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Research Article

IS MRSA REVERSIBLE? DOES E-POLYLYSINE HOLD PROMISE AS AN ADJUVANT DRUG TO TREAT METHICILLIN RESISTANT *S. AUREUS*?

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Abstract- The present study was aimed to investigate if ε-Poly Lysine (ε PL) has the potential to be repurposed for treating MRSA. Poly Lysine (ε PL) was tested for anti MRSA activity by disc diffusion assay against 5-100 µg/ml and the Minimal Inhibitory Concentration was determined by the broth micro dilution method. The disc diffusion assay was also used to do preliminary screen of ε PL to know its antimicrobial activity. All experiments were performed at sub-MIC of ε PL so that the bacteria do not come under stress (microbicidal effect). The *mecA* genotype was confirmed by PCR after every passage and the reversibility of MRSA phenotype was investigated after successive passages on agar plates by disc diffusion against Cefoxitin and Oxacillin. The morphological changes were observed under SEM. The MICs of ε PL was 12.5 µg/ml and 20 µg/ml for MSSA and MRSA isolates respectively by disc diffusion method. However, the MICs determined by broth micro dilution method were 6.25 µg/ml for both MSSA and MRSA isolates. The anti-MRSA activity of ε PL is inducible in the bacteria. After four passages, the Cefoxitin inhibition zone increased from 0 mm to 18 mm. *mecA* could be detected in cells of all passages. The cells started collapsing after the first passage and appeared elongated with indentations, lysed, distorted and membrane severely disrupted with increasing passages. ε- PL inhibited the growth of MSSA and MRSA. It also reversed MRSA phenotype and restored sensitivity of the bacteria to methicillin. It appears to act through an independent mechanism. ε PL could be re-purposed to treat MRSA and also used in co-therapy to avoid development of drug resistance.

Keywords- *Adjuvant drug, mecA, MRSA, MSSA, ε-Poly Lysine*

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Introduction

Staphylococcus aureus is a gram positive, opportunistic and a very common human pathogenic microorganism causing a variety of infectious diseases such as minor skin infections, bacteremia, osteomyelitis, endocarditis, and pneumonia [1]. Methicillin Resistant *Staphylococcus aureus* (MRSA) is one of the most antibiotic resistant bacteria which is also endowed with multiple virulence factors. The increase in the prevalence of multi-drug resistant (MDR) pathogens has severely limited the therapeutic options. Methicillin Resistant *Staphylococcus aureus* (MRSA) is a major cause of nosocomial and community infections throughout the world. MRSA is constitutively resistant to all β-lactam antibiotics because the *mecA* encodes the penicillin binding protein PBP2a which significantly reduces the affinity to several β-lactams rendering these antibiotics ineffective [2,3].

The rapid increase in antibiotic resistance coupled with a shockingly poor rate of discovery or approval of new antibiotics in the last two decades has landed the medical fraternity and society in a global crisis. It is predicted that there won't be any effective antibiotic available by 2050 if no new drug is developed or discovered. Antibiotic resistance is difficult and perhaps impossible to overcome. Nevertheless, efforts are being made in recent times to energize antibiotic discovery research. It is important that the new candidate antibiotics ideally act through mechanisms hitherto not common and against which resistance has not been reported yet. Multiple approaches are being undertaken by researchers which include i) design and development of newer scaffolds [4], ii) chemical modification of existing antibiotics to increase their effectiveness [5], iii) antimicrobial peptides (AMP) which possess broad immune modulatory activity [6], iv) repurposing existing drugs as potent antibiotic adjuvants as a cost-effective strategy [7], and v) Compounds which act as resistance breakers [8] or antibiotic potentiators [9], which may or may not have antibiotic activity but when co-administered with the antibiotic they either circumvent the bacterial resistance mechanism or enhance the antimicrobial action of the main antibiotic. Natural inhibitors with reversal activity are also currently being investigated due to their tremendous potential of resistance reversal and to avoid toxicity associated with the currently used drugs.

ε-Polylysine (ε-PL) is a cationic polypeptide, it consists of 25-35 L-Lysine residues with linkages between α-carboxyl and ε-amino group of adjacent residues, produced by Streptomyces albulus. It has several favourable properties: bio-degradable, water soluble, non-toxic, highly thermo-stable, eco-friendly, and safe for human consumption as it is an approved food preservative [10-13]. It adsorbs to and disrupts the integrity of the bacterial membrane which in addition to successfully killing the bacteria reduces the risk of development of resistance. The broad anti-microbial effectiveness of ε-PL has already been well established against a wide spectrum of microbes like yeast, fungi, and bacteria [14]. ε-PL is thus an attractive candidate in the search for novel strategies to combat antimicrobial resistance [15]. In the past decade, ε-PL derivatives have been used in cell adhesion and labelling, gene delivery and biosensors, emulsifiers, drug carriers, biodegradable fibers, highly water-absorbable hydrogels, biochip coatings [16-19]. However, its effect on drug resistant bacteria, especially MRSA, has not yet been investigated in detail or reported from therapeutics point-of-view.

Recently ε-PL has found many biomedical applications like wound dressing, sutures [12,20] but there is no report of any therapeutic application.

Though reported to be effective against both gram negative and gram-positive bacteria, ε-PL has not yet been tested on multi drug resistant bacteria especially MRSA. The focus of this study was, therefore, to evaluate the antimicrobial action of ε-PL on *Staphylococcus aureus* and MRSA. We were particularly interested to investigate if ε-PL would improve the efficacy of methicillin even on MRSA isolates and reverse the MRSA phenotype to qualify as an adjuvant drug to treat MRSA infections.

Material and Methods

Bacterial strains and reagents

The bacteria were stored in Mueller Hinton Broth supplemented with (20% v/v) glycerol at -20˚C. For experiments, MRSA type strain ATCC 43300 and a clinical MRSA isolate B-1367 were grown in MHB at 37˚C for 18 h. ε-PL (Cat No. FP-155) was purchased from Bimal Pharma Private Limited. Mueller Hinton Broth (MHB, Cat No. M391-100G), Mueller Hinton Agar (MHA, Cat No. M173-500G), Cefoxitin discs (Cat. No. SD 041) and Oxacillin discs (Cat. No. SD 088) were procured from HiMedia. Dimethyl sulphoxide (DMSO, Sigma Aldrich, Cat No. 20621LM500), Ethanol (Hayman group Limited, Cat No. F203640) were also used in this study.

Determination of Antimicrobial activity and Minimum Inhibitory Concentration (MIC)

Antimicrobial activity of ε-PL was investigated initially, and approximate MIC was determined by disc diffusion assay on MHA plates. Single colonies of the bacteria from MHA plates were inoculated into 5 ml of MHB in triplicate and grown with orbital shaking (180 rpm). The bacterial cultures of biomass 0.5 McFarland (0.08 to 0.10 A610 nm) were spread evenly using a sterile cotton swab over the entire surface of the MHA plates [21]. Sterilized Whatman filter paper discs (6 mm diameter) containing 5 µg/ ml to 100 µg/ ml of ε-PL were positioned on the agar surface. Discs coated with DMSO were used as negative control. The plates were incubated for 24 h at 37°C. The MIC was described as the minimum concentration of the drug which inhibited the growth of *S. aureus*.

Determination of Minimum Inhibitory Concentration (MIC) by broth micro dilution method

The MIC of ε-PL against the *S. aureus* isolates were determined using the CLSI broth micro dilution method [22]. Each bacterial isolate was sub-cultured and incubated overnight prior to the experiment. The bacterial suspension was adjusted to 0.5 McFarland (0.08 to 0.10 A610 nm). The assay was performed in 96 well microtiter plate. Each well contained 200 µL of broth with ε-PL concentration ranging from 0.625 µg/ ml to 75 µg/ ml. All the plates were incubated overnight at 37°C. The MIC values were calculated for the tested MRSA and MSSA isolates.

In vitro assessment of anti MRSA activity of ε PL

The effect of ε-PL on MRSA cells was tested in MHB which contained sub-MIC concentration (5 µg /ml) of ε-PL. The cultures were incubated at 37°C for 24 h with orbital shaking (160 rpm). After incubation, 100 µl of this culture was used to inoculate 5 ml of MHB for every passage in triplicate and the whole process was repeated for 8 passages. Aliquots of each passage were examined to assess the effect of ε-PL. The control cultures received equal volume of DMSO. Aliquots from each passage were subjected to drug susceptibility test by disc diffusion against Cefoxitin and Oxacillin, *mecA* gene detection by PCR and morphological analysis by SEM [23] to determine the effect of ε-PL on the cells [Fig-1].

MRSA Phenotyping

Antibiotic susceptibility test was performed by disc diffusion method. Zone diameters of Cefoxitin (30 µg) and Oxacillin (1 µg) were measured after each passage on the MHA plates.

Genotyping by *mecA* PCR)

The *mecA* gene detection for the confirmation of methicillin resistance and MRSA genotype was done using the following primers [24],
mecA P4 TCCAGATTACAACTTCACCAGG
mecA P7 CCACTTCATATCTTGTAACG
under the following conditions:
Amplification of the *mecA* gene was performed in 25 µl reaction volume. The reaction mixture comprised of 2.5 µl of 10X PCR buffer, 1.5 µl of 25 mM $MgCl_2$, 2 µl of 10 mM dNTPs, 2 µl of 10 pmol each of *mecA* forward and reverse primers, 0.2 µl of Kappa Taq DNA polymerase (5 U/ µl), 1 µl of template DNA in a final volume of 25 µl with molecular biology grade water. Initial denaturation of template DNA was done for 5 min at 94°C, followed by 35 amplification cycles, each consisting of: 1 min denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C. The final extension was 7 min at 72°C (G-Storm GT-1197). The amplicons (162 bp) were resolved in agarose (2%) gel by electrophoresis and analyzed.

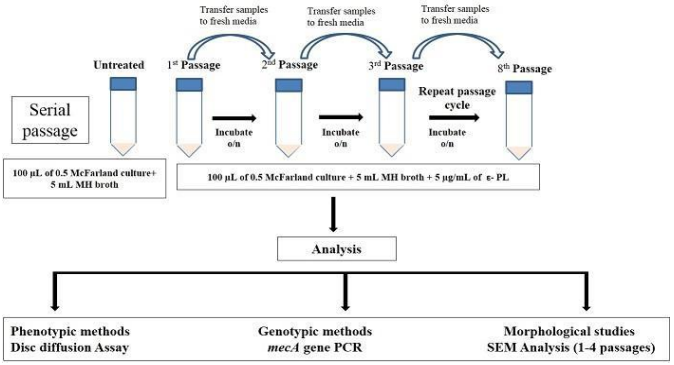

Fig-1 Sequential passage experiment to evaluate the anti-MRSA activity of ε-PL

Morphological study (SEM analysis)

The *S. aureus* cells were examined by Scanning Electron Microscopy (SEM) as described [25]. Briefly *S. aureus* cells were collected (108 CFU/ml) at '0' time and after each passage by centrifugation (1500 g, 5 min). The bacterial pellets were fixed overnight at 4˚C in 2.5% (v/v) glutaraldehyde containing 0.2 M Sodium Cacodylate Buffer (SCB pH: 7.2). The pellets were washed thrice using 0.1 M SCB buffer at 30 min interval to remove the excess fixative and dehydrated by immersing them stepwise in a gradient of (30%, 50%, 70%, 80%, 90% and 100% v/v) of ethanol (Ethyl alcohol 100%: Hayman Group Ltd., UK F204325) for 15 min. The *S. aureus* cells were then sputter sprayed with gold (Model: E-1010 Hitachi Japan) and subsequently visualized at different magnifications under a scanning electron microscope (SEM) (S3400N Hitachi Japan) at 15 KV and high vacuum (10-7 Torr) and documented.

Statistical analysis

Duplicate samples were used for each passage and each experiment was performed three times. For each treated and untreated, the data from independent replicate experiments were pooled and analyzed.

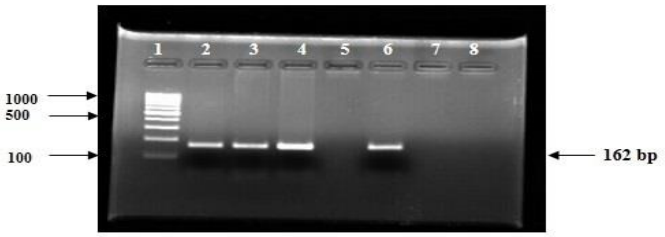

Fig-2 Demonstration of the presence *mecA* gene by PCR in clinical isolates of *S. aureus* after each passage

Results

Determination of MIC values

The minimal inhibitory concentration of ε-PL was determined by disc diffusion method using a concentration range of 5 µg/ ml to 100 µg /ml. The MIC of ε PL on MSSA isolates was 12.5 µg /ml and 20 µg/ml for MRSA isolates. Using the broth micro dilution method, the MIC of ε- PL on both MSSA and MRSA isolates was determined to be 6.25 µg/ ml.

Fig-3 Effect of ε-Poly Lysine on sensitivity to Cefoxitin (30 µg) and Oxacillin (1 µg) in MRSA (ATCC 43300): A) Untreated B) 1st Passage C) 2nd Passage D) 3rd Passage E) 4th Passage

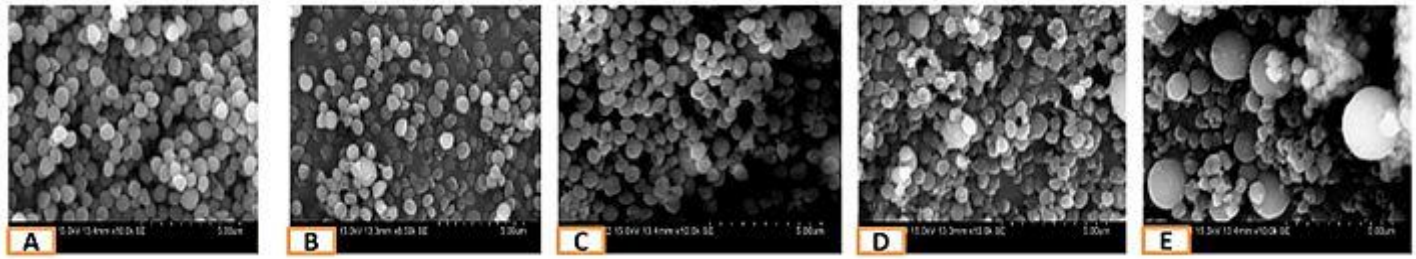

Fig-4 SEM observations on effect of ε-Poly Lysine (sub-MIC-5 µg/ ml) on morphology of MRSA (ATCC 43300) by SEM A) Untreated B) 1st Passage C) 2nd Passage D) 3rd Passage E) 4th Passage

Confirmation of methicillin resistance by *mecA* PCR

The presence of *mecA* gene, known to be a genetic marker for methicillin resistance was investigated in clinical isolates of *S. aureus* and after each passage by PCR assay and agarose gel electrophoresis [Fig-2].

ε PL restores Cefoxitin sensitivity and reverses the MRSA phenotype

The anti-MRSA activity of ε PL is remarkable as the sensitivity to methicillin increases with every passage and the methicillin resistance appears to be almost completely reversible in these cells. Bacterial cultures were subjected to Cefoxitin susceptibility test, *mecA* gene detection and SEM analysis after every passage.

These results showed that ε PL caused obvious destruction of the cell wall/cell membrane of *S. aureus* thereby altering the permeability. After four passages, the zone diameter of the Cefoxitin inhibition increased from 0 mm zone (1st passage) to 18 mm after four passages [Fig-3AE]. The presence of *mecA* gene in the cells after each passage suggests that the reversal of MRSA to MSSA is apparently a phenotypic change and not due to any genetic alteration consequent to exposure to ε-PL. SEM was used to visualize the morphological changes of *S. aureus* cultures exposed to ε- PL and remarkable morphological changes were noticed after exposure to ε-PL compared to control cells. As shown in [Fig-3A], the untreated cells appeared as typical cocci, grape like structure with smooth surface. However, some cells collapsed after the first passage [Fig-4B]. More cells collapsed in 2nd passage [Fig-4C]. The cells appeared distorted: elongated with indentations, enlarged, collapsed, and cell membrane severely disrupted indicating plasmolysis in 3rd [Fig-4D] and 4th [Fig-4E] passages.

Discussion

The occurrence of MRSA as a profile invariably underlines a MDR phenotype in the clinical isolates of *S. aureus*. This profile undermines the clinical efficacy of antibiotics and leaves limited options for the treatment of such infections. Almost all existing antibiotics were developed on shared/similar technology platforms against a handful of microbial targets. With a poor rate of success in antibiotic discovery and a dismal approval rate currently seen, alternative and unconventional strategies are required in new drug discovery to manage this serious global crisis. Repurposing approved chemicals and drugs as potential antibiotic adjuvants is currently gaining traction for human health especially in the management of Anti-Microbial Resistance (AMR). This strategy offers perhaps one of the cheapest and fastest routes for a drug which, if found effective, likely to get the approval for treating AMR infections. The World Health Organization (WHO) already published a Global Action Plan in 2015 [26]. One of the five objectives of this multifaceted plan is to increase the investment in the development of new antimicrobials with novel mechanisms of action. Further, MRSA has been listed in the high priority among the pathogens which urgently requires development of new drugs [27, 28]. There has been virtually little effort to engage the MRSA to reverse its phenotype. Since methicillin has long been an effective and extremely affordable broad-spectrum antibiotic, it makes sense to reclaim its place in the antibiotics' domain. Jain et al. reported that EDTA could reverse MRSA to MSSA, and this might have therapeutic potential [29]. However, EDTA would have undesirable side effects when administered systemically. A new compound reportedly having dual mechanism of action on both gram negative and gram-positive bacteria was evaluated and reported to be effective on MRSA persisters [30]. An ideal antibiotic would be the one against which resistance does not develop easily while retaining its prime function of being broad spectrum antibiotic. Since most of the antibiotics have been designed and developed on similar technology platform with a shared mechanism of action, resistance to any one of these antibiotics impacts the efficacy of several others [31]. This is very unfortunate as the clinician is facing "starvation amidst plenty" and is confronted by multitude of MDR infections. Compounds which enhance the susceptibility of the microbe to existing antibiotics also need to be investigated as candidates for co-therapy as this would potentially reduce the development of drug resistance while enhancing the effectiveness of therapy. Some natural products like ellagic, tannic acids [32] and trans-cinnamaldehyde and Eugenol [33] have been reported to have this potential. Against this background our study assumes significance as it reports apparently for the first time that it is possible to re-sensitize the bacteria to a multitude of beta lactam antibiotics which have been rendered in-effective due to genetic alterations in the bacteria.

ε PL is widely used as a food preservative and in this study it was able to re-sensitize the MRSA to Cefoxitin and Oxacillin when exposed to sub-MIC concentrations of ε-PL. Growth inhibition zones of Cefoxitin and Oxacillin increased from zero at 1st passage (day 1) to 18 mm for Cefoxitin and 14 mm for oxacillin at 4th passage (day 4), confirming the reversal of clinical isolates of MRSA to MSSA. In our study the sensitivity zone did not increase beyond 18 mm even at 8th passage. This could be because we used sub-MIC (5 µg/ml) of ε- PL and the zone would increase at a higher concentration. ε PL could be tried even at MIC for treating MRSA in adjuvant therapy or to reduce development of MRSA in co-therapy with methicillin. The exact mechanism by which ε- PL reverses the resistance to methicillin needs to be investigated further. It is possible that ε- PL acts through a mechanism independent of penicillin binding protein whereby it inhibits the growth of MRSA. Morphological (SEM images) changes observed provide some evidence for this hypothesis. We have demonstrated in this study that there is no plasmid curing as *mecA* genotype was unchanged when the phenotype reversed from MRSA to MSSA. It is well known that ε-PL selectively interferes and inhibits cell wall synthesis [34, 23]. Our results also indicate that ε-PL apparently altered the membrane permeability thus allowing the entry of Cefoxitin and Methicillin into the cells thereby inhibiting the growth of MRSA. The re-sensitization of MRSA has been made possible apparently by facilitating entry Cefoxitin or Methicillin into young rapidly multiplying cells and binding to the unmodified PBPs. Cells were examined after each passage and definite changes in the cell morphology of the cells were noticed under SEM.

Similar effects were reported earlier when MRSA cells were exposed to EDTA [29]. Our results highlight there is scope for re-purposing ε PL and developing it as adjunct drug for treating MRSA infections. Recent studies have shown that ε PL can serve as an effective drug carrier and a viable drug delivery system [35, 36].

Conclusion

Antimicrobial activity is effected through non-receptor-based mechanisms like enhancing the cell permeability, ε PL could not only enable methicillin to act on MRSA but also would reduce the chances of development of MRSA when administered early during antibiotic therapy. Results of our study also reinforces the reports that ε PL which is an approved food preservative, also has the potential to be an effective adjuvant drug for treating MDR in general and MRSA in particular.

Application of research: Study of ε-polylysine hold promise as an adjuvant drug to treat methicillin resistant *S. aureus*

Research Category: Antimicrobial activity

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Study area / Sample Collection: National Institute of Nutrition, Hyderabad, 500007

Strain name: *Staphylococcus aureus*

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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