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Influence of vancomycin induced increase cell size in *Staphylococcus aureus*

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Abstract
Introduction: The emergence and world wide spread of MRSA between the early 1960s and late 1990s have begun to pose serious threat to the chemotherapy of staphylococcal diseases world wide this leads to difficulty in successfully treating *S. aureus* infection.This leaves only vancomycin as the drug of choice and vancomycin-resistant MRSA has already been reported in several countries.
Methods: Fourstrains of *S. aureus* were isolated from culturing pus and blood samples collected from hospitals and diagnostic clinical samples of Gulbarga region, Karnataka, India. The vancomycin resistant was studied using different techniques such as broth macrodilution, agar dilution combined with agar diffusion, morphology cell changes by scanning electron microscopy.
Result: The multidrug resistant *S. aureus* howed stepwise adaptation when grown in increasing concentration of vancomycin eventually reaching a maximum of 45µg/ml. The resultant vancomycin resistant mutant strain was stable and did not revert to susceptibility on frequent subculturing. The response of the cells to different concentration of Vancomycin was examined by scanning electron microscope which showed that the size of the bacterium increased with increasing concentration of Vancomycin.

Keywords: vancomycin resistant *Staphylococcus aureus* (VRSA), Scanning electron microscope

Introduction

Staphylococcus aureus (*S. aureus*) infection is one of the most frequently occurring bacterial infections [1]. Eradication of the organism is extremely difficult, particularly resistant clinical isolates, due to its multiple antibiotic resistance mechanisms has affected the efficacy of traditional therapeutic approaches and adds an urgency to search for antimicrobial agents with novel chemical structures and bacterial targets [2]. Among various drug resistant microorganisms vancomycin-resistant forms of *Staphylococcus aureus* (VRSA) are a major threat causing a wide range of infections from skin to pneumonia [3, 4], The vancomycin, glycopeptide antibiotic is the drug of choice against strains of Staphylococcus aureus that are resistant to methicillin and gentamicin [5, 6]. The vancomycin resistant have emerged by cell wall modifications that trap the antibiotic before it reaches its action site peptidoglycan component of the inner layer [5]. The appearance of vancomycin resistance among clinical isolates of enterococci has raised concern about transfer of the resistance genes to highly virulent strains of MRSA [7]. The Minimum Inhibitory concentration of glycopeptide antibiotics derivatives vancomycin have influenced change in morphology and biochemical alteration of VRSA and the response of cell wall active antibiotic on bacterial morphology have been demonstrated by scanning electron studies [8]. The adaptive nature of bacteria uniquely modifies their morphology and physiological to counteract the high concentration of externally occurring changes in the environment [9]. the present paper describes the effects of antibiotic stress on the morphology in experimental populations of a stepwise adapted VRSA mutant strain isolated from clinical sample examined by scanning electron microscope.

Materials Methods Bacterial strains

In this study, a total of 4strains of *S. aureus* were isolated from culturing pus and blood samples collected from hospitals and diagnostic clinical samples of Gulbarga region, Karnataka, India. All the samples were enriched using brain heart infusion (BHI) agar (Himedia Laboratories, Mumbai, India) for 24 h at 37°C. The preliminary identification of *S. aureus* was done using mannitol salt agar which was detected by changes in color of the medium from red to yellow due to mannitol fermentation. Further, isolates were identified on the basis of microscopic morphology, and biochemical studies. Standard strain *S. aureus* (MTCC 96) was obtained from the Microbial Type Culture Collection and Gene Bank centre, Chandigarh, India and used as control.

Isolation of Vancomycin resistant mutant strains

The stepwise adaptation procedure was used for the isolation of VRSA mutant strain from the Vancomycin susceptible *S. aureus* isolates; Stepwise selection for all four vancomycin susceptible *S. aureus* was performed with the successive transfer of cells into medium containing gradually higher concentrations of Vancomycin (µg/ml) ranging from 5, 10, 15, 30to 45 Stepwise adaptation continued until a maximal resistance level of 45µg/ml of vancomycin was achieved, efforts to attain higher levels of resistance were not successful. As a confirmation of their resistance to vancomycin, colonies grown on brain heart infusion (BHI) agar were inoculated into drug-free broth and incubated overnight at 37C in a temperature controlled rotary shaker at 180 rev/min and 100µl of culture was then replated on BHI agar containing vancomycin (45µg/ml). The presence of subpopulations on this agar after 48 h of incubation at 37C confirmed the vancomycin resistance [9].

Influence of antibiotic on *S. aureus* morphology

The minor changes in cell morphology of the population that have been adapted to antibiotic stress was determined using scanning electron microscopy (S-200C scanning electron microscope). The isolated VRSA mutant and standard control MTCC 96 were grown on Brain Heart Infusion (BHI). The bacterial cells from each culture were recovered by centrifugation at 6000 RPM for 5 minutes and the cells were washed twice with potassium phosphate buffer (50 mM, pH 7.0). Bacterial cells were fixed by immersing in 2.5% glutaraldehyde in potassium phosphate buffer (50 mM, pH 7) for overnight at 4°C. Then the specimens were washed twice with buffer and dehydrated by ethanol series (v/v) ranging from 30%, 40%, 50%, 60%, 70%, 80%, 90% to 100%. For SEM analysis, all the specimens were dried to the critical point, coated with gold. The cell volume and surface area obtained from SEM photograph were directly measured and calculated by using the following equations;

$V\ (\mu\ m^3) = 4/3\ \pi r^3$,

$a\ (\mu\ m^2) = 4\pi r^2$;

Where "r" is the radius, "V" is volume and "a" is surface area. The average cellular volumes and surface areas were calculated by using 30 individual bacteria per population. Cells showing deformations were not considered. The mean values were calculated from SEM photographs by taking 30 bacteria per population. Statistics were calculated using the ANOVA using excel 2007 [9, 10, 11].

Results

Selection of vancomycin resistant mutant strains

The Vancomycin resistant mutant strains were selected through the stepwise passage of VSSA isolates in the BHI media containing vancomycin Out of four VSSA strains only one was able to grow in the presence of 45 µg/ml of Vancomycin and remained resistant even after five to six generation passages through Vancomycin free BHI agar plates. Determination of antimicrobial sensitivities confirmed that all VSSA were susceptible to vancomycin.

Changes in cell morphology and size

In order to evaluate the antibiotic response on *S. aureus* morphology SEM was carried out and summarized in Table (1). The results showed VRSA mutant cells altered their morphology with respect to VSSA. In the presence of vancomycin the mutant cells altered their morphology with respected to different concentrations of the vancomycin. In the absence of vancomycin, the cell morphology of both wild type VSSA and VRSA mutant cells were apparently normal (Fig. 1a,b), but enlarged, malformed and rough surfaced cells were observed in the adapted VRSA mutant culture(Fig. 1c, d), the enlarged cells in VRSA mutant culture containing vancomycin 30 and 45µg/ml.). The change in the morphology may be occurring due to antibiotic stress.

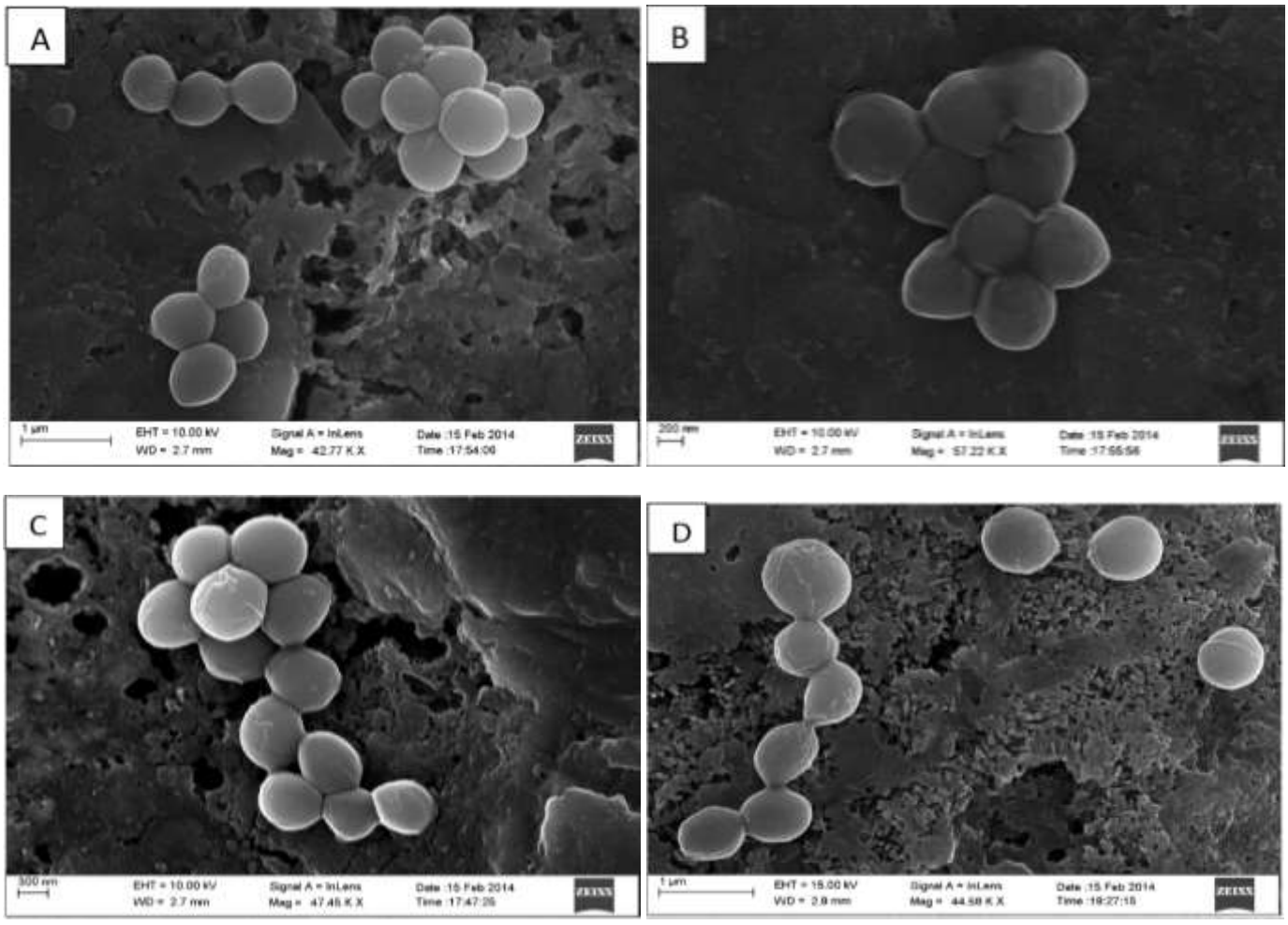

Fig 1: Scanning electron micrographs of VRSA mutant cells at various concentration of vancomycin, VSSA at 0µg/ml (a), VRSA mutant cells in presence of 0 µg/ml (b), 15µg/ml (c), 30 µg/ml (d), 45µg/ml

Table 2: Effect of different concentrations of vancomycin on cell sizes of the stepwise-adapted VRSA mutant cells.

Vancomycin (µg/ml)	Radius[a] (µm)	Volume[b](µm^3)	Surface area (µm^2)
0	0.330±0.04	0.166±0.06	1.412±0.37
15	0.369±0.02	0.211±0.04	1.721±0.25
30	0.382±0.05	0.249±0.08	1.881±0.45
45	0.412±0.06	0.310±0.14	2.172±0.70

[a] Volume, [b] surface area is calculated by using formula as described in materials and methods each mean value, was the average of 30-cell size based on the scanning electron microscopy photos.

Discussions

The morphological alterations in microorganism induced by antimicrobial agents is an adaptive response which interfere with their mechanism of action demonstrated by scanning electron microscopy [8]. To overcome the adverse environments stress by activating survival mechanisms bacteria are known to spawn reflective changes by forming resistant spores, nonsporulating microorganisms [10]. In the present investigation we have studied that the cell morphology of stepwise-adapted VRSA mutant cells under SEM provided strong evidence that the presence of a high concentration of vancomycin is stressful for the bacterial populations, characterized by the large size of cells in the exponential phase culture. The average cell volumes in stepwise-adapted VRSA mutant cells increased substantially at their corresponding selection environment and retained their changes even after five to six subcultures. The intermediate concentration of vancomycin (15and30µg/ml) also showed the increase in size of the mutants which was smaller than those adapted to 45 µg/ml concentration.

The transfer of vancomycin resistance among clinical isolates of enterococci has raised concern about transfer of the resistance genes to MRSA have become the mainstay of chemotherapy. The bottle neck of the resistant mechanism in the VRSA mutants to alter the morphology of the cell wall in contrast changes the binding of the glycopeptide at the surface of the cell from the sites of cell wall biosynthesis [8, 13].

The change in the morphology of stepwise-adapted VRSA mutant cells increased substantially at their corresponding selection environment may be occurring due to antibiotic stress; the increase in cell size reduces the relative contact surface and consequently reduces the attachable surface for organic compounds. Therefore, bigger cells can tolerate the stress conditions better than normal cells of the same. The effect of antibiotic stress on bacterial cell size and morphology is known to be concentration-dependent. Increased concentration of the antibiotic have increased cell size this predicts that the step wise adapted stress on bacteria changes the multiplication inturn unaffecting the metabolism [9].

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