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POTENTIATING EFFICACY OF ANTIBIOTIC CONJUGATES WITH ZINC OXIDE NANOPARTICLES AGAINST CLINICAL ISOLATES OF *STAPHYLOCOCCUS AUREUS*

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In the present study, the efficacy of antibiotic-nanoparticle conjugates against the clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-susceptible *Staphylococcus aureus* (MSSA) were invesitgated. MRSA and MSSA were isolated from ICU patients and successfully confirmed by molecular methods. Antibiotic disc diffusion susceptibility revealed multiple antibiotic resistances in isolates. The MRSA and MSSA were also assesed against the oxide nanoparticles of calcium, magnesium and zinc. Among the nanoparticles, the zinc oxide was found more lethal against these potent pathogens. Thus, ZnO was conjugated with antibiotic which showed a synergistic antibacterial activity. Interestingly, the 1 to 3µg/mL of methicillin conjugated ZnO particles was found as a benchmark concentration range during evaluation of MIC. However, similar concentration ranges of antibiotics alone were incapable of producing good antibacterial activity. Conclusively, the synthesized conjugates can be used as an alternative to the currently available drugs especially the ones to which bacteria are getting resistant.

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Keywords: MRSA; Methicillin-susceptible *Staphylococcus aureus*; Antimicrobial susceptibility; Minimum inhibitory concentrations; Metallic nanoparticles; ZnO particles; Antibiotic conjugates

1. Introduction

The re-emergence of infectious diseases and the continuous development of antibiotic resistance among a variety of disease-causing bacteria has posed a serious threat to public health worldwide [1, 2]. Among these pathogenic microorganisms, *Enterococcus*, *Staphylococcus* and *Streptococcus* are closely related species that causes a wide variety of infections and diseases [3]. Significantly, *S. aureus* is the frequently occurring pathogen in hospitals as well as in the community [4]. In order to have adequate information for the treatment of staphylococcal infection

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and the formulation of effective infection control measures, data on susceptibility patterns and characterization of *S. aureus* are of great importance [2].

Since the last few decades, the widespread use of antibiotics to treat staphylococcal infections has resulted in the emergence of resistant forms of these organisms [5]. Particularly, methicillin-resistant *S. aureus* (MRSA) is a major concern, as it reduces the antibiotic treatment options for both prevention and cure [6] and ultimately leading to prolonged patient hospitalization, increased mortality and treatment costs [7].

Now these days, the focus is on alternative cheap treatment regime [8]. To achieve the milestone, several studies have been suggested and carried out on different nanoparticulate formulations including inorganic oxides e.g. calcium oxide (CaO), magnesium oxide (MgO), titanium oxide (TiO_2) and zinc oxide (ZnO) as effective bactericidal materials [9, 10, 11, 12, 13, 14, 15]. But, the concentration and size related toxicity of these particles have diverted the regimen towards more friendly and effective approaches [16, 17].

Currently, we constructed rod shaped particles of ZnO nanoparticles conjugated with ciprofloxacin, erythromycin and methicillin. We found that antibiotic conjugated ZnO were comparable as bactericides and killed clinical isolates of MRSA and MSSA.

2. Material and methods

2.1 Materials and Equipment

VITEK 2 System (Version 5.01 BioMerieux), *S. aureus* ATCC 29213, ID-Gram Positive Cocci card (BioMérieux), primers (Eurofins Genomics, USA). DNA purification kit (Promega Corporation, USA), CHEF-DR III system, T100™ Thermal Cycler and DNA ladder (BioRad, USA), Ultrapure Agarose (Life Technologies). Ampicillin, azithromycin, bacitracin, chloramphenicol, clarithromycin, clindamycin, gentamicin, kanamycin, lincomycin, nalidixic acid, ofloxacin, penicillin, rifampin, streptomycin, tetracycline, and tobramycin (Remel Fisher Scientific, USA). Ciprofloxacin (980µg/mg), erythromycin (850µg/mg), methicillin (850µg/mg), vancomycin (1000µg/mg), ethidium bromide, CaO, MgO and ZnO nanoparticles and lysostaphin (Sigma Aldrich, USA), Mueller Hinton media (Fluka, USA). Spectrophotometer Fluoster Omega (BMG Lab Tech), 96-well plate reader (BioTek, USA), Plasmid Kit and RNase A (Qiagen, USA), DNA marker (GeneRuler™), (Qiagen, USA), NE Buffer 4 and *Sma*I (BioLabs), Gel documentation system GDS 8000 and Compar II version 6.6 (UVP Inc., USA).

2.2 Identification

Total 52 clinical samples (catheter tips, blood, pus and urine) of intensive care unit (ICU) patients were collected from Microbiology Laboratory, Holy Family Hospitals, Rawalpindi, Pakistan, from April to October 2013. VITEK 2 System and Gram Positive Cocci card were used for biochemical confirmation and identification. *S. aureus* ATCC 29213, SH 1000 and MRSA 252 were used as control.

2.3 Molecular identification

Molecular identification of *S. aureus* was done with PCR by targeting *nuc* genes with nucA primer (F) 5'-GCGATTGATGGTGATACGGTT-3' and nucA (R) 5'-AGCCAAGCCTTGACGAACTAAAGC-3', while the methicillin-resistance was identified by targeting *mecA* gene with mecA primer (F) 5'-AAAATCGATGGTAAAGGTTGGC-3' and mecA (R) 5'-AGTTCTGCAGTACCGGATTTGC-3'. DNA was extracted according to the manufacturer's instructions using Wizard Genomic DNA purification kit. The different amplification conditions employed were as follows: For *nucA*, initial denaturation was done at 95°C for 5 min followed by 35 cycles of 95°C for 60 sec, 61°C for 30 sec, 68°C for 30 sec in a Thermal cycler. Final extension of 68°C for 7 min was provided to the reaction mixture. For *mecA*, initial denaturation was done at 94°C for 5 min followed by 35 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 50 sec. Final extension of 72°C for 7 min was provided to the reaction mixture. Ultrapure agarose (1%) was used in the interpretation and detection of amplified PCR product for

gel electrophoresis. DNA ladder (200-10000bp) was used to compare the size of PCR amplified fragments.

2.4 Plasmid DNA and Pulse field gel electrophoresis (PFGE) profiling

Plasmid kit was used for the isolation of any plasmid DNA according to manufacturer instructions and were checked against plasmid marker 1kb DNA Ladder. For PFGE, microbial suspension of 100µl was mixed with 72µl of 2X lysis buffer, 8µl RNase A without lysozyme and 4µl of lysostaphin. Afterwards, 200µl of 1.5% molten low melting point agarose stock tempered to 50°C was added to the solution. The mixture was transferred to plug molds and solidified at 4°C for 10 min. Afterwards, the plugs were incubated overnight at 37°C in water bath with medium shaking in 2ml of 1X lysis solution containing 50µg/ml lysozyme. The plugs were then incubated with 2ml of a solution containing 10mM Tris-HCl, 100mM EDTA (pH 8.0), 1% SDS and proteinase K (100µg/ml) at 50°C for 4h. Repeatedly, the plugs were washed with Tris-EDTA buffer (TE) and then mixed with 1mM phenylmethanesulfonyl fluoride at 50°C.

Restriction digestion was carried out by incubating the plugs in 20µl of 10X restriction enzyme buffer (NE Buffer 4), 50 units of *Sma*I and 177.5µl of nuclease free water at 37°C for 3h according to enzyme manufacturer specifications. The plugs were removed from the buffer 30 min before electrophoresis and suspended in 200µl filtered 0.5X TBE. Electrophoresis was carried out at 14°C in a Bio-Rad CHEF-DR III system using 1% pulsed-field certified agarose with an included angle of 120^{o} in 0.5X TBE buffer containing 0.1% thiourea. The gels were run at 6V/cm with a ramping factor, a = -1.357, and two pulse programs, with the pulse times of 2/22 sec for 19h to separate the larger DNA fragments (50-500 kb) and 0.23/5.09 sec for 3.5h to separate the smaller DNA fragments (5-50 kb). These gels were stained with ethidium bromide and photographed using a gel documentation system. The PFGE was then analyzed with the help of Gel Compar II.

2.5 Antimicrobial susceptibility testing

Kirby-Bauer modified disc diffusion method [18] according to guidelines of clinical and laboratory standards institute (CLSI) was used to perform antimicrobial susceptible testing. The MIC of different antibiotics were determined using microdilution procedure for the quantitative measures of resistance [19]. Inoculum suspension were made in Mueller Hinton broth and 10^{6} CFU/ml per well (200 µl) of inoculum were added to a 96-well plate under continuous shaking. The optical density (OD) of the cultures was serially monitored with spectrophotometer at 600nm after every 15 min and final reading were recorded after 24h using 96-well plate reader according to British Society for Antimicrobial Chemotherapy guideline 2007.

2.6 MIC with metallic nanoparticles

Metallic particles in a concentration range of 0-30mM were used for MIC. CaO (<160 nm), MgO (<50 nm) and ZnO (<50 nm, <100 nm & <5 µm) were used to evaluate antibacterial activity against *S. aureus*. The bacterial cells exposed to metallic oxide particles were examined by using the same protocol used for the determination of MIC of antibiotics.

2.7 MIC with antibiotic conjugates

ZnO particles were coated and characterized by the Department of Applied Science, University of Arkansas, Little Rock, USA (data not shown). These ZnO particles were purified by centrifugation and used further in the reaction with various antibiotics. In order to obtain the desired conjugated compounds, the antibiotics were conjugated onto ZnO surface by the formation of electrostatic bonds between the antibiotics and ZnO surfactant. The attachment was confirmed by transmission electron microscopy (TEM) and UV/VIS spectroscopy. The concentration of all sized ZnO particles was 25µg/mol per ml and the concentration of the antibiotics were: Ciprofloxacin (1.5µg/ml), erythromycin (2.1µg/ml) and methicillin (2µg/ml). MIC was conducted with antibiotic conjugates with ZnO particles. Broth dilution protocol of MIC was followed by using 25-450 µl of conjugates (Table 1), depending upon growth inhibition.

Table 1: Concentration of antibiotic conjugates with ZnO particles used for evaluating enhance antibacterial activity

µl	Ciprofloxacin (µg/ml)	Erythromycin (µg/ml)	Methicillin (µg/ml)	Vancomycin (µg/ml)	ZnO (µM)
25	0.187	0.262	0.25	0.425	25.6
50	0.375	0.525	0.50	0.85	51.2
75	0.562	0.787	0.75	1.27	76.81
100	0.75	1.05	1.00	1.7	102.41
125	0.937	1.312	1.25	2.125	128.015
150	1.125	1.57	1.5	2.55	153.62
175	1.312	1.837	1.75	2.97	179.22
200	1.5	2.1	2.0	3.4	204.82
250	1.87	2.62	2.5	4.25	256.03
300	2.25	3.15	3.0	5.1	307.23
350	2.62	3.67	3.5	5.95	358.44
400	3	4.2	4.0	6.8	409.65
450	4.5	4.72	4.5	7.3	460.86

2.8 Statistical Analysis

All the experiments were repeated three times. To identify significance effects, $p<0.05$ was considered as statistically significant.

3. Results

3.1 Identification

Vitek identification system confirmed 20/52 (38.5%) *S. aureus* (>85%-99%), 3/52 (5.8%) *S. epidermidis*, 6/52 (11.5%) *S. haemolyticus*, 1/52 (1.9%) *S. lentus*, 14/52 (26.9%) *S. sciuri*, 5/52 (9.6%) *Enterococcus faecium*, 2/52 (3.8%) *Aerococcus viridans* and 1/52 (1.9%) *Kocuria kristinae* species. PCR successfully confirmed all *S. aureus* with the help of nucA primer giving band at 270bp. In these, 13/20 (65%) were MRSA identified by mecA primer with amplicon at 533bp.

3.2 Plasmid DNA and PFGE profiling

Plasmids were isolated from 12/20 (60%) of MRSA and MSSA, 2/12 (16.7%) were having multiple plasmids while, 10/12 (83.3%) were carrying single plasmids. Out of these, 9/12 (75%) were MRSA with plasmids of different sizes. Total staphylococci isolates were belonging to 11 PFGE groups based on bands. Moreover, 17 isolates lacked 100% band resemblance (Supplementary information, Fig. S1).

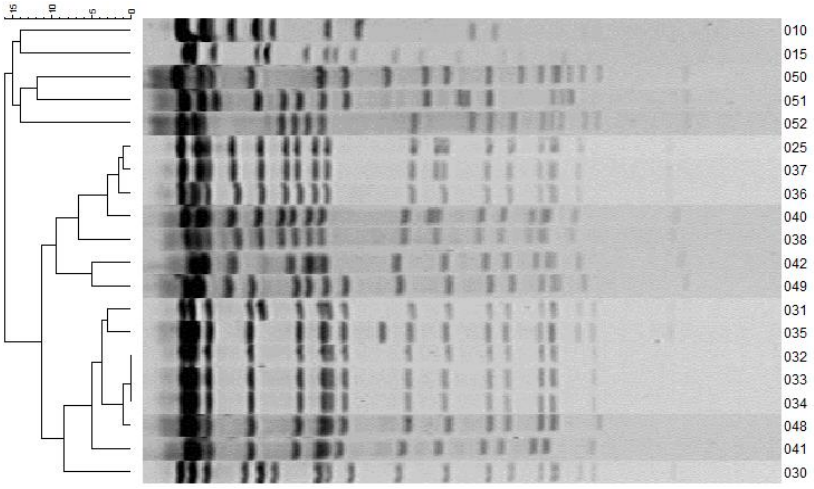

Fig. S1: Dandrogram of MRSA and MSSA isolates

3.3 Antibiotic susceptibility pattern

With disc diffusion, all the isolates were found highly resistant (80-100%) to ampicillin, ciprofloxacin, kanamycin, nalidixic acid, ofloxacin, penicillin G, streptomycin and tobramycin whereas 65-75% resistance was found against gentamicin, lincomycin, methicillin, and tetracycline. Moderate resistance of 35-50% was found with azithromycin, bacitracin, clarithromycin, erythromycin, and rifampicin and high susceptibility was confirmed with chloramphenicol (5%) and vancomycin (100%) (Fig. S2).

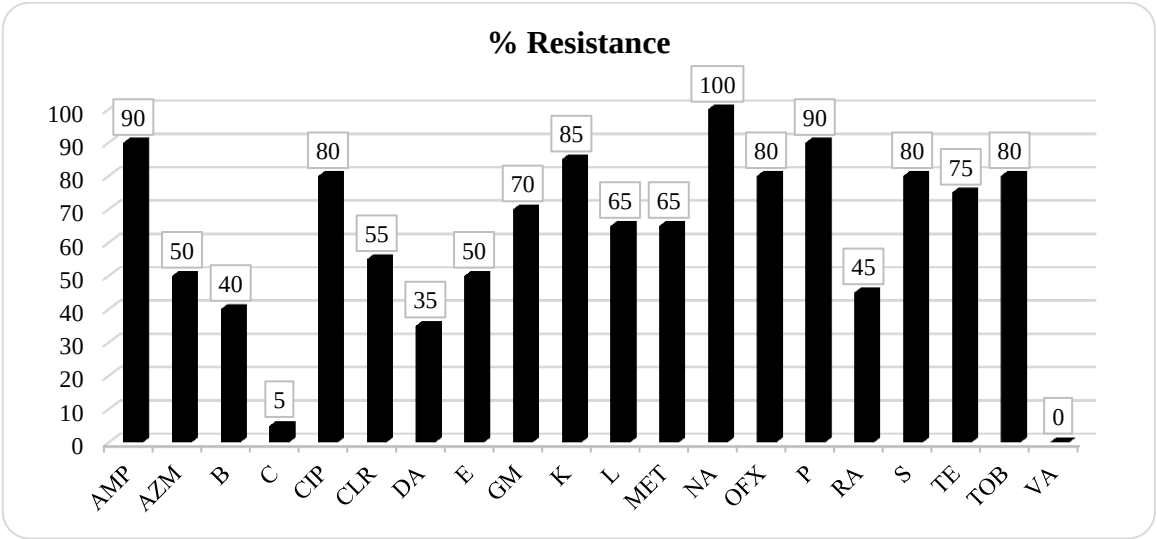

Fig. S2: Antibiotic resistant pattern of Disc Diffusion test
AMP: Ampicillin, AZM: Azithromycin, B: Bacitracin, C: Chloramphenicol, CIP: Ciprofloxacin, CLR: Clarithromycin, DA: Clindamycin, E: Erythromycin, GM: Gentamicin, K: Kanamycin, L: Lincomycin, MET: Methicillin, NA: Nalidixic acid, OFX: Ofloxacin, P: Penicillin, RA: Rifampin, S: Streptomycin, TE: Tetracycline, TOB: Tobramycin, VA: Vancomycin

The MIC with ciprofloxacin ranged from 16 to 512µg/ml, showing that majority of isolates were resistant against ciprofloxacin. Erythromycin with few susceptible isolates (20%) ranged from 0.5 to 2048 µg/ml of MIC. The MSSA isolates showed MIC range of methicillin of 1 to 4µg/ml while that of MRSA possessed an MIC range of 32 to 256µg/ml. There were no VRSA

found in the collected isolates with MIC between 1 to 2µg/ml. All the results were statistically significant with $p<0.05$ (Supplementary information, Table S1).

Table S1: MICs of different antibiotics against MRSA and MSSA isolates

Antibiotic Dilutions (µg/ml)	Ciprofloxacin	Erythromycin	Methicillin	Vancomycin
	*N (%)			
0.5	----------	3 (15)	----------	----------
1	----------†	2 (10) †	2 (10)	16 (80)
2	----------	----------	1 (5)	4 (20)
4	----------	----------	4 (20)	----------
8	----------	----------	----------†	----------†
16	1 (5)	----------	----------	----------
32	3 (15)	01 (5)	4 (20)	----------
128	6 (30)	----------	----------	----------
256	2 (10)	----------	9 (45)	----------
512	8 (40)	----------	----------	----------
2048	----------	14 (70)	----------	----------
t-Test	5.871	6.681	4.124	-74.101
P-value	p<0.05	p<0.05	p<0.05	p<0.05

Notes: †Breakpoint concentration
*N= no. of isolates and % is the killing percentage of the isolates

3.4 MIC with nanoparticles (NPs)

MIC with CaO and MgO NPs, showed effective inhibition of all the isolates at concentration of 15mM. The ZnO (<50nm) NPs were effective in a range of 1.25 to 5mM whereas, ZnO (<100nm) NPs inhibited the growth from 2.5 to 5mM and the ZnO (<5µm) particles were effective at 2.5mM (Fig. 1).

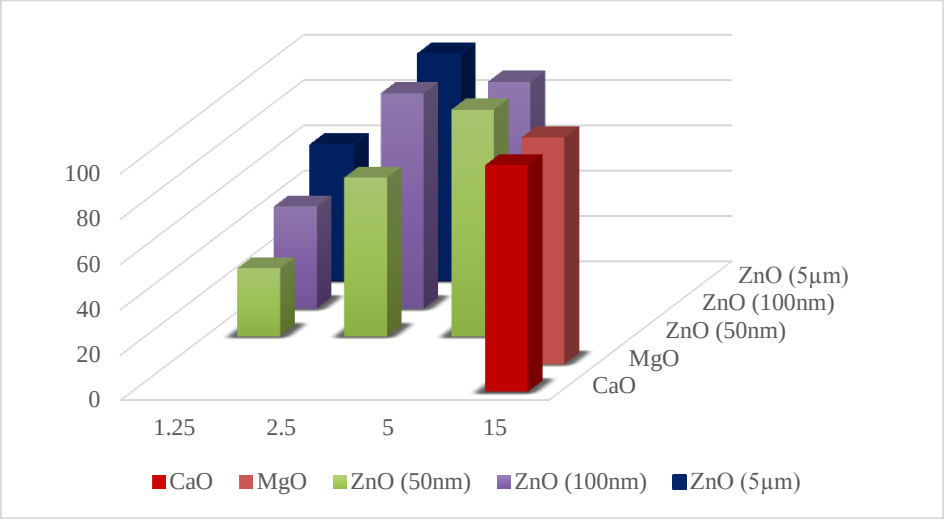

Fig. 1: MICs with NPs against MRSA and MSSA isolates

3.5 MIC with antibiotic conjugates

The antibiotic coated ZnO particles nanostructures were confirmed by TEM images. These ZnO NPs were lacking monodisperse distribution and the shape varied from nanorods to steric or even cube or hexagonal structures (Fig. 2a, b and c) with a thin shell of antibiotics covering the nanostructure surface. Moreover, the 50nm, 100nm and 5µm ZnO conjugates were rod shaped with lengths of 71.89±35.18nm, 108.59±37.44nm and 342.75±175.34nm and with diameters of 60.44±14.51nm, 110.21±22.76nm and 175.48±71.73nm respectively. The UV/VIS analysis, upon comparison with the reported literature data evidently showed that the prepared particles have typical characteristics of ZnO,[20, 21] which were known for broad-spectrum UV blocking. Fig. S3 (a, b and c) represents the UV/VIS spectrum of these conjugated antibiotics and ZnO particles with the absorption between 225nm to 380nm.

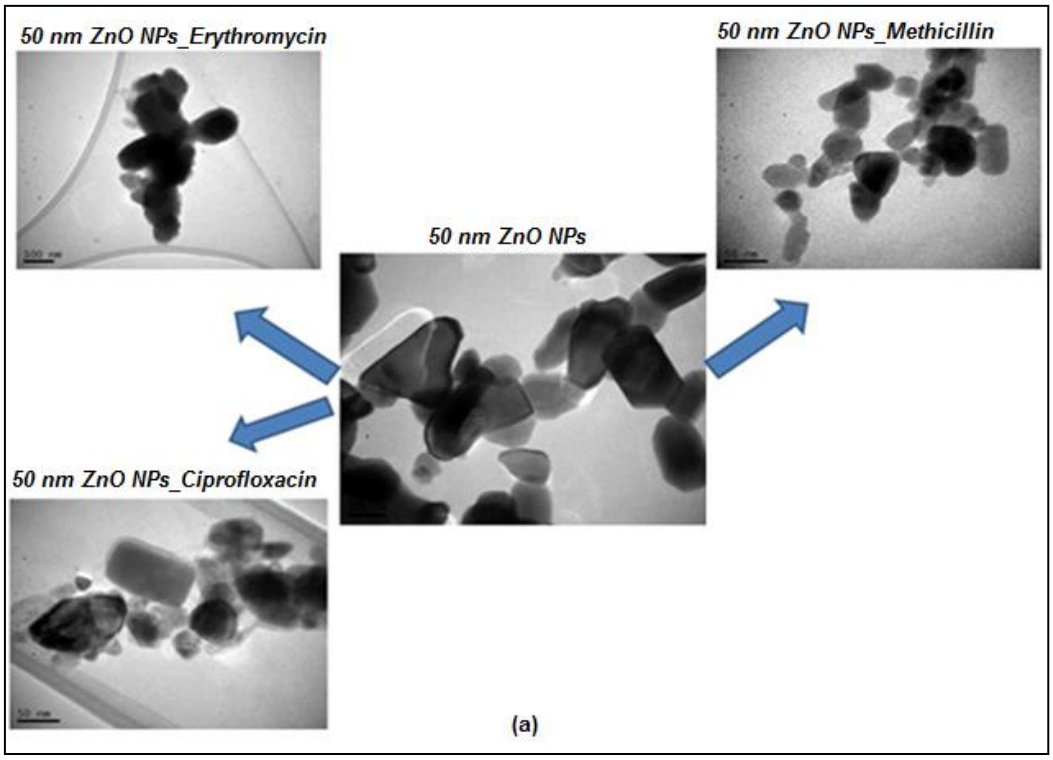

Fig. 2: TEM images of ZnO-antibiotics nanostructure of (a) <50 nm

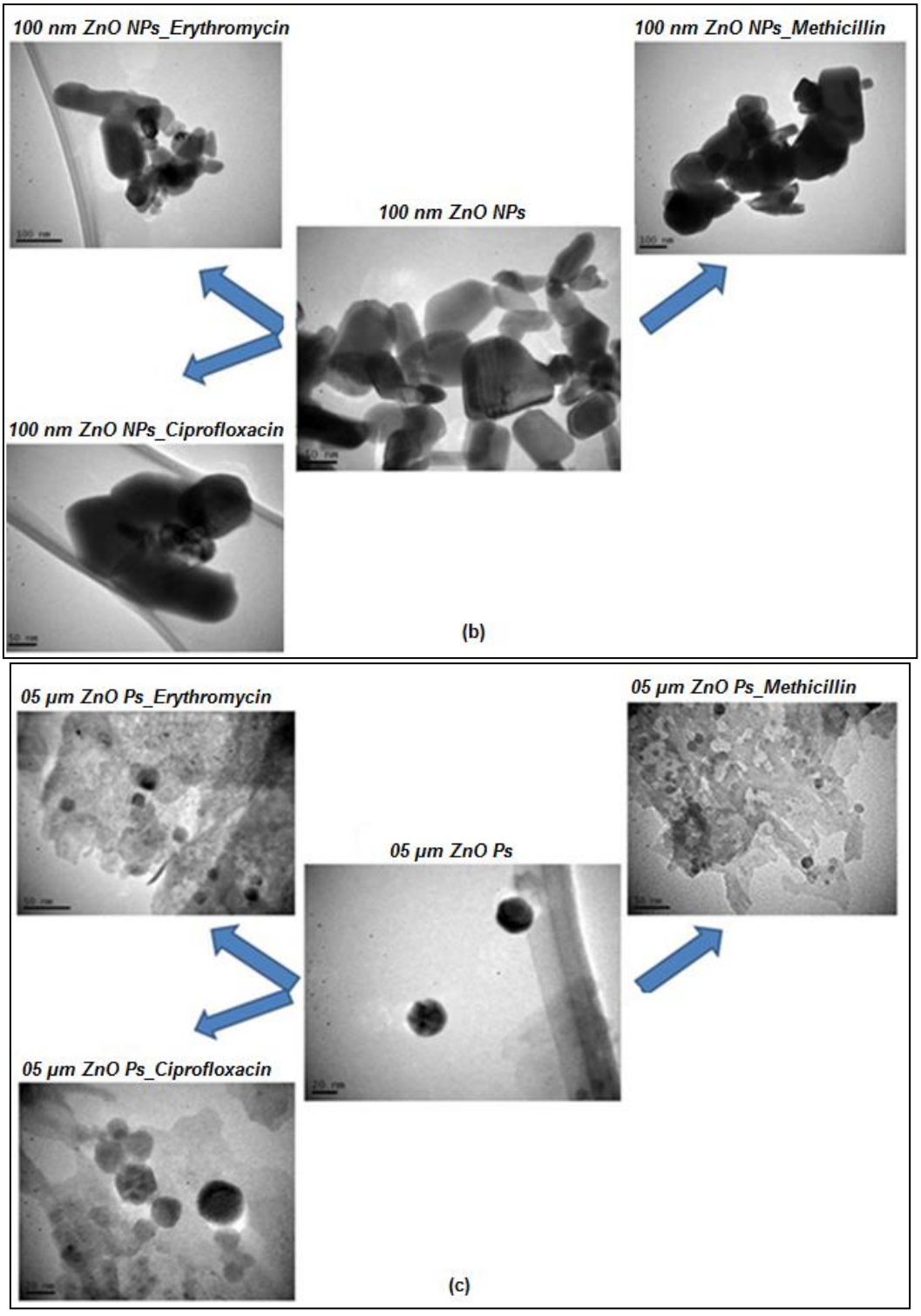

Fig. 2: TEM images of ZnO-antibiotics nanostructure of (b) <100 NPs and (c) <05 µm particles

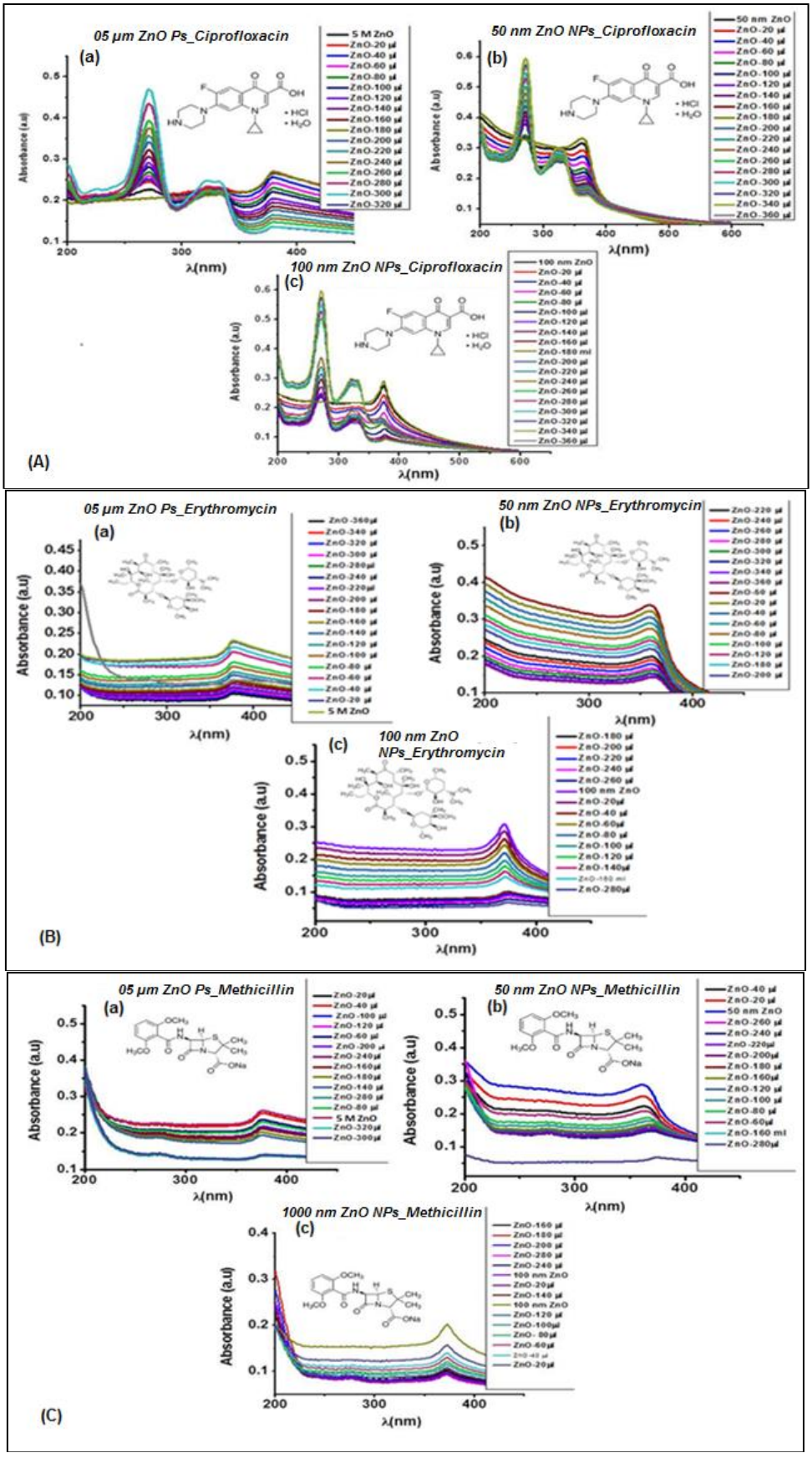

Fig. S3. UV/VIS absorption spectra confirming the adsorption of ***(A)*** *Ciprofloxacin* ***(B)*** *Erythromycin and* ***(C)*** *Methicillin on ZnO* ***(a)*** *<5µm particles,* ***(b)*** *<50nm and* ***(c)*** *<100nm NPs*

Antibacterial susceptibility testing showed that antibiotic coated ZnO particles effectively inhibited all the isolates at very low concentrations. For ciprofloxacin coated ZnO particles, the MIC ranged from 0.56 to 1.31µg/ml with ZnO concentration from 102.4 to 204.82µM. However, an effective inhibition was observed with ZnO ≤50nm NPs ranging from 0.56-1.125µg/ml (Table 2). Similarly, erythromycin coated ZnO particles showed MIC range from 0.26 to 2.1µg/ml with ZnO particles concentration ranging from 102.4 to 358.4µM. Again, the effective erythromycin MIC was found with ZnO ≤50 nm NPs (0.26 to 1.57µg/ml). For methicillin coated ZnO particles, MIC were in the range of 1 to 3µg/ml with ZnO concentration from 102.4 to 307.2µM.

Table 2: MICs of antibiotic conjugates against MRSA and MSSA. Final concentration of ZnO particles and respective antibiotic in the antibiotic conjugates. Percentage killing of each formed conjugates against the clinical isolates.

ZnO	Ciprofloxacin				Erythromycin				Methicillin			
Conc. (µM)	Conc. (µg/ml)	50 nm	100 nm	5µm	Conc. (µg/ml)	50 nm	100 nm	5µm	Conc. (µg/ml)	50 nm	100 nm	5µm
		*N (%)				N (%)				N (%)		
102.4	0.56	3 (15)	ND	ND	0.26	1 (5)	ND	ND	1.00	3 (15)	3 (15)	3 (15)
128	0.75	8 (40)	8 (40)	3 (15)	0.52	2 (10)	1 (5)	1 (5)	1.25	ND	ND	ND
153.6	0.94	7 (35)	3 (15)	9 (45)	0.78	ND	2 (10)	ND	1.5	6 (30)	1 (5)	ND
179.2	1.125	2 (10)	8 (40)	8 (40)	1.05	3 (15)	ND	2 (10)	1.75	ND	ND	ND
204.8	1.313	ND	1 (5)	ND	1.31	11 (55)	7 (35)	ND	2.0	1 (10)	4 (20)	5 (25)
256	1.5	ND	ND	ND	1.57	3 (15)	6 (30)	11 (55)	2.5	8 (40)	3 (15)	3 (15)
307.2	1.87	ND	ND	ND	1.84	ND	4 (20)	ND	3.0	2 (10)	9 (45)	9 (45)
358.4	2.25	ND	ND	ND	2.1	ND	ND	6 (30)	3.5	ND	ND	ND

*N= no. of isolates and % is the killing percentage of the isolates

4. Discussion

Multidrug resistant staphylococci isolated from clinical samples of ICU patients were identified and used to study the antibacterial activity of metallic particles and their conjugates with antibiotics.

VITEK ID successfully confirmed all the isolates endorsing previous study.[22] Molecular identification of *S. aureus* by PCR was successful by using strain-specific gene primers *nucA* and MRSA with methicillin-resistant gene primer *mecA* and they were found in accordance with the study of Murakami *et al*., 1991 [23] and Louie *et al*., 2002 [24].

Additionally, all the isolates were highly resistant to most of the tested antibiotics with the exception of a few, to which the resistance was moderate (Table S1). Moreover, all the isolates were found susceptible to vancomycin. Previously, it was found that many of the isolates (37.5%) were resistant to antibiotics [25] including multidrug resistance in MRSA [26] and similarly, 100% susceptibility with vancomycin were in accordance with our findings [27]. The MIC data also revealed that all isolates were resistant to ciprofloxacin. Previous reports showed 16 to 256 mg/L MIC range with ciprofloxacin and 1 to 2 mg/L with vancomycin [28]. MRSA showed that the methicillin MIC was greater than the breakpoint [29] and also, strains resistant with erythromycin showed higher MICs range [30].

Previously, CaO, MgO and ZnO NPs were found to have tremendous antibacterial properties [31, 32, 33]. In the current study, the evaluation showed that ZnO NPs (≤50 nm) effectively inhibited 70% of *S. aureus* isolates with an MIC of 2.5mM. It was found that the size and shape of nanoparticles have significant role in antibacterial activity. However, in previous studies, it was found that the antibacterial activity of ZnO NPs was because of the intracellular accumulation with a concentration ranging from 3 to 10mM [20, 31, 32, 33]. Furthermore, Padmavathy and Vijayaraghavan (2008) [34] reported that ZnO NPs (12nm) significantly inhibited Gram-negative bacteria at 1mM concentration. However, in the present study, it was found that higher concentrations and large size of ZnO NPs (≤50 nm) have efficiently inhibited the Gram-positive organism. This might me also be because of the difference in the bacterial group and it needs further evaluation. Furthermore, both CaO and MgO nanoparticle were found lower in efficacy upon comparison with ZnO particles. According to Makhluf *et al*., (2005) [32], smaller sized MgO NPs (25nm) were found with an MIC of 1mg/mL (24.8mM) against *S. aureus* and *E. coli* while in the present study, low concentration and large-sized MgO nanoparticle (≤50nm) have successfully inhibited *S. aureus*. Similar results were accredited with large sized CaO NPs (≤160nm).

Interestingly, the MIC of ZnO particles of nano and micro size were more effective in lowering the MIC at lower concentrations than CaO and MgO NPs. Thus, it led us to conjugate the three sized ZnO particles with three different antibiotics. Evidently, promising results were found with these conjugated particles. Currently, it was found that ciprofloxacin conjugated with ZnO NPs (<50nm) inhibited the isolates at lower MIC ranges (0.56 to 1.125µg/ml) than the ciprofloxacin alone (16 to 256mg/L). Moreover, ciprofloxacin conjugated with large sized ZnO particles (100nm & 5µm) also showed similar results with slightly higher concentration range. The results were in accordance with a recent study by Patra *et al*., (2014) [35] where they found similar enhanced antimicrobial effect of ciprofloxacin conjugated ZnO NPs. Similarly, erythromycin conjugated ZnO particles represented the similar kind of results. Interestingly, erythromycin conjugated ZnO NPs (≤50nm) lowered the MIC of resistant strains from 2048 mg/L to a range of 0.26 to 1.575µg/ml. Similarly, methicillin conjugated ZnO particles also exhibited an excellent inhibition with lower MIC range of 1 to 3µg/ml.

5. Conclusion

Conclusively, safe and cost effective drugs are promptly demanded to eradicate bacterial infections from almost every corner of the world especially to which the currently available drugs are getting resistance e.g. MRSA. Currently, the conjugates of ciprofloxacin, erythromycin and methicillin with ZnO particles exhibited enhanced bactericidal activity against multidrug resistant

clinical strains of MSSA and MRSA with dose dependency. MIC concentration of 1 to 3µg/mL of methicillin coated ZnO micro and nanoparticles were the benchmark concentration that successfully inhibited the resistant strains of MRSA, while a high concentration of similar antibiotics were lacking the antibacterial activity. Similar effects were observed with ciprofloxacin and erythromycin conjugates against isolated resistant strains.

Thus, this new strategy with the employment of nanotechnology has proved their potential for the optimum treatment of MRSA, in *in-vitro* conditions. In this milieu, the currently used approach will be helpful in eradicating not only MRSA but also other deadly pathogens. Thus, the current approach will contribute in the development of cheaper, safer and highly efficacious drugs for the improved public health in clinical settings.

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