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Study on *In Vitro* Exposure of *Staphylococcus aureus* to Human Neutrophil Pepitde-1

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Abstract: This study involves the exposure of a *Staphylococcus aureus* (*S. aureus*) strain to the well studied antimicrobial peptide (AMP), human neutrophil peptide-1 (hNP-1). This was performed in order to understand whether serial exposure to an AMP, which is considered as potent alternative for antibiotic-resistant pathogens, can lead to development of resistance for the study AMP. For this, we subjected *S. aureus* ATCC 29213 to serial passage in the presence of sub-inhibitory concentrations of hNP-1 *in vitro*, for several hundred generations. The study *S. aureus* strain did not show any change in susceptibility pattern on continued exposure of the peptide. This indicates that prolonged exposure of susceptible *S. aureus* to hNP-1 might not result in decrease in its susceptibility. Further, it suggests the sustainability of therapeutic importance of hNP-1 to treat antibiotic-resistant pathogens.

Keywords: Antimicrobial peptides, Serial passage, Human neutrophil peptide-1 , *S. aureus* membrane, Adaptations

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Introduction

The recent, alarming emergence of multi-drug resistant pathogens and the associated infectious diseases highlight the need for new therapeutics. *Staphylococcus aureus* is the frequent causative agent of hospital and community acquired infections (Hafeez *et al*., 2021). The decline in the potency of current therapies has brought antimicrobial peptides (AMPs) in the scenario and has gained much attention in the search for antimicrobial alternatives to chemicals and commonly used antibiotics (Luo and Song, 2021). They are either constitutive or inducible and are recognized as important components of innate defense mechanism. The cationic and amphipathic nature of the AMPs is associated with their activities. The target of the AMPs is specifically the bacterial membrane, owing to the fundamental difference in design of the microbial membrane from that of multicellular animals (Mukherjee *et al*., 2021; Büyükkiraz and Kesmen, 2022). Despite their ancient lineage, AMPs have remained effective defensive weapons, contradicting the general belief that bacteria, viruses and fungi can and will develop resistance to any conceivable

substance. Appreciating this prospect, few AMPs are already in clinical use and some of them are in clinical trials. Polymyxins, pexiganan, omiganan, LTX-109, are some AMPs that are developed for local applications. There are a few AMPs aimed for systemic administration, namely hLF1-11, novexatin, alpha-MSH (Mahlapuu *et al*., 2016). Some defensins and defensin-mimetic molecules have been already used as templates for the development of pulmonary pharmaceuticals.

However, it is argued that usage of AMPs in therapeutic amounts over an extended period of time might lead to reduced susceptibility along with adaptive changes in phenotypic and genotypic characteristics of the organism (Mahlapuu *et al*., 2016). It is also speculated that sublethal exposure to particular environmental conditions and antimicrobial substances may result in the development of increased resistance and promotion of cross-resistance to antimicrobial compounds (Gorr and Bechinger, 2017). Unfortunately, several *in vitro* studies and recent reports in clinical outcome supported the argument that on continuous exposure, bacteria might evolve means of lessening the impact of AMPs and thus develop resistance against them. For example, within few years of approval of daptomycin in medical therapy in 2003, reports of *S. aureus* strains, *Enterococcus* species with reduced susceptibility to the AMP began to appear (Kaatz *et al*., 2006). Another study showed the *in vitro* selection of daptomycin resistant *S. aureus* strain after subjecting a well-known MRSA strain to 20-day serial passage of sublethal concentration of daptomycin. The *in vitro* selected strains displayed alterations in various membrane parameters including an increase in cell wall thickness (Mishra *et al*., 2009). Perron *et al*, (2006) showed *in vitro* evolution of resistance in *E. coli* and *P. fluorescens* against pexiganan through continued selection method. Few other studies have also reported the study of the propensity of bacteria to generate resistance or how they behave on serial exposure to antimicrobial agents (Pollard et al. 2012). In a recent study, it was observed that exposure of *S. aureus* FDA209P to vancomycin resulted in induction of vancomycin-survivor (VSV) strains, accompanied by acquired mutations in the *ileS* gene, which encodes isoleucyl-tRNA synthetase (IleRS) (Singh *et al*., 2020). Resistance to AMPs arises from a range of nonspecific mechanisms, including secretion of proteases and exopolymers, biofilm formation, activation of efflux pumps, and modification of the cell envelope, including its charge (Lazzaro *et al*., 2020).

In this complicated scenario of limited therapeutic option for treating infections caused by *S. aureus,* it is of paramount importance to study and understand the sustainability of these AMPs before their therapeutic introduction. In this context, the objective of this study was to determine whether or not *S. aureus* on continuous exposure to sub-lethal concentrations of the well studied AMP, defensin (hNP-1) alter their susceptibility and whether resistant strains are selected. In this study a selection protocol was designed, where *S. aureus* was grown for several hundred generations in media containing increasing concentration of the peptide and then examined for the development of resistance.

Materials and Methods

Bacterial strain, growth media and antimicrobial peptide:

S. aureus strain ATCC 29213 was used in the study. The strain was stored at -80°C in 15% (v/v) glycerol until subcultured onto brain heart infusion (BHI) (Himedia Laboratories, India) agar plates or Mueller Hinton Broth (MHB) (Himedia Laboratories, India) for further analysis. hNP-1 (Ala-Cys-Tyr-Cys-Arg-Ile-Pro-Ala-Cys-Ile-Ala-Gly-Glu-Arg-Arg-Tyr-Gly-Thr-Cys-Ile-Tyr-Gln-Gly-Arg-Leu-Trp-Ala-Phe-Cys-Cys), disulphide bridges: 2-30, 4-19, 9-29) was purchased from Sigma-Aldrich Chemicals Pvt Ltd (St Louis, MO) and its concentration was determined spectrophotometrically (Shimadzu UV- 2450 spectrophotometer).

Selection of antimicrobial peptide resistant strain:

Two cell lines were established for the AMP – one

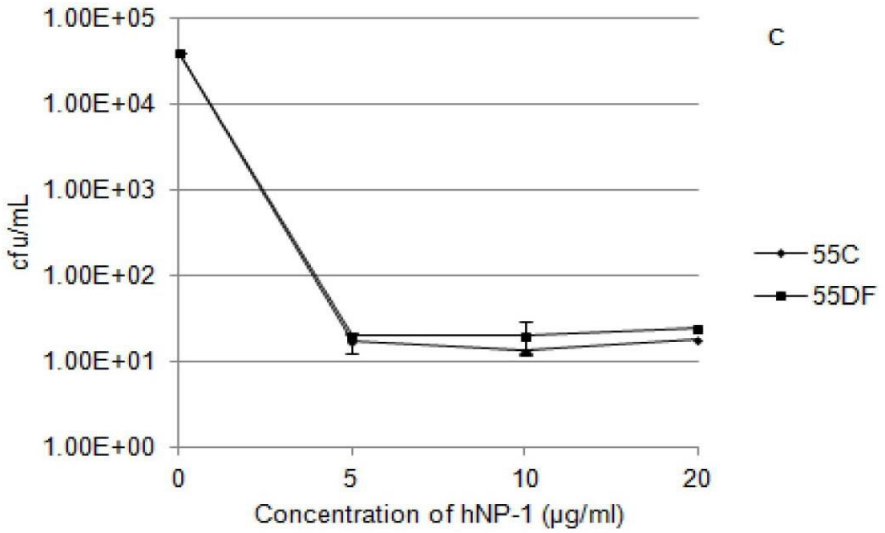

Fig. 1: *In vitro* susceptibility of *S. aureus* by bactericidal assay against different concentrations of hNP-1. The peptide was incubated for 1 h at 37°C in 10 mM PB (pH 7.4) containing mid-logarithmic phase bacterial cells. Serial dilutions were then plated on BHI agar and colony counts were performed after 18 h of incubation at 37°C. Survival of *S. aureus* was determined by counting of the colony and was presented as cfu/ml. The results represent the mean (±SD) from three independent experiments. Symbols: diamonds, control strain; squares selected strains, **indicates P ≤ 0.01 (one-way ANOVA, Minitab™).

grown in the presence of peptide, hereafter referred to as positive selection line and the other grown in an environment devoid of peptide for the entire duration of the experiment, referred as the control selection line (Perron *et al*., 2006). Bacterial cultures were grown overnight in MHB in shaking incubator at 37°C. *S. aureus* cell lines were transferred daily by inoculating 20 µl of stationary phase culture to 2 ml fresh MHB, to give a daily dilution of 100. At every transfer ancestral clones were preserved at -80°C in 15% (v/v) glycerol. Every transfer was given strain designation to indicate serial passage number. Both control and positive selection lines were initially grown in a pristine environment free of AMP for 20 transfers. At 21st transfer, the medium of the positive selection line was supplemented with a non-inhibitory concentration of hNP-1 (0.5 µg/ml). Later, either the concentration of the AMP was doubled ev 5 transfers or continued with the same depending on the growth showed by the positive selection line. The experiment was conducted for 55 serial passages, constituting 600-700 bacterial generations.

In vitro susceptibility testing:

The evolution of resistance was determined by performing bactericidal assays as described by Xiong *et al.* (2005). In brief, mid-logarithmic phase *S. aureus* cells were used. After being harvested cells were washed in PB (potassium phosphate buffer, pH 7.4). Mid-logarithmic *S. aureus* cells were exposed to various concentrations of the peptide. At selected time points, aliquots were plated on BHI agar and were incubated at 37°C overnight. Survival of *S. aureus* was determined by counting of the colony and was presented as cfu/ml (Madhuri *et al*., 2009). Evolution of resistance was identified as a significant increase in cfu/ml in positive selection line compared to that of control selection line.

Results and Discussion

Since the discovery of AMPs there has been an increasing interest in the potential of these peptides as antimicrobial agents for therapeutic use. Along with this interest, the concern of

consequences of possible development of resistance to these AMPs has emerged (Bell and Gouyon 2003). Therefore, it seems imperative to check if pathogenic organisms like *S. aureus* can alter its susceptibility under continuous selection pressure of AMPs and its adaptations in the changed conditions. With this objective *S. aureus* ATCC 29213 was subjected to subinhibitory and step-wise increasing concentrations of the well studied defensin, hNP-1. It was found that susceptibility to the AMP did not decrease till the 55th passage. The strain at the 55th passage (55DF) was equally susceptible as the control strain (55C) in all tested concentrations of the AMP (Fig. 1). Hence, hNP-1 resistant *S. aureus* strain could not be selected.

Along with pore formation in the microbial membrane, defensins are widely accepted to exert bactericidal activity via other modes, involving membrane-independent mechanisms and targeting of intracellular components. Therefore, to develop resistance against this multi-targeted defensin may be a difficult and an expensive task for *S. aureus*. hNP-1 is kind of a more robust peptide due to the presence of intramolecular disulphide bridges, connecting the cysteine residues. Although the disulphide bridges are not necessarily essential for the antimicrobial activity of defensins, they do render peptides more resistant to proteolysis. This makes the peptide more stable; it could be also one of the reasons why serial exposure to the increasing concentration of hNP 1 in this study, did not alter the susceptibility of the strain (Zasloff, 2019; Gao *et al*., 2021; Solanki *et al*., 2021).

Conclusion

On a positive note, the present study indicated the failure of *S. aureus* to develop resistance against hNP-1 under continuous *in vitro* exposure. This is an encouraging observation in regard to developing defensin like molecules as therapeutic applications. In conclusion, considering the urgent need of introducing AMP as an alternative therapy to combat bacterial infection, a study of this kind is of utmost importance to minimize emergence of resistant organisms against AMPs and to develop AMPs as potentially useful antimicrobial agents.

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