Electric stimulations mediated beta lactam resistance reversal and correlation with growth dynamics of community acquired methicillin resistant \textit{Staphylococcus aureus}

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The community associated methicillin resistant \textit{Staphylococcus aureus} (CA-MRSA) is a serious issue of public health. Here, we conducted an experimental approach to determine: (i) the optimal significant stimulation range of electrical current for effective checking of CA-MRSA growth; (ii) the effect of electrical stimulations on methicillin susceptibility and possible beta lactam resistance reversal; and (iii) the variation in the level of ATP as function of exposure to electric current. An 8 chambered electrical system was developed for DC flow in control and test sets, with and without drug (oxacillin 4 mg/ml). Measurement of growth by CFU/ml and spectrometry, susceptibility and ATP levels were calculated and interpreted. Linear pattern in reduction of ATP was observed with respect to the intensity of electric current (EC) and an enhanced inhibitory effect was explicit with 1000 microampere (μA) with 30 min exposure. At 4000 μA exposure to DC at 180 min and in combination of drug (μA+D), the growth of CA-MRSA was substantially checked to 0.23 absorbance in comparison to current without drug and the effect of DC electrical current to the culture showed that 10 μA, 100 μA and 4000 μA current exposure in combination of oxacillin (μA+D), markedly reduced the CFU to an average of 256.4. ATP level was linearly reduced with exposure to EC.

The widespread emergence of community acquired methicillin resistant \textit{Staphylococcus aureus} (CA-MRSA) across the globe since last decade has posed a serious threat to the public health\textsuperscript{1,2}. Its ability to form biofilms in the environmental surfaces and substrates further augments the problem. Electrical stimulations have been in practice for healing purpose by different mechanisms\textsuperscript{3,4}. Investigations on the effect of electrical current (EC) on pathogenic bacteria with different stimulation types are not uncommon\textsuperscript{5,8} and detachment of biofilms from the steel surfaces\textsuperscript{9}. Swimming pools and occasionally natural lakes get contaminated with potential pathogens by various sources and behave like reservoir of pathogens. CA-MRSA and similar gram positive bacteria have property to produce exopolysaccharides which in turn mediates development of biofilms, the micro environment of microbes. Besides the ability of EC to detach this biofilm from the surface, \textit{in vitro} attempts have been made to document the fact that it has bacteriostatic property for CA-MRSA. It further enhances the antimicrobial efficiency of quinolones, aminoglycosides on gram positive and negative pathogenic bacteria by bioelectric effect\textsuperscript{10-14}. However, data are limited on the effect of electrical current of different strength on drug efficiency and growth dynamics of the bacteria simultaneously with the assessment of ability of ATP production in real time.

In the present study, we explored: (i) the optimal clinically significant stimulation range of electrical current to significantly reduce growth of CA-MRSA strain; (ii) the effect of electrical stimulations on methicillin susceptibility of same strain and possible beta lactam resistance reversal; and (iii) the variation in the level of ATP during electrical exposure.

\textbf{Materials and Methods}

\textit{Bacterial strain—Community acquired methicillin resistant \textit{Staphylococcus aureus}} with SCCmec A gene type IV with positively producing penicillin binding protein (PBP2a) was obtained from the culture preserved in Laboratory of Microbiology from a community study\textsuperscript{15}. Bacteria were grown in a trypticase soy broth (Himedia). These bacteria were isolated from the local rural population of Himalayan region, Uttarakhand, India.
mecA gene detection by PCR—The staphylococcal DNA was isolated using phenol: chloroform extraction method (Sambrook et al., 1989). Primers used for this study were 5'-GTAGAAATGACTGACGTCCGA TAA-3' and 5'-CCAATTCCACATTGGTTTCTAA-3' as described earlier16.

The PCR conditions used for cycling were earlier denatured at 94°C for 4 min; 30 cycles of amplification (denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 30 s); and final extension at 72°C for 2 min. Gel bands were obtained by loading 10 μl of the PCR product in 1.8% agarose gel in TBE containing 0.5 μl/ml of ethidium bromide and visualized by UV transilluminator at 290 nm. DNA fragments of 310 bp were corresponded to the mecA gene.

Multiplex PCR was done for SCCmec typing as described earlier17. ATCC 33591 (mec A +ve) and ATCC 25923 (mec A –ve) were used as control strain.

Growth of bacteria—CA-MRSA was grown to exponential growth phase in broth medium at 37°C till the concentration of bacteria reached to the 3-4 on the McFarland Scale18. The growth medium was subjected to spectrometer measurement at a wavelength of 580 nm and a ratio of bacterial density and broth absorption was determined.

Adenosine triphosphate (ATP) concentration of viable cells was determined by reacting it with a luminous reagent. An ATP analyzer, Model AF-100 (Toa Electronics Ltd., Tokyo) was used to estimate the ATP activity. The pre existing or free ATP present in the sample drawn from stock medium was decomposed with apyrase which acted as ATPase.

Instrumental setup for electrical stimulations—The 8-channeled polycarbonate study chamber (LWH: 92×32×40 mm) were designed and developed in-house. Four such control and 4 test chambers were operated in triplicate simultaneously with fissure openings at 4 cm beneath the top to add fresh medium continuously with and without beta lactam drug methicillin separately (Fig. 1). All the 4 test channels were connected to a specified electric current generator with a particular DC supply ranging 10-4000 μA. A pulse interval of 2 s was applied after every 5 s of current supplied to avoid the generation of excessive heat, damage to the membrane of cells and froth production in media. Two ports were designed in the lid of apparatus for electrodes anode and cathode vertically submerged in the medium and at least 2 cm apart from each other.

The electrodes used were 60 mm long stainless steel or graphite cylinders of 1.5 mm in diameter as used in earlier studies19. An electric current of 10, 100, 1000 and 4000 μA DC was continuously passed through the test chambers with pause of 2 s after every 5 s. One ml of medium was taken from each chamber after every 15, 30, 45, 60, 75 and 90 min and was adjusted to 0.5 Mcfarland of turbidity. About 10 μl of this was subjected to antimicrobial susceptibility testing for methicillin by Kirby Bauer method in strict accordance with CLSI guidelines 2010 and expression pattern of SCCmec A gene by PCR. A spectrometric measurement of each test chamber was analyzed at every 20 min to track the effect of electric current on growth dynamics.

Statistical analysis—A Wilcoxon signed ranks test and ANOVA with SPSS 16.0 package at P<0.05 was performed to determine a possible main effect for (a) beta lactam susceptibility for electric current treated test samples and untreated ones, and (b) variation, if any, in the growth pattern of treated and non treated samples.

Results
McFarland scale was calculated at a frequency of 580 nm for CA-MRSA strain for each of the 8 chambers and respective triplicates. A calibration curve was then obtained by plotting absorbance against set concentrations of bacteria. Correlation was 0.96 which computed an R² of 0.954. Further study was conducted at the given calibration on the McFarland scale.

Fig. 1—Experimental unit of 8 channeled system setup. The unit was replicated 8 times. Of which, 4 were connected to DC supply as shown above while rest were controls.
Inhibition zone of CA-MRSA (diameter in mm) is given in Table 1 for two variables: duration of exposure of electrical current and strength. Each entry represents an average of 3 culture plates. An enhanced inhibitory effect was observed with 1000 μA with 30 min exposure. However, 10 μA DC current showed the same effect after 60 min of exposure. DC 100 μA was not found significant in terms of inhibitory effect at any time duration. At 4000 μA, results varied on successive days, and hence were not taken into consideration for ANOVA.

Changes in absorbances during the bacterial growth with and without administration of oxacillin at the same concentration of 4 mg/mL are shown in Fig. 2. At 4000 μA exposure to DC at 180 min and in combination of drug (μA+D), the growth of CA-MRSA was substantially checked to 0.23 absorbance in comparison to current without drug (1.23) (μA–D) and control (1.32) which was statistically significant (P <0.05) (Fig. 2d). At 10 μA no significant difference was observed and all the 3 groups (control, μA+D and μA–D) followed almost same growth trend (Fig. 2a). At 100 μA with drug at 300 min absorbance dropped to 2.08 in comparison to group without drug (μA–D) that showed value of 3.04 and the control group, 3.93 OD. At 1000 μA and at 180 min growth was 0.4 significantly less (P <0.05) than other two groups.

The study of the bactericidal or bacteriostatic effect of DC electrical current to the culture showed that 10, 100 and 4000 μA current exposure in combination of oxacillin (μA+D) as shown in Fig. 3, markedly reduced the colony forming units to an average of 256.4 which was statistically significant (P <0.01) compared to μA–D group.

ATP levels were reduced consistently with duration of exposure and the current strength. A decline was found to be statistically significant with respect to time and strength (P <0.005) as shown in table 2. Molecular studies for detection of SCCmec gene IV for all the test and control strain was found positive with 180–220 kb mec gene. However, it needs to be ascertained that the consistent reduction in ATP by electric stimulations is solely or partly responsible for reduced growth and increased zone of inhibition.

**Discussion**

Disposal of unsterilized swabbing, samples, bandages and surgical instruments to the environment e.g., soil or rivers and lakes leads to the emergence and spread of CA-MRSA to the healthy population. Colonization with MRSA imposes a great risk for
development of infection\textsuperscript{20}. By avoiding the chance of developing drug resistance, application of weak electric current to the artificial water storage systems and swimming pools may be of immense value in future. The present study has demonstrated that the exposure duration and strength of electric field had notable effects on CA-MRSA growth and susceptibility to methicillin. A maximum inhibitory effect was observed with 1000 μA for 30 min and 10 μA for 60 min which was presumably due to the gradual and more stable impairment to the membrane constituents. The inhibitory effects were not at all impressive at 100 μA and 4000 μA results of ANOVA depicts that the inhibitory effect was primarily due to the strength of electrical current applied rather than the duration of exposure, although variance in inhibitory effect was seen at 4000 μA. Although enhanced zone of inhibition was observable on 100 μA and 4000 μA sets at 60 min which makes it in agreement with 10 μA set, and hence it was thought that the effect of electric current on inhibition is a slow effect rather than abrupt. Merriman et al.\textsuperscript{21} had also reported an inhibitory effect at 500 μA for 1 h and our results are partlyagreement with their findings i.e., the effect was at weak current and prolonged duration i.e., 1 to 2 h.

Control plates were unaffected and read the same zone diameter as no electric current supplied to each corresponding broth sets. It indicates that the electric current damaged the cell wall of the bacteria by interfering in their cell wall constituents. Gram positive bacteria are more resistant to electric stimulations than gram negative bacteria and hence electric current has bacteriostatic effect on \textit{S. aureus} rather than bactericidal\textsuperscript{22}. We further propose to use different electrode types to investigate the possibilities of occurrence of inhibition as solely or partly due to the electrodes used in this study.
Although to avoid such margins of error and cost effectiveness, we performed the experiment using stainless steel as used in earlier studies

Effect of electric stimulations on growth dynamics were evident as at 4000μA exposure to direct current at 180 min with oxacillin 4 mg/mL (μA+D) where the OD measured was 0.23 and showed statistical significance at $P < 0.03$ with control and μA–D groups. Possibly, the current supply facilitated the drug administration. Further, no such significant differences were observed at weak electric current i.e., at 10 μA. It emphasizes the fact that slightly higher voltage is required for drug action. Hence, electric stimulations acted on CA-MRSA by two possible ways: (a) bacteriostatic; and (b) facilitated administration of oxacillin. However, charge on antibiotic is associated with the bioelectric effect and the polarity of electrodes may be a factor to focus on as done by other study group.

Taking variation in two major physical factors, duration of exposure to electrical current and its strength among several other factors like type of bacteria i.e., gram positive MRSA, inoculum size, pH and temperature, etc., we analyzed the efficiency of bioelectric effect by measuring CFU/ml. At 10, 100 and 4000 μA current exposure with oxacillin (μA+D), the number of colonies showed a marked reduction (Fig. 3a-d) compared to the control (10 μA–D). Additionally, $R^2$ was calculated for the control strain (Fig. 4). This signifies the role of electric stimulation types in growth inhibition of S. aureus. Our results are in agreement with the findings of results of other research groups who used static electric fields in their studies. In our study, we have proposed that at higher electric current the bacteriostatic effect may solely be due to the current supplied and not due to the antibiotic. This effect of growth inhibition have been well explained by other study groups earlier where they used static electric current without antibiotics. One way ANOVA showed the increasing strength of electrical current to be the main reason for drop in CFU than exposure duration. Here, in our study, we have demonstrated that administration of drug and simultaneous application of electric current ranging 10-100 μA result in better growth inhibition of CA-MRSA in short time (30-60 min) rather than the electric current alone.

Investigation on the effect of electric stimulation types on ATP levels was done in real time. It showed significant reduction of ATP level proportional to the duration of exposure and strength compared to the electric current untreated control sets (no current and no drug D). The nonparametric wilcoxon signed ranks test revealed significant (2 tailed) decrease in ATP levels of all 4 strength sets and all 6 exposure duration sets to the control set indicating disturbance to the ATP molecules by electric current and hence decreased growth of cell.

The SCCmec A positivity of all the tests and control strains indicates either production impairment of the PBPs or induced functionality loss of PBPs which occurred either by change in stereotype or by charge interaction of weak current and protein. However, it requires further investigation.

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Disclosure
Authors declare no conflicts of interests.

References


