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In Vitro Activity of Nisin and Evaluation of Guar Gum as A Potential Drug Delivery System against Methicillin- Resistant *Staphylococcus aureus* Isolated from Diabetic Foot Ulcers

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Abstract :

Introduction: Diabetic foot ulcer is one of the most preventable long term complication of diabetes mellitus, *Staphylococus aureus* being one of the frequently isolated organism. Presence of infection by MRSA implies future amputation of the limb. These bacteria have ability to produce biofilm which is resistant to the action of most antibiotics. The antimicrobial peptide(AMP) nisin has been investigated for new therapies and guar gum has been tested as drug delivery system

Aim: To determine the antimicrobial activity of nisin against biofilm producing *S. aureus* isolated from diabetic foot ulcers. To evaluate guar gum as a drug delivery system

Materials & methods: This study was conducted at Saveetha Medical College & Hospitals ,Tamil Nadu using 16 isolates of biofilm producing MRSA from diabetic foot ulcers. Inhibitory potential of nisin against 16 *S. aureus* isolates collected from DFU patients was evaluated. The minimum inhibitory (MIC), bactericidal (MBC) concentrations were determined for nisin diluted in HCl and incorporated in guar gum gel separately. Inhibitory activity of nisin incorporated in guar gum gel for a period of 6 months was tested.

Result: All isolates tested are considered susceptible to nisin. For nisin diluted in HCl, mean value for MIC and MBC were 72.30±37.67 µg/mL & 446.15±161.32 respectively . Regarding the nisin incorporated in guar gum gel, mean value for MIC & MBC were 143.84±110.87 µg/mL & 676.93±252.17 µg/mL respectively. Statistical differences were observed between MIC & MBC for nisin-HCL & nisin- guar gum. Inhibitory activity of nisin in guar gum for 6 months was observed to be positive.

Conclusion: Results show the importance of nisin as a substitute or complementary therapy to the current antibiotics used for treating DFU infections. Guar gum represents innovative therapeutic strategy & shows a promising delivery system for AMP, allowing the development of novel topical therapies as treatments for bacterial skin infections.

Key Words : diabetic foot ulcers, *Staphylococcus aureus*, biofilm, nisin, guar gum, minimum inhibitory concentration

INTRODUCTION:

Diabetes mellitus is common throughout the world. Among all the several complications of Diabetes mellitus, diabetic foot ulcer (DFU) is one of the most preventable long term complications. Diabetic foot ulcers are associated with significant morbidity and mortality [1]. Mild diabetic foot infections are usually monomicrobial whereas severe diabetic foot infections commonly yield polymicrobial growth [2]. *Staphylococcus aureus* and beta hemolytic streptococcus are the first organisms that colonize wild infections [3].

Staphylococcus aureus can cause a wide range of illness, from mild skin infections to severe bacteremia and sepsis. Methicillin resistant *Staphylococcus aureus* (MRSA) strains are most commonly associated with hospital acquired infections. It is one of the most common pathogen isolated from diabetic foot ulcers and post surgical wound infection [4,5]. Presence of infection by MRSA implies future amputation of the limb [6]. The production of biofilm by these organism reduce the healing mechanism. Thus, diabetic foot infections needs to be treated properly with appropriate wound care and the initiation of novel treatment strategies are required.

Nisin is a heat stable cationic lantibiotic produced by *Lactococcus lactis* subspecies *lactis*. It is an antimicrobial peptide (AMP) belonging to class 1 bacteriocin [7]. It is the only bacteriocin which has been legally approved as safe for use in food and beverage industry. It is classified as Generally Recognized as Safe (GRAS) and the Food Safety Authority has established an acceptable daily intake of 0.13mg/kg [10]. Additionally nisin also has antimicrobial activity. It is effective against Gram positive organisms and also prevents growth of *Clostridium* and *Bacillus* species [8,9]. Treatment of infected diabetic foot ulcers with nisin requires an effective drug delivery system. This is because antimicrobial peptide can be easily degraded before reaching its target site at therapeutic concentrations [11]. Thus Guar gum has been tested as potential delivery system in case of antimicrobial peptides.

Guar gum is naturally occurring gum also called Guaran. It is derived from the plant *Cyamopsis tetragonobolus*. It has gained increasing popularity due to its non toxic and bio degradable nature. It is also cost effective. Hence it is widely used in drug delivery in almost all areas. The use of guar gum in drug delivery is due to its structural attribute [12].

The present study was done to find out the antimicrobial activity of nisin against biofilm producing MRSA isolated from diabetic foot ulcers and to evaluate guar gum as a potential drug delivery system for nisin.

MATERIALS AND METHODS:

This study was conducted at Saveetha Medical College and Hospital, Tamil Nadu for a period of one year (June 2016-June 2017). Patients with diabetic foot lesions from surgery ward and OPD were screened. Discharge from the diabetic foot ulcers were collected using sterile swabs. Pus aspirates from abscesses & debrided necrotic material were collected for culture.

Bacterial strains: 59 isolates of *Staphylococcus aureus* was obtained. These were characterised according to their antimicrobial resistance and biofilm producing ability. 16 isolates were found to be strong biofilm producing MRSA which was used for the present study. A reference strain *S. aureus* ATCC 29213 was used as a control, being a known biofilm producer.

Confirmation of methicillin-resistant *S. aureus* (MRSA) : Confirmation of MRSA strains was done using cefoxitin disc diffusion. Kirby Bauer method was used in accordance with Clinical Laboratory Standards Institute (CLSI) using ATCC S.aureus 25923 as control. The medium used was Cation adjusted Mueller-Hinton Agar. Inoculum was direct colony suspension equivalent to 0.5 McFarland standards. The media was incubated at 37 degree Celsius at ambient air for 16 to 18 hours. The strength of the cefoxitin disc was 30microgram.

Tissue culture plate method: This quantitative test described by Christensen et al. [13] is considered the gold-standard method for biofilm detection. Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose.

Broths were incubated at 37 ^{0} C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom polystyrene tissue culture treated plates (Sigma-Aldrich) were filled with 200 µL of the diluted cultures. The control organisms were also incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth. The plates were incubated at 37^0 C for 24 h. After incubation, contents of each well were removed by gentle tapping. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removed free floating bacteria. Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%). Excess stain was removed by using deionized water and plates were kept for drying. Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times. The interpretation of biofilm production was done according to the criteria of Stepanovic et al. [14].

Preparation of nisin & Guar gum incorporation: a nisin stock solution(1000µg/ml, corresponding to 40,000 IU/ml) was prepared as described in previously done studies. 1 g of nisin powder was dissolved in 25 ml of HCL(0.02M). This was filtered and stored at 4˚C. A set of dilutions were prepared using distilled water: 900, 800, 700, 600, 500, 400, 300, 200, 100, 40, 10, 5 µg/ml

A Guar gum gel of 1.5% was prepared by dissolving 0.75g of Guar gum in 50ml of sterile distilled water and heat sterilized by autoclave. A set of dilution of nisin were incorporated within the gel in a proportion of 1:1.

Determination of Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): MIC and MBC were determined in triplicate using the microtitre broth dilution [15]. Bacterial suspension were diluted in brain heart infusion broth at a concentration of 10^7 CFU/ml.

Dilutions of nisin were distributed in 96 well microtitre plates, 25µl in case of nisin in HCL and 50 µl when combined with guar gum. All wells were inoculated with 150 µl of bacterial suspension except for negative control. The plates were incubated for 24 hours at 37˚C. MIC was interpreted as the lowest concentration of nisin that inhibited the microbial growth visually. MBC was determined using Brain heart infusion agar plates. 3 µl of the suspension from well showing no visible growth was inoculated into the BHI agar plates and incubated at 37˚C for 24 hours. MBC was interpreted as the lowest concentration of nisin that showed no growth on the agar plates.

Statistical Analysis : Statistical analysis was performed using t – test. Significance of the variables under study and *p*-value < 0.05 was considered to be statistically significant. Quantitative variables, were expressed as means ± standard derivation.

RESULTS :

Results of the MIC and MBC values of nisin diluted in HCL and nisin combined with guar gum are shown in Table 1 and summarized in Figure 1 & 2

All 16 isolates tested were considered susceptible to nisin. The reference strain S. aureus ATCC 29213 was also susceptible. For nisin diluted in HCl, mean value for MIC and MBC were 72.30±37.67 µg/mL & 446.15±161.32 µg/mL respectively. Regarding the nisin incorporated in guar gum gel, mean value for MIC & MBC were 143.84±110.87 µg/mL & 676.93±252.17 µg/mL respectively.

MIC values ranged between 10 to 100 µg/mL and MBC values ranged between 300 to 800 µg/mL for nisin diluted in HCL (Figure 1). 10 isolates showed MIC values of 100 µg/mL. MIC and MBC values were between 5 to 300 µg/mL and 300 to 900 µg/mL respectively (Figure 2).

Difference between MIC values for nisin-HCL & nisin- guar gum were significantly different (p-value<0.05). Statistical difference was also observed in case of MBC values for nisin-HCL & nisin-guar gum.

TABLE : 1 MIC and MBC values of nisin diluted in HCL and nisin combined with guar gum

Organism	Nisin-hcl		Nisin-guar gum	
	MIC(µg/ml)	MBC(µg/ml)	MIC(µg/ml)	MBC(µg/ml)
ORGANISM 1	40	300	40	400
ORGANISM 2	100	300	40	900
ORGANISM 3	100	500	100	900
ORGANISM 4	100	500	200	900
ORGANISM 5	40	400	300	800
ORGANISM 6	100	300	200	300
ORGANISM 7	10	300	40	300
ORGANISM 8	100	400	200	800
ORGANISM 9	100	700	10	900
ORGANISM 10	100	800	300	900
ORGANISM 11	40	500	40	400
ORGANISM 12	100	500	100	800
ORGANISM 13	10	300	300	500
ORGANISM 14	40	400	200	600
ORGANISM 15	100	500	200	500
ORGANISM 16	100	700	40	500
ATCC 29213	100	500	200	800

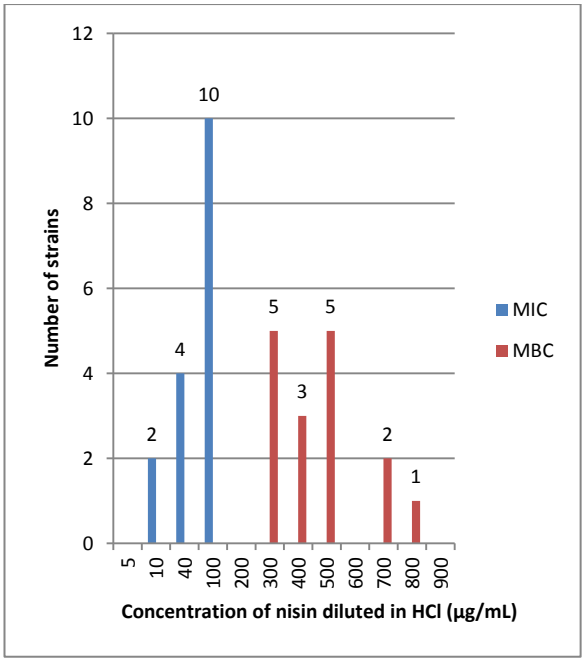

FIGURE 1: MIC & MBC values for nisin diluted in HCL aginst S. aureus DFU isolates

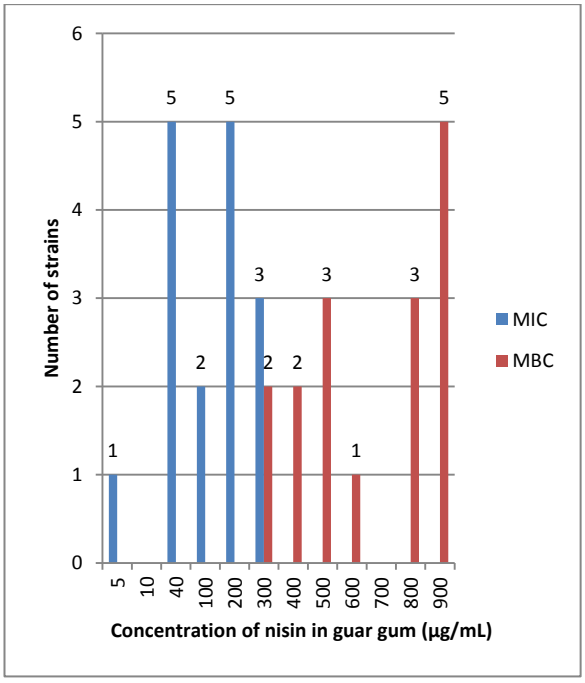

FIGURE 2: MIC & MBC values for nisin incorporated in guar gum gel against S. aureus DFU isolates

DISCUSSION :

Foot ulcers are the most common medical complications of patients with diabetes, with an estimated prevalence of 12-15% among all individuals with diabetes. Diabetic foot ulcers are responsible for more hospitalizations than any other complication of diabetes. Ulcerations can have potential devastating complications as they cause up to 90% of lower extremity amputations in patients with diabetes. Several factors are involved in the decreased healing potential of a diabetic foot, presence of infection being one of the most important factor. Several studies have reported *S. aureus* as the most common organism associated with the DFU. In one of the studies conducted previously at Annamalai university, Chidambaram, Tamil Nadu, the most common organism isolated was *Staphylococcus aureus* (40.9%) followed by *Pseudomonas* (22.7%) [16].

The biofilm producing capacity of the infecting organism is also another major contributing factor to healing impediment. In our study 16 isolates were selected based on their abilty to produce biofilms using tissue plate method. And all the isolates were resistant to cefoxitin and carriers of mec A gene.

In our study, the objective was to find out the ability of nisin to control *Staphylococcus aureus* DFU isolates when incorporated in guar gum, a natural galactomannan polymer, with the ultimate aim of identifying its efficacy as a topical drug delivery system.

As shown in the results all isolates were susceptible to nisin when incorporated in guar gum as well as when diluted in nisin. This was in accordance with one of the previous studies Raquel *et al.* Nisin diluted in HCL showed an average MBC values 6 times higher than the MIC values[17]. Antimicrobial agents are usually classified as bacteriocidal if MBC values are not more than four times its MIC values[18]. Thus our study shows that nisin is a bacteriostatic agent against the tested isolates. Similarly nisin when incorporated with guar gum, the MBC values were 4.5 times higher than the MIC values. Hence stating that nisin-guar gum also worked as bacteriostatic agent but even more efficient than nisin-HCL.

In one of the studies done earlier by Raquel Santos *et al*, nisin presented high level of antimicrobial activity towards planktonic bacteria [17]. Okuda and collabrators investigated the effect of diverse bacteriocins on MRSA clinical isolates and demonstrated that nisin showed a higher bactericidal activity against both free floating and biofilm cells[19].

Guar gum thus kept its antimicrobial activity towards all tested isolates. Guar gum is considered as a promising drug delivery system as it confers high viscosity. Due to its thickening and binding nature it finds application as a safe system for delivery of the antimicrobial peptide. This shows its potential as a topical therapeutic administration. Also, the minimum concentrations required to inhibit the isolates are well below nisin's acceptable daily intake. This shows safety of nisin at its therapeutic range.

CONCLUSION:

Results show the importance of nisin as a substitute or complementary therapy to the current antibiotics used for treating DFU infections. Nisin is considered GRAS for oral consumption. Guar gum represents innovative therapeutic strategy & shows a promising delivery system for AMP, allowing the development of novel topical therapies as treatments for bacterial skin infections. Considering the overall clinical and economical burden caused by such virulent strains, AMP have attracted great interest in their potential use as new antibacterial agent mainly due to their high antibacterial activity and low AMP resistance development.[20,21]

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