intermediate results. We, therefore tried to find out electronmicroscopically the localization of slime in relation to the bacterial cell wall and look for the effect, if any of the slime location on the staphylococcal adherence as well as on the quantum of slime production.

Methods: A total of 132 coagulase negative staphylococci from cases of infectious keratitis were identified as S. epidermidis following the recommended protocol. Slime was detected both by Christensen’s tube method and congo red agar plate method. Antibiotic sensitivity testing was performed by standardized disc diffusion method. Adherence of the organisms to artificial surfaces was determined by a quantitative method and transmission electron microscopy was carried out by the conventional techniques.

Results: Of the total 132 isolates, 57 (43.2%) were slime positive and 75 (56.8%) were slime negative. Twenty seven (47.4%) of the 57 slime producing organisms were multi drug resistant as compared to only 12 (16%) of 75 nonslime-producing organisms (P<0.001). A majority i.e., 45 (78.9%) of 57 adherent organisms were slime producers as against 12 (16%) of 75 nonadherent organisms. Electron microscopic study revealed a thick viscid layer of slime anchoring to the bacterial cell wall, especially in adherent organisms and those yielding positive slime test. Some of the organisms showed loose nonadherent slime and those were mostly nonadherent to artificial surfaces.

Interpretation & conclusion: Slime and multi drug resistance were the important virulence factors of S. epidermidis in bacterial keratitis. It was the adherent slime (i.e., slime in intimate association with the bacterial cell wall as shown by electron microscopy) only, which was responsible for resistance to multiple antibiotics and for the adhesion phenomenon observed in the quantitative slime test.

Key words Adherence - electron microscopic study - multidrug resistance slime - Staphylococcus epidermidis

Infectious keratitis is a leading cause of ocular morbidity and blindness1. Staphylococcus epidermidis, a commensal of the eye, accounts for 45 per cent of total cases of bacterial keratitis. However, the pathogenic potential of this commensal organism which is responsible for such a high percentage of keratitis, has been explored only recently3.
A polysaccharide slime, earlier reported to be one of the virulence factors of *S. epidermidis* in extraocular infections\(^3,5\), has been shown to be produced by isolates from keratitic lesions, rather than by control organisms\(^3\). The role of slime in the pathogenicity of *S. epidermidis* in bacterial keratitis, is mainly due to slime mediated adherence\(^6\). These observations warranted reproducible routine method for the detection of slime in *S. epidermidis*. In an earlier study\(^7\), we compared two *in vitro* methods for the detection of slime, *i.e.*, the Christensen’s tube method\(^8\) and the Congo red agar (CRA) plate method\(^9\). Each had its own merits and demerits. However, the only drawback in both the methods was that some isolates produced inconclusive results in the Christensen’s test, while there were intermediate results in the CRA test.

Thus, the present study was conducted to find out if there were ultrastructural differences amongst ocular isolates of slime positive, and slime negative *S. epidermidis* phenotypes and to know exactly, if such adhesion inducing staphylococcal slime was loosely expressed into the medium or is firmly anchored to the bacterial cell wall, and secondly to know whether the location of slime in relation to the bacterial cell wall could affect the slime test results.

**Material & Methods**

**Bacterial isolates**: A total of 132 consecutive isolates from the corneal scrapings of equal number of patients of infectious keratitis, who were either out patients or were admitted to the wards of Dr Rajendra Prasad Centre for Ophthalmic Sciences, All India Institute of Medical Sciences, New Delhi, during 1999 to 2001 were studied. Cultures yielding mixed growth were excluded.

**Isolation, identification and antibiotic sensitivity**: The procedure of isolation from the clinical material (corneal scrapings) and identification were as per the recommended methods\(^10,11\).

Antibiotic sensitivity testing was performed by the standard disc diffusion technique\(^12\). The antibiotics (Hi-media, Mumbai, India) tested with their respective concentration/disc(\(\mu g\)) were tetracycline(30), chloramphenicol (10), cloxacillin (1), ciprofloxacin (5), tobramycin (10), vancomycin (30) and cephazolin (30). Isolates showing resistance to 3 or more antibiotics were regarded as multidrug resistant\(^3\).

**Detection of slime by Congo red agar method**: The method developed by Freeman et al\(^9\) was followed with minor modifications, as described earlier\(^7\).

**Detection of slime by tube method**: The method by Christensen et al\(^8\) was adhered to with some modifications\(^7\).

**Adherence test**: Adherence of each isolate to smooth (quartz) surfaces was determined quantitatively by a method earlier standardized in our laboratory\(^9\). Briefly, overnight cultures of bacteria in trypticase soy broth (Hi-media, Mumbai, India) were diluted 1:100 in fresh trypticase soy broth (TSB) and 1ml volume of each was taken in separate quartz cuvettes. After overnight incubation at 37°C, the cuvettes were washed 4 times with phosphate buffered saline (PBS), pH 7.2 and then fixed with Bouin’s fluid and stained with crystal violet. Excess stain was removed by decanting the cuvettes first, and then rinsing them gently with tap water. The optical density (OD) of the stained bacterial biofilm was read with the help of a spectrophotometer (Spectrocolorimeter 103, Systronics, Baroda, India) at 570 nm. The cut-off OD was calculated as 3 X standard deviation above the mean OD of 10 blanks stained exactly by similar procedure.

**Transmission electron microscopy**: Randomly selected slime positive (n=9), intermediately slime positive (n=3) and slime negative (n=4) *S. epidermidis* isolates were incubated in TSB at 37°C overnight. Reproducibility of these isolates for slime production was seen earlier\(^7\). Cultures were washed in PBS and fixed in 2.5 per cent glutaraldehyde in 0.1M PBS for 30 min at 4°C. After centrifugation at 120g the pellets were collected, resuspended in PBS and centrifuged again. The pellets were postfixed in 1 per cent Osmium tetroxide for 30 min, dehydrated in ascending grades of acetone, infiltrated and embedded in araldite CY 212 (TAAB, UK). Thin sections (60-70 nm) were cut, stained with uranyl acetate and alkaline lead citrate for 1min and observed under a Philips CM10 transmission electron microscope at an operative voltage 80 KV) Netherlands.

**Statistical analysis**: Chi-square test was employed for all statistical analysis of data.

**Results**

Of the 132 corneal ulcer isolates of *S. epidermidis*, 57 were slime positive and 75 were slime negative. While 27 (47.4%) of 57 slime positive isolates were found to be multi drug resistant, only 12 (16%) of 75 slime negative isolates showed multi drug resistance. This difference was statistically significant (\(P<0.001\)) (Table I).
A few selected slime positive and negative isolates were subjected to transmission electron microscopy. Fig. A shows thick viscid layer of slime covering the bacterial cell, whereas Fig. D shows loose slime. Bacteria without any slime were seen quite clearly with distinct cell wall (Figs B and C). Some of these cocci also showed binary fission (arrowhead). Majority of nonadherent bacteria had either loose slime or no slime as evidenced by electron microscopy. Those showing thick viscous slime on the cell wall were all slime positive by both the methods and all of them were adherent to smooth surfaces and were multi drug resistant.

**Discussion**

In coagulase negative staphylococci, a cell wall bound exopolysaccharide layer (slime) has been reported in the past. The production of slime was related to *in vivo* colonization of surgical materials such as indwelling artificial devices like orthopaedic appliances, prosthetic joints, prosthetic cardiac valves and intravascular devices etc., by these organisms. In the previous studies virulence of slime producing *S. epidermidis* has been reported in extraocular infections. However, in our earlier study we demonstrated the significance of slime production by...
S. epidermidis in ocular infections such as bacterial keratitis.

In addition to slime, multi drug resistance has also been observed as one of the virulence determinants in S. epidermidis, in the same clinical situation like bacterial keratitis. Amongst the slime positive isolates in our study, multi drug resistance was detected in 47.4 per cent. This finding was in good agreement with the observations of Younger et al., who demonstrated multi drug resistance in 45 per cent of coagulase negative staphylococcal isolates from patients with ventriculo-peritoneal shunt infections. Slime production was observed in 78.9 per cent of the adherent organisms in our study similar to previous studies, which put forth the view that slime not only facilitated the colonization of bacteria to host tissue by preventing access of host defense mechanisms, but also protected them from being killed or arrested by antibiotics.

It was further observed that significantly higher number of slime positive isolates (data not shown) were isolated from patients with moderate to severe degree of ulcerative keratitis than from those with mild keratitis (ulcer severity was determined according to our Centre’s grading criteria), suggesting that clinical severity had an association with slime positivity and the role of slime in the pathogenicity of S. epidermidis in bacterial keratitis was mainly due to slime mediated adherence.

Our ultrastructural study demonstrated cell wall attached slime in the adherent bacteria and loose slime or no slime in majority of nonadherent bacteria. Sanyal and Greenwood observed the effects of antibiotic exposure on bacterial cells under transmission electron microscopy. Their findings showed that bacteria exposed to teichoplanin often showed abnormal septation and double layered cell wall.

Our electron microscopic study was conducted on normal cells without any antibiotic exposure. According to our observations, the thick viscid film outer to the cell wall was the true slime layer and the thin minute projectile fibrils could be loose slime. Our findings were in agreement with those of Baselga et al., who reported that a majority (85%) of slime producing and only 5 per cent of nonslime producing S. aurius isolates showed a condensed exopolysaccharide matrix (slime) surrounding the bacterial cell wall as revealed by electron microscopy and immunofluorescence.

In conclusion, our findings showed that the adherent slime i.e., slime firmly attached to the cell wall was responsible for resistance to multiple antibiotics as well as for the adhesion phenomenon. The loose slime could possible give rise to inconclusive results in the tube test (Christensen’s method) and intermediate results in the congo red agar test.

However, the isolates devoid of any adherent slime or loose slime could be yielding negative results. Thus, there is a definite impact of the bacterial ultrastructure on the in vitro slime test results.

References


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