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Effect of Enhanced ROS Production on Methicillin Resistant Staphylococcus Aureus (MRSA)

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Abstract: *Methicillin Resistant Staphylococcuus aureus, (MRSA) is one of the greatly feared strains as it has become resistant to most β- lactam antibiotics due to certain protective enzymes. [1,2]. Alternatively, generation of Reactive Oxygen Species (ROS) creates oxidative stress which can be lethal to such cells. Recently, number of chemicals and dyes found to stimulate the production of ROS in bacterial cells [3,4,9]. Malachite green (MG) is an example of nonflourescent and photosensitizer dye that can be targeted to a particular cellular site and generates singlet oxygen radical i.e., ROS which also helps in the sensitization of antibiotic resistant bacteria. In this study different concentration of Malachite green were used (100-1000 ng/ ml) to find the sensitivity of MRSA. 760 ng/ ml of MG was found to be lethal against MRSA.*

Keywords: *OH radical, Oxidative stress, Malachite green, Methicillin Resistant Staphylococcus aureus, Reactive Oxygen Species.*

I. Introduction

Staphylococcus aureus (*S. aureus*), also known as "golden staph, is one of the five most common causes of nosocomial infections and postsurgical wound infections. MRSA is a leading threat to public health, causing more death in USA. Today more than 90% of *S. aureus* are resistant to penicillin, methicillin and, to some extent, to many commonly used antibiotics. Despite this, MRSA generally remained an uncommon finding, even in hospital settings, until the 1990s, when there was an explosion in MRSA prevalence in hospitals, where it is now endemic[2].

Staphylococcal resistance to penicillin is mediated by penicillinase enzyme which cleaves the β-lactam ring of the penicillin molecule, rendering the antibiotic ineffective. Penicillinase-resistant β-lactam antibiotics, such as methicillin, nafcillin, oxacillin, cloxacillin, dicloxacillin, and flucloxacillin, are able to resist degradation by staphylococcal penicillinase. Resistance to methicillin is mediated via the mec operon, part of the staphylococcal cassette chromosome mec (SCCmec). Resistance is conferred by the mecA gene, which codes for an altered penicillin-binding protein (PBP2a or PBP2') that has a lower affinity for binding β-lactam antibiotics. Resistance to these antibiotics has also led to the use of new, broad-spectrum anti-Gram-positive antibiotics, such as linezolid, because of its availability as an oral drug. First-line treatment for serious invasive infections due to MRSA is currently glycopeptide antibiotics (vancomycin and teicoplanin) which has many problems associated with it [1, 2]. Generation of ROS can serve more safer and novel way to answer this problem asrecent studies have shown that by forcing bacteria to produce large amount of Reactive Oxygen Species (ROS), can either kill bacteria or make them susceptible to antibiotics. Small amount of ROS don't hurt them because of certain protective enzymes within the bacteria but too much production of the ROS can lead to oxidative stress [3, 5].

Recently, a number of antibiotics have been demonstrated to stimulate the production of Reactive Oxygen Species (ROS) in bacterial cells. Reactive oxygen species are reactive by-products formed by the partial reduction of molecular oxygen[6] The enzymatic defense system against ROS comprises of specific enzymes, like superoxide dismutase, catalase, and peroxidase, which decrease the steady-state level of reactive oxygen[3,4]. Redox cycling of various chemical substance including some antibiotics, affect the ROS produced by cells during oxidation process [6, 7]. Other example of such chemicals can be dyes. Malachite green is a nonfluorescent photosensitizer dye that can be targeted to particular bacterial cellular site and acts on their iron acquisition process thereby making bacteria susceptible to targeted therapy [11].

When bacteria were treated with malachite green, out of 207 genes, 167 genes belongs to oxidative stress, virulence, carbohydrate metabolism, heat shock protein were upregulated while 37 genes involved in iron acquisition, filamentous growth, mitochondrial respiration were found to be down regulated. Malachite green triggered depletion of intracellular iron pools and enhanced ROS levels[12,13]. It is mainly used in Photodynamic therapy and generates singlet oxygen radical and acts as the main photodegradation mechanism due to the OH radical and electron transfer reaction [8,10]. Toxic effects of the dye (MG and LMG) have been tested on mice and rats in 28-day administration and it was found that the median lethal dose for malachite green in rats is 275 mg/ ml [14].

II. Material and Methods

The colonies of golden coloured pigment of *S. aureus* were isolated from the lab culture stock on Nutrient Agar plate by Streak plate technique and was confirmed by doing biochemicals* . To check the sensitivity of the strain to various antibiotics, 18 hr old of *S. aureus* was used to swab the sterile Nutrient agar plates with the help of sterile cotton swabs on which sterile antibiotic discs of methicillin (5mcg), penicillin (10 mcg), and an octodisc (Himedia) were placed and then plates are incubated at 37^0C for 24hrs.

* As per Bergy's Manual of Systemic Bacteriology vol.

To check for the ROS generation using Malachite Green (MG) dye, the minimum inhibitory concentration (MIC) against *S. aureus* was checked in the range of 100-1000 ng/ml of MG. A tube free from Malachite green serves as growth control. Each of the tubes is then inoculated with a standardized bacterial suspension and incubated at 37^0C for 24 hrs. At the end of the incubation period, the tubes were visually examined for turbidity. At this point, to distinguish between the bactericidal or bacteriostatic effect of Malachite green and to determine the effective concentration of the same against MRSA, after performing the MIC, 0.1 ml from each of these tubes were inoculated in 5 ml of Sterile Nutrient broth (NB) and again incubated at 37^0C for 24 hr the tubes were checked for turbidity[7,9].

To confirm the result of the MIC, iodonitrotetrazolium chloride (INT) assay was performed wherein 0.5 ml of 0.2% INT dye was added in each MIC tubes. INT dye is pale yellow in color, but when its react with superoxide radical, the color is changes from pale yellow to dark pink color. This colored compound is nothing but the Formazan derivatives. Reactive Oxygen Species i.e., Superoxide radical are responsible for a color change from INT into Formazan. All the tubes were kept at ambient temperature for an hour (i.e., 2-3hrs) to check the colour change in the tubes. After color change, the reading has been taken within 40 mins in UV- vis Spectrophotometer (WPA Biowave II) at 490 nm [13].

Note: All media used are from Hi Media and all chemicals are from Loba Chemi unless otherwise stated.

III. Results and Discussion

The antibiotic sensitivity studies indicated that the above bacterial culture was Penicillin Resistant *Staphylococcus aureus* (PRSA) and Methicillin Resistant *Staphylococcus aureus* (MRSA)#.

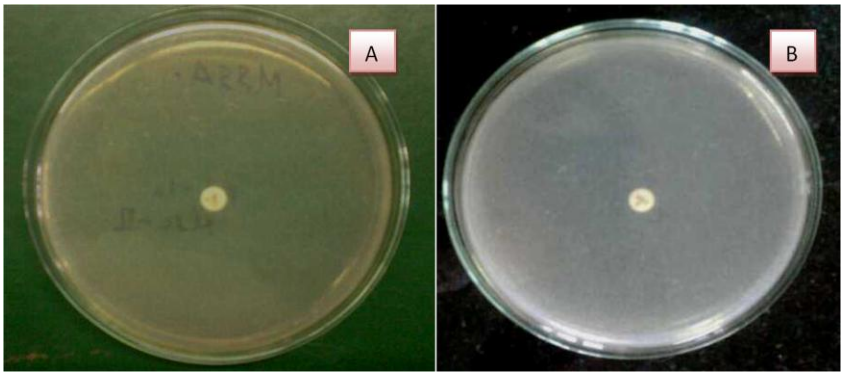

Fig I A: Methicillin Resistant S. aureus B: Penicillin Resistant S. aureus

As per Kirby Bauer Test for Antibiotic Susceptibility

Nanomolar concentration of the dye was used in investigating Minimum Inhibitory concentration (MIC) against the culture under study which was found to be between **760 ng/ml -770 ng/ ml**. This concentration was confirmed to be bactericidal by presence of growth in the NB tube.

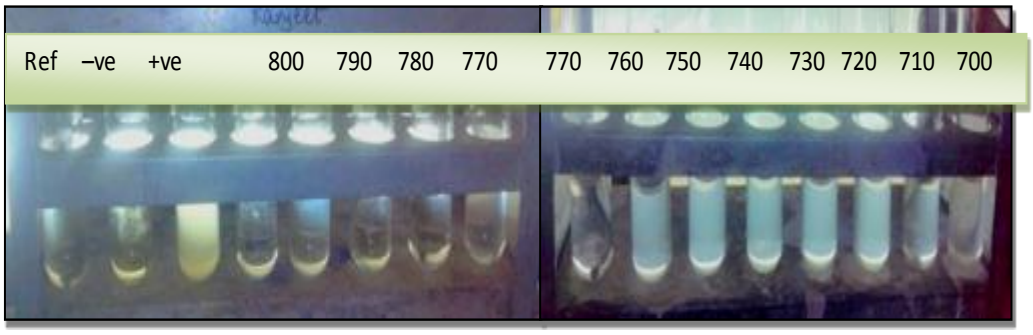

Fig II MIC test of Malachite Green dye

The zone of inhibition as per AST of MRSA after 760 ng/ ml conc of Malachite green treatment was found to be **1.9cm** which was more than the same without treatment. This indicates that malachite green treatment is making the strain sensitive to antibiotic.

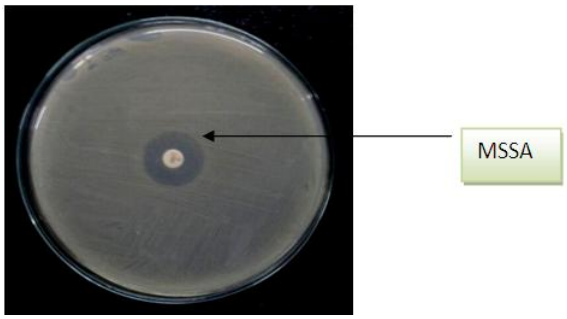

Fig III AST after MG treatment

In INT tube assay, pink color solution was observed till seventh tube i.e., 760 ng/ ml concentration containing tube, which suggests that ROS was successfully generated in the bacterial cell. The color change was observed only in the tubes that contains malachite green dye but not in its absence as no color change was seen in the control tubes i.e., absence of Malachite green dye. This indicates that the Malachite green dye was able to induce ROS in the *S. aureus*.

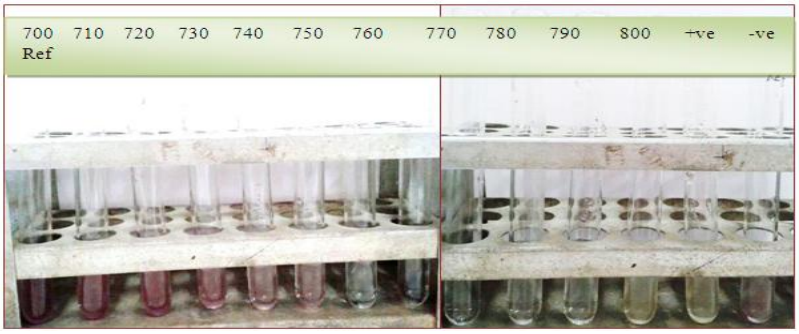

Fig IV INT assay with Malachite green treatment

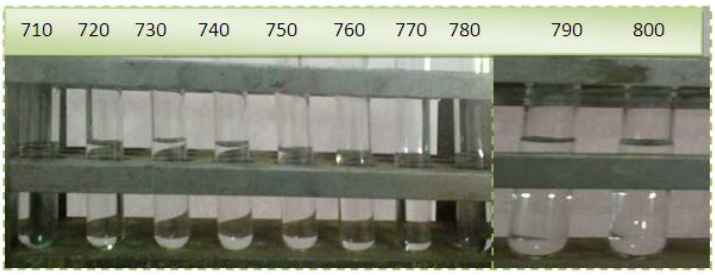

Fig V INT assay without Malachite green treatment

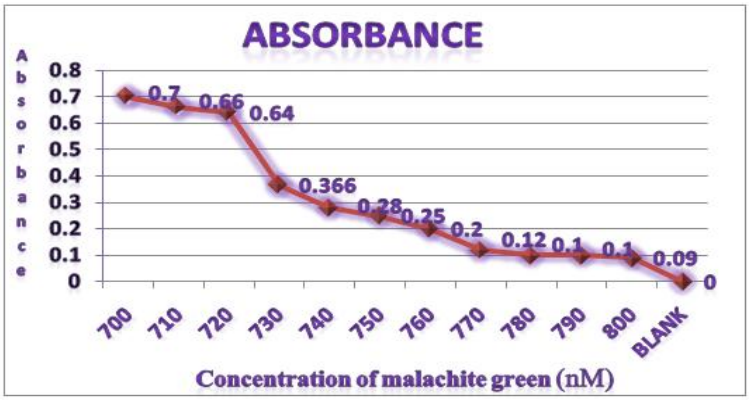

Fig VI Effect of Malachite green dye on MRSA with INT Dye using UV spectrophotometer

The bacteriocidal effect of Malachite green dye was observed in the range of 760 ng/ ml- 770 ng/ ml as no turbidity was seen above that concentration which suggests that the dye acts as bacteriostatic agent against MRSA but not as bacteriostatic as complete inhibition of MRSA takes place.

The growth of *S. aureus* was completely Inhibited at 760 ng/ ml concentration of Malachite green dye. This bacterial inhibition is also confirmed by testing MDR strain of *S .aureus* and it is observed that the *S aureus* which are resistant to Ceftaxime, Cephalexin, Amoxyclav, Norfloxacin, Gentamycin, Co- trimazole, Ciprofloxacin, Chloramphenicol and Methicillin shown sensitivity in presence of Malachite green dye at that concentration. As S. aureus was inactive in the concentration of 760 ng/ ml of Malachite green. So by that concentration the nosocomial infections caused by MDR strain can be treated by the use of Malachite green caused by multi drug resistant strain of bacteria. Hence, by performing AST assay, it is observed that the *S. aureus* which are resistant to Ceftaxime, Cephalexin, Amoxyclav, Norfloxacin, Gentamycin, Co- trimazaole, Ciprofloxacin, Chloramphenicol and Methicillin starts showing sensitivity on treatment with 760 ng/ ml conc of Malachite green.

Table I Antibiotic Susceptibility Test of MDR strain[#]

Name of the antibiotic and concentration/disc	Observation Zone of inhibition (cm)	
	AST without Malachite green (760 ng/ml) treatment	AST with Malachite green (760 ng/ ml) treatment
Ceftaxime (30 mcg)	Resistant	Sensitive (25mm)
Cephalexin (30 mcg)	Resistant	Sensitive (21mm)
Amoxyclav (10 mcg)	Resistant	Sensitive(26mm)
Gentamycin (10 mcg)	Resistant	Sensitive (18mm)
Co-trimazole (25 mcg)	Resistant	Sensitive(15mm)
Ciprofloxacin (10 mcg)	Resistant	Sensitive (23mm)
Chloramphenicol (30 mcg)	Resistant	Sensitive(19mm)
Norfloxacin (10 mcg)	Resistant	Sensitive(18 mm)
Methicillin (5mcg)	Resistant	Sensitive(19mm)

[##] As per Himedia Octodisc

IV. Conclusion

By simply increasing the concentration of ROS in the MRSA with the help of Malachite green dye, sensitizes MRSA to Methicillin. This study is an attempt to provide a simple cost effective method to answer the problem of increasing health concern due to various drug resistant microorganisms.

Future prospects

Hand washing is one of the preventive measures of avoiding nosocomial infection so by adding malachite green in hand washes we can simply reduce the risk of MRSA and other nosocomial infection in the hospital area. Toxicity study should be carried out before applying it for the routine use. We can also add MG in disinfectants and detergents which are frequently used in the cleaning of hospital floors, bed sheets or blankets.

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