



Resistance Modulation of *Staphylococcus aureus* Isolates of Dairy Cattle through Metallic Oxide Nanoparticles

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ABSTRACT

The rise in prevalence and drug resistance in *Staphylococcus aureus* of dairy animals require an alternative to antibiotics. Nanoparticles particularly metallic nanoparticles are a wise approach to modulating drug resistance. The current study thus focuses on the replacement of antibiotics with ZnO, MgO, and Fe₂O₃ nanoparticles. To achieve this goal, 200 cattle milk samples were collected aseptically and screened for subclinical mastitis using the purposive sampling technique. Following Bergey's manual of determinative bacteriology, *S. aureus* was identified and antibiotic susceptibility was tested using clinical laboratory and standard institute guidelines. On the other hand, resistant strains were put to antibacterial testing assay against metallic nanoparticles both by well diffusion and broth microdilution method. To analyze the data, both probability and non-probability statistical tools were applied using SPSS version 22 of statistical software at 5% probability. The current study showed 24.56% of *S. aureus* positive from commercial dairy while the resistance of these isolates against gentamicin, enrofloxacin, levofloxacin, and vancomycin was found to be 50, 40, 30, 30%, respectively. On the other hand, a disc diffusion assay was concluded with 24.525±0.806 mm (ZnO) followed by 16.475±0.950mm (MgO) and 13.150±1.392mm (Fe₂O₃). MgO stood first to contribute lowest Minimum inhibitory concentration (MIC) (1.302±0.564 mg/mL) followed by Fe₂O₃ (2.930±1.691 mg/mL) and ZnO (3.906±0.000 mg/mL). MIC of ZnO was recorded to be 18.23±11.93 mg/mL, 15.63±0.0 mg/mL, 13.02±4.5 mg/mL, 7.81±6.77 mg/mL, 5.21±2.26 mg/mL, and (3.906±0.000 mg/mL) at 04, 08, 12, 16, 20 and 24th h of incubation. A similar trend was followed by other nanoparticles at different hours of incubation. The study thus concludes a rise in resistant strains of *S. aureus* in bovine milk while metallic nanoparticles as effective alternatives.

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Authors' Contribution

MA, MM, AM and AIA executed methodology, collected data, did initial analysis and wrote initial draft. MA, SH and SM did formal analysis and revised draft. SRK, AIA, KA and AMQ conceived the idea, validated findings and revised manuscript.

Key words

Staphylococcus aureus, Bovine milk, Antibiogram, ZnO, MgO, Fe₂O₃

INTRODUCTION

Microorganisms such as *Staphylococcus aureus* have a symbiotic relationship with certain animals and can colonize in them various places (Shoib *et al.*, 2020). Bovine subclinical mastitis disease (BSM) is common around the world, being one of the diseases causing the greatest losses to the dairy industry (Gomes *et al.*, 2016). Nonetheless, *S. aureus* is the most common cause of subclinical mastitis in cattle, goats, and bovine in Pakistan

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(Javed *et al.*, 2021) and around the globe. Milk output and quality have decreased in the cattle industry because of mastitis caused by *S. aureus*. *S. aureus* has become a multidrug-resistant bacterium that equally affects other than udder of the animals (Sarwar *et al.*, 2021) reflecting potential contagiousness for a wider range of infections (Tong *et al.*, 2015).

Methicillin-resistant *S. aureus* (MRSA), often known as resistant staph or “superbug,” is one of the most common strains of *S. aureus* responsible for human infections in hospitals and the community. The World Health Organization has designated MRSA as a high-priority microbe for future investigation and treatment (Tacconelli *et al.*, 2018). *S. aureus*'s ability to parasitize intracellularly and formation of biofilms shield them from host immune responses and antibiotic effects, which pose significant therapeutic challenges. *S. aureus* is able to survive and multiply in cells by inhibiting the combination of phagosome and lysosome, subverting autophagy, and other methods (Foster *et al.*, 2014). This bacterium causes considerable economic losses, such as a significant drop in milk production, reproductive difficulties, expenditures associated with the culling of animals, higher veterinary medicine costs, and substituting contaminated milk. Antibiotics are frequently used to treat Staphylococcal infections while resistance has become global issue. Nanoparticles' antibacterial action is influenced by their stability in infected cells and their ability to reach the target site predictably.

The nanoparticles contain phagocytosis (zipper-like and trigger-like) and no phagocytosis transport pathways into cells (Geiser, 2010). There have also been promising results with inorganic nanoparticles applied to treating *S. aureus* infections. Nanomedicines have recently been touted as a viable solution to the MRSA problem (Kalhapure *et al.*, 2015). Metallic nanoparticles have shown strong antimicrobial properties against multiple bacteria species in multiple studies (Dizaj *et al.*, 2014). Nanoparticles of zinc oxide had bactericidal effects against gram-positive and gram-negative bacteria as well as spores that are resistant to high temperatures and high pressures. As reported in the studies, MgO nanoparticles cause the disruption of cellular membranes, which causes leakage of cellular contents and leads to the death of the bacterial cells (Jin and He, 2011).

The current study was purposed to check the prevalence of *S. aureus* in milk, antibiogram of MRSA against a wider range of antibiotics, and to evaluate the antibacterial activity of metallic nanoparticles against *S. aureus*.

MATERIALS AND METHODS

Sample collection site

The present research was focused on the region of districts Khanewal and Lodhran of South Punjab, Pakistan because it is an emerging commercial dairy setup. A total of n=200 cattle milk samples using the purposive sampling method (Thrusfield, 2018) were collected from commercial farms. Each milk sample was checked for subclinical mastitis using the Surf Field Mastitis Test (Muhammad *et al.*, 2010) while positive samples were moved to the Cholistan University of Veterinary and Animal Sciences, Bahawalpur.

Isolation of S. aureus and MRSA

Milk samples were centrifuged at 6000g/5 minutes, and sediment was incubated for 24 h at 37°C in sterile nutrient broth. The sediment from the incubated nutrient broth was centrifuged again and swabbed on blood agar aseptically. The pinpoint round colonies were picked up and streaked on differential medium, mannitol salt agar, after 24 h of incubation at 37°C. Following a 24 h incubation period at 37°C, the round colonies on yellow-colored media were selected for gram staining and other biochemical assays (Bergey and Holt, 1994).

All *S. aureus* isolates were phenotypically identified for MRSA using the oxacillin disc diffusion technique (CLSI, 2018). *S. aureus* growth was distributed at 10⁸ CFU/mL on Muller Hinton agar. To discover MRSA, a 10 µg oxacillin disc was put on the surface of the zone of inhibition, the diameter of which was measured and compared to standards supplied by the Clinical and Laboratory Standard Institute.

Antibiogram of MRSA

Antibiotic susceptibility of MRSA was determined using linezolid, enrofloxacin, chloramphenicol, gentamicin, ciprofloxacin, levofloxacin, fusidic acid, septran, and vancomycin. In a nutshell, new MRSA growth was distributed aseptically at 10⁸ CFU/ml on Muller Hinton agar. Selected antibiotics were put on agar aseptically, stored at 37°C for 18-24 h, and the diameter of inhibitory zones was measured. Isolates were classified as resistant, intermediate, or sensitive using standards from the Clinical Laboratory and Standard Institute (CLSI, 2018).

Preparation of nanoparticles

Metallic nanoparticles were obtained from the Department of Chemistry, University of Agriculture Faisalabad. For this purpose, Magnesium chloride (MgCl₂·6H₂O), sodium dodecyl sulfate (SDS), sodium

hydroxide (NaOH), Zinc acetate dihydrate (Zn (CH₃COO)₂·2H₂O), polyethylene glycol (PEG), urea (NH₂CONH₂), iron chloride tetrahydrate (FeCl₂·4H₂O) and ammonia were purchased from Sigma-Aldrich USA. A chemical method was applied to prepare nanoparticles (Parashar *et al.*, 2020).

Well diffusion method

Standard culture (10⁸CFU/ml) was aseptically disseminated on Mueller Hinton agar. The well-borer was drilling 6-8 mm holes. The suspension of antibiotic-coated nanoparticles was made individually for each nanoparticle that was poured into wells and incubated for 24 h at 37 °C the formation of inhibition zones around wells showed the preparation's antibacterial activity (Anwar *et al.*, 2020).

Minimum inhibitory concentration (MIC)

A standard inoculum (10⁵ CFU/mL) was used in the broth microdilution procedure to determine the MIC. Mueller Hinton broth in 96 wells microtitration plate was filled. Each preparation was 2-fold diluted, starting at 500 mg/mL, with one positive control and one negative control. For a total of five isolates, the test was performed in triplicate. At 37 °C, the plates were incubated for 24 h, and optical density (OD) readings were determined using a spectrophotometer at 590nm wavelength before and after incubation. The lowest concentration showing inhibition of growth was termed minimum inhibitory concentration (Anwar *et al.*, 2020).

Statistical analysis

The prevalence was computed using the (Thrusfield, 2018) formula. When comparing the means of two groups, the student t-test was used, while ANOVA for more than three groups and tukey test to find significance among groups were applied on quantitative data. SPSS version 22 of the computer program was used at 5% probability.

RESULTS

Prevalence of *S. aureus* and MRSA

Prevalence of *S. aureus* from the study area was noted to be 24.56% while minimum was found as 17.14% to the highest as 25.71%. MRSA was found 16.07% in the study area with the lowest being 0% and the highest noted to be 25% (Table I). Study showed non-significant association ($p>0.05$) of farms with *Staphylococcus aureus* and same was observed in case of methicillin resistant *Staphylococcus aureus*.

Antibiotic susceptibility

Antibiogram of the current study found higher percentages of intermediate isolates. Unusual than routine susceptibility profile was also observed in case of antibiotics considered as solution therapy like linezolid and vancomycin (Table II). Highest resistant *S. aureus* were noted against gentamicin (50%) and chloramphenicol (50%) followed by enrofloxacin (40%), and septran (33%), vancomycin (30%), levofloxacin (30%), cefoxitin (30%). The study noted higher prevalence of intermediate susceptible *S. aureus* against septran (36.67%), vancomycin (30%), enrofloxacin (20%), and fusidic acid (16.67%). Highest percentage of sensitive isolates were shown against linezolid (80%), and fusidic acid (70%).

Resistance modulation by nanoparticles

Zone of inhibition (ZOI)

Current study showed significantly highest zone of inhibition by ZnO followed by MgO and Fe₂O₃ showing 24.53±0.80, 16.48±0.95 and 13.15±1.39mm, respectively. Difference of zone of inhibition was noted to be significant ($p<0.05$) when ZnO compared with MgO and Fe₂O₃. Similarly, MgO was statistically significant different than those of ZnO and Fe₂O₃. Same goes for Fe₂O₃ in comparison with MgO and ZnO (Fig. 2).

Table I. Percentage prevalence of *Staphylococcus aureus* and methicillin resistant *Staphylococcus aureus* (MRSA) isolated from commercial dairy cattle milk.

Dairy farm	Sample tested (N)	Cattle milk <i>S. aureus</i>				Cattle milk MRSA			
		Positive (n)	Percentage (n/N*100)	CI (95%)	p value	Positive (x)	Percentage % (x/n*100)	CI (95%)	p value
A	35	9	25.71	14.16-42.06	0.140	2	22.2	1.58-18.6	0.938
B	35	6	17.14	8.1-32.68		1	16.66	0.51-14.54	
C	42	7	16.67	8.32-30.61		1	14.28	0.51-14.54	
D	34	15	44.12	28.89-60.55		2	13.33	1.58-18.6	
E	23	5	21.74	9.66-41.9		0	0	0-0	
F	24	6	25.00	12.00-44.90		1	16.66	0.51-14.54	
G	35	8	22.86	12.07-39.02		2	25	1.58-18.6	

Table II. Antibiogram of *Staphylococcus aureus* isolated from dairy cattle milk.

Antibiotics	R	I	S
Vancomycin (VA30)	30.00	30.00	40.00
Levofloxacin (LEV)	30.00	10.00	60.00
Cefoxitin (CXT30)	30.00	10.00	60.00
Linezolid (LNZ30)	10.00	10.00	80.00
Enrofloxacin (ENR10)	40.00	20.00	40.00
Gentamicin (CN10)	50.00	10.00	40.00
Fusidic acid (FA10)	13.33	16.67	70.00
Septran (SXT25)	33.33	36.67	30.00
Chloramphenicol (C30)	50.00	16.67	33.33

R, resistant; I, Intermediate; S, sensitive.

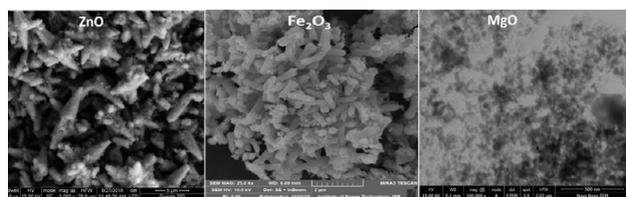


Fig. 1. Scanning electron microscopy (SEM) analysis of (i, ZnO), (ii, Fe₂O₃), (iii, MgO).

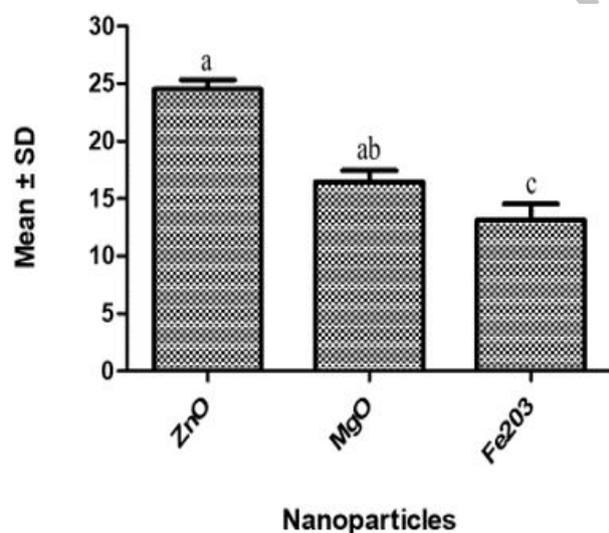


Fig. 2. Comparison of zones of inhibitions (mm) expressed by different nanoparticles (Zinc oxide, Magnesium oxide, and Ferric oxide). Different superscripts among nanoparticles indicate significant differences.

MIC (mg/mL) of *S. aureus*

The present study for ZnO nanoparticles highest MIC

(18.23±11.93) against *S. aureus* was noted at 4h followed by 12 h, 8h, 16h, 20h, and 24h. All values show a non-significant difference ($p < 0.05$) with all other MIC of the different time intervals. Value at 24h showed lower MIC (3.906±0.000) compared with 4h. For MgO nanoparticles highest MIC (11.72±6.77) against *S. aureus* was noted at 4h followed by 8h, 12 h, 16h, 20h, and 24h (Table III).

Comparison of incubation intervals for significant change in MIC was found different in all three nanoparticles (Fig. 3). ZnO showed significant difference of MIC when 4th incubation was compared with 8th, and 12th h of incubation. Similarly, 8th h of incubation was non-significant difference with 16th h of incubation. 12th h of incubation proved to be non-significantly different with all incubation periods. In case of MgO, significant difference started at/after 8th h of incubation while starting from 12th h onwards significant difference was not found particularly 16th, 20th and 24th h of incubation was non-significant with each other but significantly lower than those of 4th and 8th h incubation. Considerable variation in MICs was noted among different time intervals in case of Fe₂O₃ in that significant difference started right after 4th h incubation. Middle order incubation h (12, 16, 20) were non-significant to each other and similarly 16th h onwards was non-significant with each other but significantly differing with initial 3 incubation intervals. These findings reflect potential antibacterial activity at various hours.

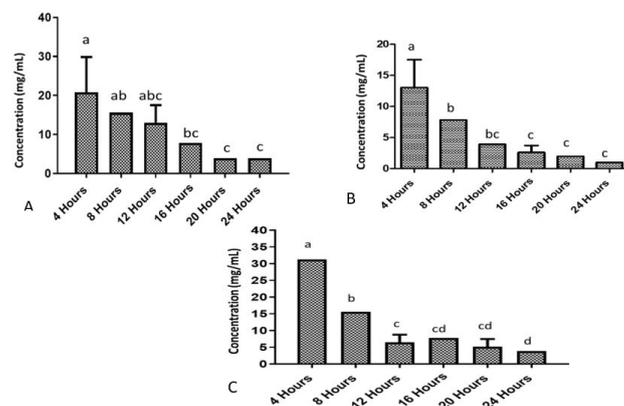


Fig. 3. Comparison of MICs (mg/mL) among incubation periods (h) for each metallic oxide nanoparticle.

A, MIC of ZnO nanoparticle at different time intervals; B, MIC of MgO nanoparticle at different time intervals; C, MIC of Fe₂O₃ nanoparticle at different time intervals. NB, Different superscripts among different time intervals of incubations (4, 8, 12, 16, 20 and 24 h) indicate significant differences ($p < 0.05$).

SEM analysis

SEM image of ZnO nanoparticles is shown in Figure 1. (i) Polygonal star-like microparticles are observed in Table III. Comparison of minimum inhibitory concentration (mg/mL) exhibited by metallic oxide nanoparticles against methicillin resistant *Staphylococcus aureus* at different hours of incubation.

	4 h	8 h	12 h	16 h	20 h	24 h
ZnO	20.83±9.02 ^a	15.63±0.00 ^a	13.02±4.51 ^a	7.81±0.00 ^a	3.90±0.00 ^a	3.90±0.00 ^a
MgO	13.02±4.51 ^{ab}	7.81±0.00 ^b	3.90±0.00 ^{ab}	2.60±1.12 ^a	1.95±0.00 ^b	0.97±0.00 ^b
Fe ₂ O ₃	31.25±0.00 ^b	15.63±0.00 ^c	6.51±2.26 ^b	7.81±0.00 ^{bc}	5.21±2.26 ^a	3.90±0.00 ^a

Different superscripts within column indicate a significant difference ($p < 0.05$).

this figure. This star-like particles are aligned randomly and are not fused properly. Particle borders are distinct, and each particle is made up of several spikes that are linked at the center, much like a star. The length of spikes is not equal. Every star-like particle is about 4-8 μm in diameter. Spikes' terminal ends are blunt rather than pointy. Rod-shaped nanoparticles of Fe₂O₃ are observed in Figure 1. (ii) These rod-shaped particles are randomly aligned with each other. The length of the rods is around 2 μm while the width is around 100 nm. The terminal ends of rods are not pointed. It seems that rods have emerged from spherical nanoparticles because few spherical-shaped minute particles have adhered to the surface of rods. The rods are not aggregated so their surface is available for interacting with the environment. SEM images of MgO nanoparticles synthesized by the hydrothermal method are given in Figure 1. (iii) The product is comprised of spherical/oval-shaped nanoparticles which are fully dispersed. Aggregation is not observed in this image. The size of MgO nanoparticles lies in the 30-80 nm range. Contrast is not observed which shows that particles are compact and not hollow from the inside.

DISCUSSION

Contrary to the findings of current study no MRSA was detected by Ren *et al.* (2020) while *S. aureus* (41.5%) was found higher than our findings. On the other hands, current study is closely related to the findings of Li *et al.* (2017) who reported 23.6% prevalence of *S. aureus* while contrary to current study were findings of (Wang *et al.*, 2018) who reported 6.3% *S. aureus* (Liu *et al.*, 2017) reported 61.1% of *S. aureus*. Similarly, Turkey reported 83% *S. aureus* in raw milk (Bartolomeoli *et al.*, 2009) while in Malaysia 66.7% (André *et al.*, 2008), 56% in Brazil (Gundogan and Avci, 2014), Iran 12.4% (Jamali *et al.*, 2015) and Italy reported 41.0% (Traversa *et al.*, 2015) of *S. aureus*. In many parts of the world, milk and dairy products are contaminated by *S. aureus*, especially those strains carrying an MDR phenotype and possessing the ability to produce biofilms and toxins (Cavicchioli *et al.*,

2015; Wang *et al.*, 2018). Dairy products contaminated with *S. aureus* have led to food-borne poisoning outbreaks, demonstrating the public health significance of *S. aureus* (Rong *et al.*, 2017). In addition, dairy farms suffer economic losses as a result of mastitis, since the disease lowers milk production, raises health care costs, and increases culling rates (Hemekinne *et al.*, 2012). Susceptibility to different antibiotics was found 11.1% and 7.41%, against gentamicin and chloramphenicol, respectively (Liu *et al.*, 2017). Higher resistance against vancomycin in current study was in line with findings of (Liu *et al.*, 2021). Jamali *et al.* (2015) showed 5.5% of isolates resistant to cefoxitin. The lack of success of *S. aureus* therapy can be explained by its capacity for intracellular persistence within phagocytes as well as its antimicrobial resistance.

Antibacterial activity in terms of zone of ZOI of ZnO of current study was in line with findings of previous studies (Banoee *et al.*, 2010; Jagathesan and Rajiv, 2018). The latter noted 23 mm ZOI in case of ZnO against *S. aureus* which is bit higher than our study. Similarly, studies regarding MIC values of nanoparticles against *S. aureus* showed similarities while somewhere contradiction was also found. The variation might be due to the differences in the study of *S. aureus* as well as test conditions (Sadiq *et al.*, 2017). In another study, pathogens were completely inactivated after 12 h of incubation (Akbar *et al.*, 2019) which is coinciding findings of current study. According to Jones *et al.* (2008), ZnO-NPs showed a significant decrease in bacterial viability compared to other metallic nanoparticles. According to Ren *et al.* (2009), ZnO- and CuO-NPs show distinct antimicrobial activity in a range of concentrations. Nanoparticles made from zinc oxide, or ZnO, are biocompatible and safe molecules, and they are primarily used as a means to deliver drugs (Vickers, 2017). It is speculated that nanoparticles hold unique mechanism of action different to those of antibiotics and exhibit promising results against (Fisher *et al.*, 2018).

CONCLUSION

S. aureus isolated from commercial dairy was found

to be multi-drug resistant. Gentamicin, enrofloxacin, levofloxacin, and vancomycin were found least effective compared to other antibiotics. Metallic nanoparticles zinc oxide, magnesium oxide, and ferric oxide have shown antibacterial activity against multiple drug resistance *S. aureus* both in well diffusion and broth microdilution assay. Metallic nanoparticles showed antibacterial activity by staging MgO to be more effective followed by Fe₂O₃ and ZnO. The study proposes an *in-vivo* trial for dose optimization and validation of safety parameters.

Statement of conflict of interest

The authors have declared no conflict of interest.

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