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Synergistic effects of Zinc oxide nanoparticles and conventional antibiotics against methicillin resistant *Staphylococcus aureus*

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Abstract

Background: Methicillin resistance in *Staphylococcus aureus* (MRSA) is creating crises in therapeutic options for the treatment of *S. aureus* associated infections, worldwide. Nevertheless, Zinc oxide nanoparticles (ZnO-NPs) are providing a source of an attractive broad-spectrum antibiotic. The aim of the present study was to investigate the synergistic effects of ZnO-NPs and antibiotics against *mecA* positive MRSA isolates.

Methods: Antibiogram of *S. aureus* was determined by Kirby Baur disc diffusion assay. The minimum inhibitory concentration (MIC) of antibiotics and ZnO-NPs was determined by using the broth dilution method. The *mecA* gene in *S. aureus* was detected by PCR amplification with gene specific forward and reverse primers. The effects of subinhibitory concentration of ZnO-NPs on conventional antibiotics was determined by combined disk diffusion assay.

Results: Out of two hundred clinical specimens, twenty-eight showed the growth of *S. aureus*. Antibiogram of the isolates showed that *S. aureus* have acquired resistance to the majority of the conventional antibiotics. However, no isolate showed resistance to vancomycin. The confirmed methicillin resistant *S. aureus* isolates were sensitive to ZnO-NPs. The antibacterial activity of ZnO-NPs appeared in a dose and time dependent manner since higher dose produced stronger effects in two hours than the effects produced from lower dose in three hours. Furthermore, ZnO-NPs enhanced the antibacterial activity of levofloxacin significantly ($p < 0.001$).

Conclusions: *S. aureus* has acquired strong resistance to multiple antibiotics. ZnO-NPs have potential synergism with levofloxacin antibiotic against the multiple drug resistant *S. aureus* including MRSA.

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Introduction

Staphylococcus aureus are Gram-positive bacteria that cause a variety of infections including skin and soft tissue, burn and wound infections in both community and hospital settings [1]. *S. aureus* becomes resistant to β-lactam antibiotics including methicillin and/or oxacillin due to the independent acquisition of staphylococcal cassette chromosome mec (SCCmec) [2]. Consequently, the infections caused by *S. aureus* have become a challenge to public health worldwide. However, a marked geographical variation in the burden of MRSA has been found which is attributed to the diversity of local clones of MRSA strains and the locality wise differences in infection control programmes and practices [3].

Due to clonal diversity among MRSA and their specificity in the acquisition of SCCmec, it is essential that the antibacterial activity of conventional antibiotics in combination with alternative therapeutic options should be investigated using the local clones of MRSA. One of these options is to use ZnO-NPs for the treatment of MRSA associated infections. ZnO-NPs have reportedly shown activity against a wide range of bacteria [4]. The antimicrobial activity of ZnO-NPs is associated with a novel toxicity mechanism. Electron-spin resonance measurements have revealed that an aqueous suspension of ZnO-NPs produces amplified levels of reactive oxygen species, namely hydroxyl radicals [5]. Recently, a complex mechanism of antimicrobial action that involves multiple metabolic pathways has been suggested for the anti MRSA activity of ZnO-NPs [6]. ZnO-NPs possess a number of dermatological properties and are among the highly desirable anti MRSA agents [7]. In fact, ZnO-NPs are under consideration for the treatment of MRSA associated infections. The specificity to the host cell, the lowest activity against human cells while having selective toxicity against target cells make them an ideal source of anti MRSA agent.

Taken together, the clonal diversity among MRSA, the challenge imposed by multidrug resistant MRSA strains and the anti MRSA potential of ZnO-NPs, the present study was designed to characterize local MRSA clones and to evaluate the effects of ZnO-NPs on the antibacterial properties of conventional antibiotics including levofloxacin. Levofloxacin is a fluroquinolone that can be used as an alternative option to treat MRSA associated infection. However, levofloxacin resistance in MRSA brings a catastrophe to public health [8]. Therefore, the potential of synergism of ZnO-NPs with levofloxacin antibiotic may open doors for the future research to investigate synergy and the mechanism of synergism between these two antibacterial agents and the development of the strategies of future combinational therapy against a top priority pathogen, MRSA.

Methods

This is a cross sectional study conducted during a period of two years. The present study was approved from advanced studies and research board, Quaid-i-Azam University, Islamabad.

Bacterial strains, media, and growth conditions

All the media used in the present study, Muller Hinton agar medium (MHA), Nutrient agar (NA) and Nutrient broth (NB), and Mannitol salt agar (MSA), were purchased from Oxoid, UK. Bacterial cultures were grown aerobically at 35 ± 1ºC for 24 hours. ZnO-NPs was purchased from Sigma, Aldrich.

Isolation and the preliminary identification of *S. aureus* isolates

In the present study, 28 *S. aureus* strains were isolated from clinical samples such as pus swabs, throat swab, ETT tips (n=200) collected from a diagnostic laboratory connected with a tertiary care hospital of Rawalpindi, Pakistan. The isolation of *S. aureus* was based on their colony morphology on MSA medium. Identification was done using Gram-staining and standard biochemical tests such as mannitol fermentation, hemolysis, and catalase, coagulase, urease, DNase production tests.

Antibiotic sensitivity patterns of *S. aureus*

The antibiotic susceptibility of the clinical isolates of *S. aureus* (n=28) was determined using Kirby Baur disk diffusion assay according to CLSI guidelines and Zone of inhibition around an antibiotic disc was measured and compared with "Disc diffusion supplement table" [9]. The commercially available antibiotic discs used in the present study and their concentrations are listed in table 1.

Detection of *mec*A gene

The template DNA of the all *S. aureus* strains (identified as MRSA based on antibiotic susceptibility assay) was obtained by using Wizard Genomic DNA extraction kit (Promega Inc., Madison, WI, USA). The procedure for DNA extraction was performed according to the recommendations of manufacturer. The quantity and quality of the extracted DNA was assessed using the Nanodrop-2000 spectrophotometer and agarose gel electrophoresis, respectively. PCR was performed using MRSA template DNA and a pair of primers reported previously [10]. The primers were mecA_1 (AAA-ATC-GAT-GGT-AAA-GGT-TGG-C) and mecA_2 (AGT-TCT-GCA-GTA-CCG-GAT-TTG-C), used to amplify 533bp fragment corresponding to the *mecA* gene. A well characterized MRSA strain from our laboratory was used as a control strain [11].

Determination of the minimum inhibitory concentration (MIC) of antibiotics

The MIC of ampicillin, cefotaxime and levofloxacin was determined by an agar dilution method using the two-fold dilutions of antibiotics. The final concentration of the levofloxacin, cefotaxime and ampicillin in different plates was 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 and 0 µg/ml.

Determination of the anti MRSA activity of ZnO-NPs

The antibacterial activity of ZnO-NPs was assessed against the MRSA isolates (*mecA* positive) of the present study (n=13) by a broth dilution method using NB medium. Two concentrations of ZnO-NPs (100µg/µl and

200µg/µl) were prepared and sterilized. Sonication was done to prepare uniform colloidal suspension. A single colony of test MRSA strains was grown overnight with shaking (200 rpm) at 35 ± 1ºC. The overnight cultures were diluted to achieve 1.5×10^8 cfu/ml cells. Then, the MRSA cultures were grown in liquid medium in a total volume of 5 ml supplemented with ZnO-NPs colloidal suspensions with respective final concentration of 10µg/ml (low dose), and 20µg/ml (high dose). The culture without the treatment of ZnO-NPs served as a control. The culture turbidity was used as a qualitative measure of cell growth. The experiments were performed in triplicate and repeated at least twice.

Effects of ZnO-NPs on the activity of antibiotics

To determine the combined effects, each standard antibiotic paper disk was further impregnated with 20µl of ZnO-NPs (final concentration of 500-2000 µg per disk). The sensitivity of MRSA isolates against the combined disks (an ineffective concentration of a conventional antibiotic and a subinhibitory concentration of ZnO-NPs) was determined by disc diffusion assay and a checkerboard was established.

Results

Antibiogram of *S. aureus* isolates

A total of 28 *S. aureus* isolates were recovered from the clinical samples (n=200). The antibiogram of *S. aureus* isolates showed that they have acquired resistance to oxacillin/methicillin and can primarily be considered as MRSA isolates. Antibiotics from other major classes such as cephalosporins and quinolones were also ineffective to *S. aureus* isolates (Table 1). All *S. aureus* isolates of the present study were resistant to levofloxacin (100%) while linezolid was effective against 92.86 % (n=26) *S. aureus* isolates. Fortunately, no vancomycin resistant *S. aureus* was found in the present study suggesting that vancomycin remains an effective antibiotic against MRSA and can be used as a last resort therapy for the treatment of MRSA associated infections.

Antibiotics (Concentration, µg)	*S. aureus* (n=28)					
	Sensitive		Intermediate		Resistant	
	(n=)	%	(n=)	%	(n=)	%
Amoxicillin (10)	0	0	0	0	28	100
Ampicillin (10)	0	0	0	0	28	100
Amoxicillin/ clavulanic acid (30/10)	0	0	0	0	28	100
Cefoxitin (30)	0	0	0	0	28	100
Ceftazidime (30)	0	0	0	0	28	100
Cefuroxime (30)	0	0	0	0	28	100
Ceftriaxone (30)	0	0	0	0	28	100
Cefixime (5)	0	0	0	0	28	100
Erythromycin (15)	04	14.29	0	0	24	85.71
Gentamycin (10)	04	14.29	04	14.29	20	71.42
Levofloxacin (5)	0	0	0	0	28	100
Linezolid (1)	26	92.86	02	7.14	0	0
Methicillin (5)	0	0	0	0	28	100
Oxacillin (5)	0	0	0	0	28	100
Vancomycin (30)	28	100	0	0	0	0

Table 1: Antibiogram of *S. aureus* isolates of the present study.

Molecular characterization of MRSA

Of the 28 *S. aureus* isolates, 13 (46.43%) were *mecA* positive (Figure 1). Data showed that the rate of acquisition of multiple drug resistance was higher in the confirmed MRSA isolates. The MIC of ampicillin, cefotaxime, and levofloxacin was much higher for the MRSA isolates (Table 2). The higher MIC values highlight the presence of strong antibiotic resistance in the *mecA* positive MRSA isolates.

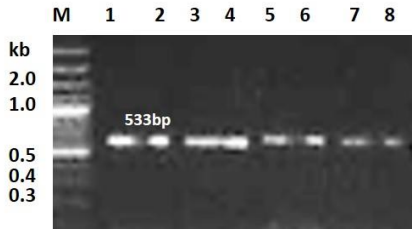

Figure 1: Agarose gel electrophoresis analysis of PCR production (533bp) corresponding to the *mecA* gene Lane M: marker (100bp), lane 1 *mecA* positive control, lane 2-8: seven MRSA isolates showing the *mecA* positive genotype.

S. aureus isolates	Minimum Inhibitory concentration of antibiotics (µg/ml)					
	Amoxicillin		Cefotaxime		Levofloxacin	
CLSI breakpoints	S ≤ 4	R ≥ 8	S ≤1	R ≥4	S ≤0.12	R ≥2
Ms_1	128		256		128	
Ms_2	256		>512		16	
Ms_3	32		01		04	
Ms_4	32		256		64	
Ms_5	128		>512		08	
Ms_6	64		>512		32	
Ms_7	512		512		32	
Ms_8	32		512		16	
Ms_9	64		32		32	
Ms_10	128		16		16	
Ms_11	64		>512		>512	
Ms_12	256		32		32	
Ms_13	256		>512		>512	

Tables 2: The MIC of ampicillin, cefotaxime, and levofloxacin for the *mecA* positive MRSA isolates (n=13).

Anti MRSA activity of ZnO-NPs

Furthermore, the antibacterial effects of ZnO-NPs against MRSA were observed in dose dependent manner. The effects of low dose of ZnO-NPs (0.10 µg/ul) on the growth of *mecA* positive MRSA were observed after 3hrs of ZnO-NPs treatment and high dose of ZnO-NPs (0.20 µg/ul) showed killing of the MRSA cells at 2hrs post treatment in the liquid culture (Figure 2). However, the curve gradually returned to increase, and culture started to become turbid after 5 hrs, suggesting that these concentrations can decrease the load of MRSA, but the MIC would be much higher than these doses. The MIC experiments showed that the concentration between 500-1000µg/ml was required to inhibit the growth of the majority of *mecA* positive MRSA isolates while disk diffusion assay showed that >2000 µg/disk ZnO-NPs was effective against *mecA* positive MRSA isolates.

Effects of ZnO-NPs in combination with conventional antibiotics against MRSA

The combined effects of ZnO-NPs and the conventional antibiotics were determined by using combined disc diffusion assay. It was observed that the subinhibitory concentration of ZnO-NPs enhanced the antibacterial

activity of amoxicillin/clavulanic acid (30/10µg) and levofloxacin (5µg) against 69.23 (n=09) and 76.92 (n=10) MRSA isolates, respectively (Table 3). However, no effect of ZnO-NPs was found on the activity of ampicillin (25µg), cefoxitin (30µg) and oxacillin (1µg). The data showed that the 2000µg of ZnO-NPs was able to increase the efficacy of levofloxacin antibiotic significantly ($p < 0.001$) (Table 4). The synergy between ZnO-NPs (2000 µg) and levofloxacin (5 µg) was found in 76.92% (n=10) confirmed MRSA isolates. Our results suggest that the ZnO-NPs with levofloxacin is synergistic with respect to multidrug MRSA killing.

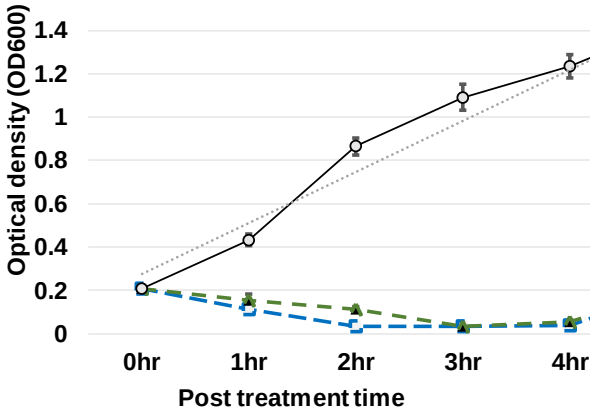

Figure 2: Anti MRSA effects of ZnO-NPs determined by optical density (OD_{600}) for at least 4 hrs of post treatment with ZnO-NPs. High dose ZnO-NPs, 20µg/ml (□), Low dose ZnO-NPs, 10µg/ml (▲), and control (○). Error bars represent mean ± SEM (n=3 replicates).

S. aureus isolates	Levofloxacin (5µg)	Amoxicillin /clavulanic acid (30/10 µg)
Ms_1	-	-
Ms_2	+	+
Ms_3	+	-
Ms_4	+	+
Ms_5	+	+
Ms_6	+	+
Ms_7	-	-
Ms_8	+	+
Ms_9	+	+
Ms_10	+	+
Ms_11	+	+
Ms_12	+	+
Ms_13	-	-

Table 3: The combined effects of ZnO-NPs and antibiotics

Concentrations of ZnO-NPs (µg)	Size of zones of inhibition of MRSA (mm)		
	ZnO-NPs	ZnO-NPs +Levofloxacin	Levofloxacin
500	0	0 (0	0
1000	0	5.10 ± 1.64	0
2000	0	7.70 ± 1.61	0

Table 4: Average size of zones of inhibition observed from the combined effects of subinhibitory concentration of ZnO-NPs and Levofloxacin.

Discussion

One of the dangerous phenomena of the current age is the increasing antibiotic resistance which is multiplied and intensified by the lack of the introduction of new antibiotics in the armory [12]. Currently, the first line therapy has almost failed to provide the treatment of bacterial infections. One example is of ineffectiveness of methicillin/ oxacillin in the treatment of *S. aureus* associated infections due to the high frequency of the prevalence of MRSA worldwide [13, 14]. Therefore, researchers are focusing on the identification and development of alternative therapeutic approaches. In this wave, ZnO-NPs have been reported to possess exceptionally beneficial safety profile and no toxicity detected when taken at different nano sizes alone [15]. Moreover, this compound is an extremely safe compound and may be taken into consideration for combinational therapy against *S. aureus*, due to its possible synergistic effect with important antibiotics. In view of these facts, the present study was conducted to explore the effects of ZnO-NPs on the efficiency of currently available antibiotics in respect of kill multidrug MRSA. Our findings showed that all the oxacillin resistant *S. aureus* were not *mecA* positive. Although presence of *mecA* gene in *S. aureus* is traditionally considered as marker for MRSA, *mecA* negative oxacillin resistant *S. aureus* have been reported, recently [16, 17].

The synergistic effects of ZnO-NPs to different antibiotics against some pathogens have been determined recently [18]. However, their synergistic effects against antibiotic resistant pathogens such as MRSA remained to be explored. In the present study, we have determined the synergistic effects of ZnO-NPs to different antibiotics against *mecA* positive MRSA isolates recovered from medical settings. The antibacterial activity of ZnO-NPs were tested using three different concentrations which were chosen to assure that the antibacterial effect produced was due to the combined effect of ZnO-NPs and antibiotics as suggested in previous report [19]. Antibiotics were carefully chosen because they represent the key classes of antibiotics (penicillins, cephalosporin, fluoroquinolones, and glycopeptides). However, our data showed that all antibiotics representing the key classes of antibiotics (penicillins, cephalosporin, fluoroquinolones, and glycopeptides) act differently in the presence of ZnO-NPs. In Agreement with a previous study, antibacterial activity of ampicillin, cefotaxime and oxacillin in combination with ZnO-NPs against MRSA neither increased nor decreased [19]. Interestingly, the activity of levofloxacin and amoxicillin-clavulanic acid was increased in the presence of ZnO-NPs against the test MRSA strains. The increase in the diameter of the zone of inhibition of MRSA growth suggested that ZnO-NPs has positive synergistic effects on levofloxacin in terms of killing the levofloxacin resistant MRSA isolates. These findings are comparable to the results found previously [20], whereas the activity of vancomycin was reduced slightly against MRSA which is similar to previous study in which the vancomycin zone diameter was reduced from 19mm to 18mm in the presence of ZnO-NPs [19]. Our data suggests that the ZnO-NPs provide synergism selectively presumably depending upon the different mode of action of antibiotics.

Levofloxacin, an L-form isomer of ofloxacin, is a renowned member of fluoroquinolones which has broad spectrum activity against Gram-positive and Gram-

negative bacteria [21]. Like other fluoroquinolone, levofloxacin has ability to enter bacterial cells and to act on bacterial topoisomerase II (DNA gyrase) resulting the inhibition of super helical twists in bacterial DNA. Given that there are nitrogen atoms in quinolone ring in levofloxacin, the hydroxylated surface of ZnO-NPs can bring the possibility of ionic interactions between them and may cause stabilization of levofloxacin and ZnO-NPs system [22, 23]. In conclusion, ZnO-NPs have the potential of providing synergism to antibiotics which may open the doors for a future strategy of using combinational therapy against MRSA.

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Conflict of interest

The authors declare that they have no competing interests.

Author Contributions

MS conducted the research work and analyzed the data. SAT analyzed the results and contributed in the compilation of the manuscript. SB performed statistical analysis and wrote the manuscript.

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