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RESEARCH ARTICLE

Resistance Modulation of Dairy Milk Borne *Streptococcus agalactiae* and *Klebsiella pneumoniae* through Metallic Oxide Nanoparticles

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ABSTRACT

Dairy udder and milk equally remain susceptible to bacterial contamination posing significant threat to food safety and security. Adding to this is overlooking some of bacterial pathogens e.g. *K. pneumoniae* and *S. agalactiae* for their infection, drug resistance, and demand of alternative therapeutics. Keeping in view the scenario, current study was planned to assess response of *K. pneumoniae* and *S. agalactiae* against wider range of antibiotics, and resistance modulation through metallic oxide nanoparticles of iron, zinc, and magnesium. Nanoparticles were prepared and characterized as per set protocols. *In-vitro* disc diffusion test for antibiotics, well diffusion for nanoparticles' antibacterial activity, and finally finding minimum inhibitory concentration expressing antibacterial activity was carried out. Parametric and nonparametric statistics was applied to confer outcomes using SPSS version 22 of statistical software at 5% probability. Study found 6 out of 16 antibiotics showing minor variation in means of ZOI against *K. pneumoniae* and *S. agalactiae* indicating limited scope of antibiotics to cover infection against these bacteria. When these bacteria were tested against MgO, ZnO, and Fe_2O_3 nanoparticles, significant antibacterial activity was noted. Fe_2O_3 showed significant higher antibacterial activity as compared to others against both of bacterial strains. Minimum time for maximum activity was found variable for each of nanoparticles against *K. pneumoniae* and *S. agalactiae* with a range of 8^{th}-20^{th} hour of incubation as significant response compared to 4^{th} hour of incubation. Study thus concluded, narrow window of antibiotic susceptibility against *K. pneumoniae* and *S. agalactiae* while wider scope of metallic oxide nanoparticles as antibacterial alternatives.

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INTRODUCTION

Food safety and food security are often breached by bacterial contamination of food items particularly milk which is highly perishable commodity. The dairy udder is primary safety concern which is impaired by clinical and subclinical presentations of infections (Cantekin *et al.*, 2019). The economic losses depend upon microbes involved that may lead to decrease in milk production, affect quality of milk, treatment costs, reduction in growth of newborn, loss of suckling calf, culling of infected animals, discard of milk due to residual time of antibiotics, extra labor costs, and increased threat to public health. Subclinical Mastitis has also significant impact on reproductive performance of dairy animals (Liu *et al.*, 2018). Due to subclinical mastitis, milk production is reduced by 10% to 26% (Dhakal, 2006). Clinical cases are most likely progress to chronic cases (Langoni *et al.*, 2015), a non-reversible outcome of mastitis in most.

Among food borne bacteria, Salmonella spp. and *S. aureus* from poultry (Sulieman *et al.*, 2020; Elmossalamy *et al.*, 2020), and *S. aureus* (Elbayoumy *et al.*, 2020) from mastitis are recently considered as major pathogens. The latter is now being identified in different forms as MRSA

(Parveen *et al*., 2020) and VRSA (Riaz *et al*., 2021) from different sources which indicates its possible transfer at animal-human-environment interface. Other than these bacteria are *E. coli*, *Corynebacterium, S. dysgalactaie, K. pneumoniae* and *S. agalactiae* that affect food and food producing animals. The latter two bacteria are commonly overlooked for its low prevalence as reported in past studies. *K. pneumoniae*, gram negative, is responsible for clinical mastitis (CM) as well as subclinical mastitis (SCM) and becomes sever than to *E. coli* at various circumstances. *S. agalactiae* on the other hands is a highly contagious, obligate bacteria of the cattle mammary gland that creates significant rise in somatic cell count (SCC) and decrease in dairy production (Chen *et al*., 2005). Reduction in milk production is significant attribute of this pathogen so is the reason for its specie name as "*agalactia*" means "no milk production". Prevalence of these bacteria varies from country-to-country e.g *Klebsiella pneumoniae* was found 20-40% in china (Han and Zhang, 2022), 30-40% in Pakistan (Ahmad and Khan, 2022) and 60% in Egypt (El-Razik and Arfa, 2021).

Another threat to the food safety and security is rise in antimicrobial resistance. It is pertinent to note that antibiotics are non-judiciously used in animal treatment which are directly and indirectly enhancing drug resistance and compromising one health (Mullard, 2018). Alternatives are required to either replace antibiotics or to lower burden of antibiotics. Among such alternatives are the nanoparticles whose mode of action is perceived to be different to those of antibiotics while they can support reduction in drug resistance by serving as vehicle for antibiotics, synergistic effects with antimicrobials, and itself antimicrobials. Nanoparticles with a diameter of more than 10 nm engage with the cell wall and membrane, triggering cell death and disintegration (Niño-Martínez *et al.,* 2019). Moreover, metals like Zn, Iron, Mg, Mn, etc. are used as trace elements in various feed formulations as these are vital for various body functions. Metallic oxide nanoparticle of zinc, iron, and magnesium have been reported against other bacteria like *S. aureus* and *E. coli* (Anwar *et al*., 2020; Lodhi *et al*., 2021) while studies are scarce for their use against *K. pneumoniae* and *S. agalactiae*. Using these nanoparticles in feed may also play role as nutraceutical for optimum health and production of animals.

The two-dimensional studies are required to cope up emerging pathogens of dairy milk 1 (to find regular epidemiological pattern of existing bacterial contaminants along with their response to antibiotics, 2) to explore alternatives to antimicrobials in addition to evidence based novel drug combinations. Current study thus focused on assessment of metallic oxide nanoparticles as an effective alternative against *K. pneumoniae* and *S. agalactiae*, the emerging bacterial contaminants of dairy milk.

MATERIALS AND METHODS

Procurement of *K. pneumoniae* and *S. agalactiae:* Commercial dairy settled in district Khanewal was approached for aseptic collection of milk samples following standard protocols as mentioned by national mastitis council (NMC, 1996). Milk samples were screened for subclinical mastitis by surf field mastitis test (SFMT) as per protocol described by Muhammad *et al*. (2010) and positive samples, maintaining cold chain, were shipped to Laboratory of Department of Food Science and Technology, Cholistan University of Veterinary and Animal Sciences, Bahawalpur. Samples were processed as per directions of Bergey's manual of determinative bacteriology (Bergey and Holt, 1994).

Estimation of response *K. pneumoniae* and *S. agalactiae* against antibiotics: An activated growth of *K. pneumoniae* and *S. agalactiae* bacteria adjusted at 10^8 CFU/mL was aseptically swabbed on Mueller Hinton (MH) agar. Total of n=16 antibiotics were applied aseptically on (MH) agar (Bauer, 1996). The assembly was kept at 37°C/24hrs while zones of inhibition (ZOIs) were measured in millimeters (mm) by vernier calipers. For confirmation of findings, test was run thrice before processing of data for analysis.

Preparation of metallic oxide nanoparticles: Metallic oxide nanoparticles were prepared by chemical method as described by (Parashar *et al*., 2020)**.** Briefly, $MgCl_2 .6H_2O$, sodium dodecyl sulfate, NaOH, Zn $(CH3\ COO)_2\ 2H_2\ O)$, polyethylene glycol, $NH_2\ CONH_2$, $FeCl_2\ .4H_2O)$ and ammonia were used in preparation process. The chemicals were bought from Sigma-Aldrich USA. Prepared metallic oxide nanoparticles (Mgo, ZnO, Fe_2O_3) were characterized by XRD (X-ray diffraction) and SEM (Scanning Electron Microscopy) analysis to assess shape and dimensions of nanoparticles.

Antibacterial assays for nanoparticles

Well diffusion assay (Bauer 1996): Working solution of nanoparticles was prepared as 1mg/mL which was poured into wells prepared in Mueller Hinton agar through well borer (6-8mm). Before application of nanoparticles, activated culture of *K. pneumoniae* and *S. agalactiae* set at 10^8 CFU/mL was swabbed homogeneously on agar. Following incubation of 24hr at 37°C, ZOIs were measured in millimeters (mm) by vernier calipers against each nanoparticle for both bacteria.

Broth Microdilution method (CLSI, 2018): Briefly describing, an activated growth of 10^5 CFU/mL was aseptically poured in 96 wells sterile microtitration plate. The plate was first filled in by nutrient broth and then two-fold diluted nanoparticles were starting from 10mg/mL onwards until 11^{th} column. The trial was conducted in triplicate to rule out any extraneous error. Optical density was measured at 695nm wavelength through ELISA reader. Net OD values was calculated by subtracting OD value after incubation from 0hr incubation. In this trial, net OD value was calculated every 4hrs of incubation until 24hrs**.** MIC was measured by comparing net OD value with cut off value. The OD value less than or equal to cut off value was considered as inhibition of growth. Two-dimensional analysis was performed for this trial:

1. Comparison of MICs of different nanoparticles at each segment of incubation
2. Assessment of minimum time interval for significant reduction in MIC for each nanoparticle

The later can be perceived as effect of incubation period on MICs of each nanoparticle.

Statistical analysis: Descriptive statistics were applied to measure means and standard deviations for ZOIs of antibiotics while parametric tests (ANOVA with Tukey's test as post hoc test) were applied to conclude difference among ZOIs, and MICs expressed by nanoparticles against *K. pneumoniae* and *S. agalactiae*. SPSS version 22 of statistical software was applied to analyze data setting probability 5%.

RESULTS

Empirical estimation of susceptibility response of bacteria against antibiotics: The study showed considerable variation in zone of inhibitions for each of antibiotics (Table 1). Out of 16 antibiotics, mean of ZOI of both bacteria against 6 antibiotics (Enrofloxacin, Clindamycin, tetracycline, gentamicin, Teicoplnin, Tobramycin) was found near to each other while there was higher standard deviation (Table 1). Mean zones of inhibition of rest of 10 antibiotics differed more than half with each other. Antibiotics showing lower minimum standard deviation in ZOI indicate uniform response while greater variation speak of multiple categories of susceptibility (Fig. 1).

Characterization of nanoparticles: ZnO nanoparticles were polygonal star like arranged randomly with distinct borders and spikes not equal in length which was 4-8µm in diameter. Fe_2O_3 nanoparticles were rod shaped having 2µm length and 100nm width. MgO nanoparticles were 30-80nm range and spherical cum oval shaped but densely dispersed. The detailed image and description can be seen in previous work (Zaheer *et al*., 2022).

Antibacterial assays of metallic oxide nanoparticles: ZOIs exhibited comparison of antibacterial potential of different nanoparticles while MICs expressed comparisons among nanoparticles at each incubation period and effect of incubation time on minimum inhibitory concentration for each nanoparticle (Table 2).

Zone of inhibitions (ZOIs): The study found significant difference (P<0.05) among three nanoparticles regarding zone of inhibitions (ZOI) measured in millimeters (mm) against *K. pneumoniae* (Table 2). Highest ZOI (mm) were found in case of Fe_2O_3 followed by ZnO and MgO presenting 14.50±0.50, 10.33±0.58, and 6.47±0.50mm, respectively. It is evident from the findings that Fe_2O_3 gave rise to more than 55.30% higher antibacterial effect than MgO while this percentage increase was nearly 28.76% when compared with that of ZnO. On the other hands, ZnO produced 37.37% higher ZOI compared to that of MgO against *K. pneumoniae* in current study. Response of nanoparticles against *S. agalactiae* was found different to that of *K. pneumoniae* in that Fe_2O_3 and ZnO nanoparticles did not show significant difference (P>0.05) while MgO showed on the other hands significantly (P<0.05) lower ZOI as compared to Fe_2O_3. ZnO and MgO differed non-significantly (P>0.05) with each other. Percentage increase in ZOI of Fe_2O_3 was observed 17.02 and 3.12% compared to that of ZnO and MgO, respectively. However, 14.35% increase in ZOI was observed in case of ZnO when compared with that of MgO showing in Table 2.

Table 1: Comparative zones of inhibition (mm) produced by bacteria against different antibiotics

Antibiotic	Concentration	Mean ±Standard deviation(mm)	
		Klebsiella pneumoniae	*Streptococcus agalactiae*
Oxacilin OX5	5µg	25±4.58	1.33±0.94
Cephazoline Kz30	30µg	3±5.19	12±7.12
Enrofloxacin ENR5	5µg	30±3.46	33.33±2.49
Clindamycin DA10	10µg	27.5±6.06	22±5.89
Levofloxeon LEV5	5µg	23.5±15.89	33.33±2.494
Vancomycin VA30	30µg	18±3.74	4.33±0.47
Azithrmycin AZM15	15µg	15±13.89	29.33±0.94
Tetracycline TE30	30µg	17.5±9.42	20.67±4.7
Gentamicin CN10	10µg	27±1	29.67±4.64
Coistin sulfate CS10	10µg	6±1.41	2.67±1.89
Teicoplnin TEC30	30µg	17.5±5.45	20±0
Tobramycin TOB10	10µg	26.5±4.33	28.67±2.49
Tetracycline TEC30	30µg	17.5±5.54	2.67±0.94
Meropenem MEM10	10µg	6.5±0.87	35.33±0.94
Enefloxacin ENR10	10µg	32±2	1.33±0.94

Table 2: Zone of inhibitions (mm) of nanoparticles against bacteria

Nanoparticles	Mean ±Standard deviation (mm)	
	Klebsiella pneumoniae	*Streptococcus agalactiae*
Fe_2O_3	14.50±0.50[a]	13.16±2.62[a]
ZnO	10.33±0.58[b]	12.750±2.22[ab]
MgO	6.47±0.50[c]	10.92±0.90[b]

Different superscripts within column indicate significant difference (P<0.05)

Minimum inhibitory concentrations (mg/mL): *Streptococcus agalactiae*: The study found significant (P<0.05) lower MIC (0.3125±0.000 mg/mL) after 24 hours of incubation with *S. agalactiae* in case of Fe_2O_3 compared to that of ZnO (0.6250±0.000 mg/mL) while this difference stood non-significant (P>0.05) when compared with MgO (1.667±0.722mg/mL) (Table 3). To assess effect of incubation period for significant reduction in MICs showed in that the post 12 hours of incubation remained non-significant (P>0.05) in case of ZnO, while for MgO and Fe_2O_3 the post 8th hour of incubation period was found non-significant (P>0.05) indicating minimum time of maximum efficacy.

***Klebsiella pneumoniae*:** Minimum inhibitory concentration (MIC) of different nanoparticles (after 24 hours) against *K. pneumoniae* presented significant (P<0.05) lower values (0.2083±0.0902 mg/mL) in case of Fe_2O_3 compared to ZnO (0.6250±0.000 mg/mL) while with MgO (1.042±0.361 mg/mL) the difference stood non-significant (P>0.05), (Table 4). The same was observed after 20 hours incubation. However, comparison of nanoparticles at 4^{th,} 8th, and 16th hour of incubation did not come up with significant difference (P>0.05). On other hands, effect of incubation period for each nanoparticles presented variable response in that after there was significant difference (P<0.05) of MICs at 16th hour of incubation compared to the previous hours for ZnO nanoparticle. MgO showed significant difference (P<0.05) at 20th hour of incubation compared to that of 4th hour incubation (Table 3 and 4).

DISCUSSION

Drug resistance by *K. pneuomonie* and *S. agalactiae*: Several studies have reported findings in line with current study while some others deviated which is due to previous exposure of antibiotics, hygiene, farm consultancy

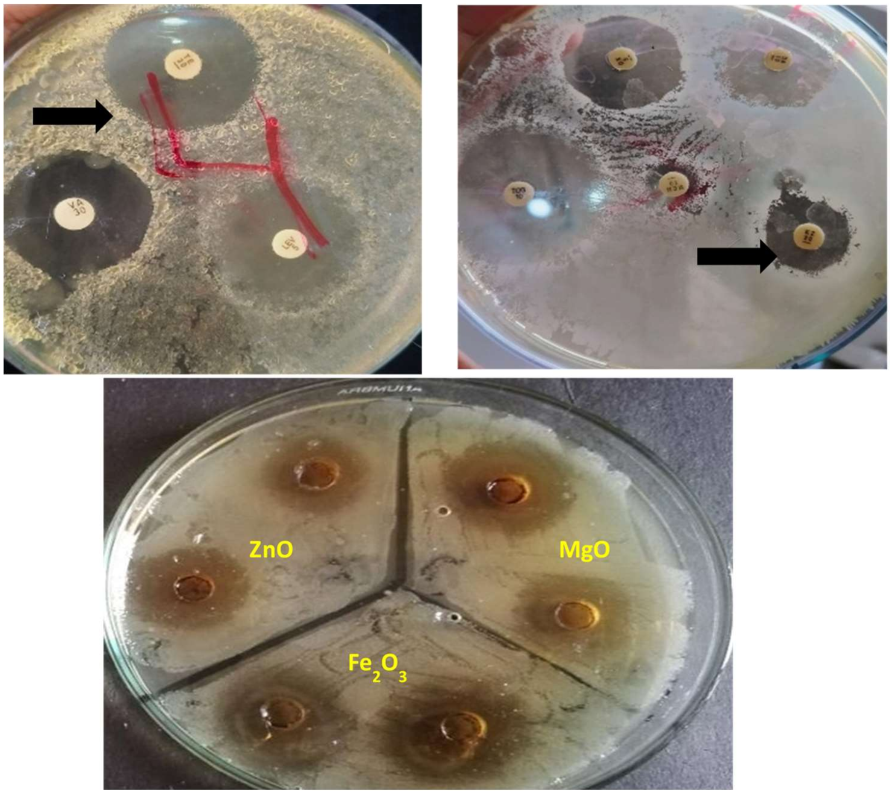

Fig. 1: Zone of inhibition of antibiotics (upper) and nanoparticles (lower) against bacteria; Arrows (black) are indicating zone of inhibitions on petri plates.

Table 3: Minimum inhibitory concentration (mg/mL) of different nanoparticles against *Streptococcus agalactiae*

Nanoparticles	4 Hours	8 Hours	12 Hours	16 Hours	20 Hours	24 Hours
ZnO	$^{A}8.33\pm2.89^{a}$	$^{AB}6.67\pm2.89^{a}$	$^{ABC}4.167\pm1.443^{a}$	$^{BC}2.083\pm0.722^{a}$	$^{C}1.042\pm0.361^{a}$	$^{C}0.6250\pm0.0000^{a}$
MgO	$^{A}6.67\pm2.89^{a}$	$^{AB}4.167\pm1.443^{a}$	$^{AB}3.333\pm1.443^{a}$	$^{B}2.500\pm0.000^{ab}$	$^{B}2.500\pm0.000^{b}$	$^{B}1.667\pm0.722^{ab}$
Fe_2O_3	$^{A}4.167\pm1.443^{a}$	$^{AB}3.333\pm1.443^{a}$	$^{ABC}2.500\pm0.00^{a}$	$^{BC}1.042\pm0.361^{b}$	$^{C}0.521\pm0.180^{b}$	$^{C}0.3125\pm0.000^{b}$

Different superscripts (small alphabets) within column indicate significant difference (P<0.05) for comparison among nanoparticles: Different superscripts (capital alphabets) within row indicate significant difference (P<0.05) for comparison among time intervals.

Table 4: Minimum inhibitory concentration (mg/mL) of different nanoparticles against *Klebsiella pneumoniae*

Nanoparticles	4 hours	8 hours	12 hours	16 hours	20 hours	24 hours
ZnO	$^{A}4.167\pm1.443^{a}$	$^{AB}3.333\pm1.443^{a}$	$^{ABC}2.500\pm0.000^{a}$	$^{BC}1.667\pm0.722^{a}$	$^{BC}1.042\pm0.361^{a}$	$^{C}0.6250\pm0.0000^{a}$
MgO	$^{A}4.167\pm1.443^{a}$	$^{A}4.167\pm1.443^{a}$	$^{AB}2.083\pm0.722^{a}$	$^{AB}2.083\pm0.722^{a}$	$^{B}1.250\pm0.000^{ab}$	$^{B}1.042\pm0.361^{ab}$
Fe_2O_3	$^{A}4.167\pm1.443^{a}$	$^{A}3.333\pm1.443^{a}$	$^{AB}2.083\pm0.722^{a}$	$^{AB}1.667\pm0.722^{a}$	$^{B}0.6250\pm0.0000^{b}$	$^{B}0.2083\pm0.0902^{b}$

Different superscripts (small alphabets) within column indicate significant difference (P<0.05) for comparison among nanoparticles: Different superscripts (capital alphabets) within row indicate significant difference (P<0.05) for comparison among time intervals.

with professional veterinarian, mastitis control program and health of animal. In Europe and United States, Klebsiella spp. isolated from mastitis cases showed only low levels of resistance to tetracycline (5.6–19.5%) and β-lactam antibiotics (0–6.9%). In China, a study revealed relatively high rates (10–32%) of resistance to cefquinome, kanamycin, ceftiofur, polymyxin B, and tetracycline among Klebsiella spp (Song and He, 2022). The antibiogram pattern of *S. agalactiae* revealed greater resistance to chloramphenicol (90 percent), following by clindamycin (78 percent), with enrofloxacin (72 percent) and Gentamicin being the most susceptible antibiotics. *S. agalactiae* antibiogram pattern revealed great resistance.

Zone of inhibitions by nanoparticles against *K. pneuomonie* and *S. agalactiae*: The strong antimicrobial activity of nanoparticles was observed against gram-positive bacteria compared to gram-negative bacteria. 09.17±0.23 and 23.41±0.50 mm, respectively. In Gram-positive bacteria, the lowest zones of inhibition were observed against *S. aureus*, and the highest zone of inhibition was noticed against *E. faecalis* – MTCC 439. The zone of inhibition against Gram-negative bacteria was observed in the range of 04.24±0.31 – 14.35±0.72 mm. In Gram-negative bacteria, the highest zone of inhibition was observed against *E. coli* – MTCC 41, and the lowest zone of inhibition was noticed against *K. pneumoniae* – ATCC 13883 (Rosaiah and Mangamuri, 2022). Florfenicol coupled with silver nanoparticles showed potential efficacy against both of gram positive and gram-negative bacteria (Youssef *et al*., 2020).

Minimum Inhibitory concentration of nanoparticles: The minimum inhibitory concentration (MIC) showed wider range of antibacterial activity when silver/copper nanoparticles in suspension (20–300 mg/L) (Qi and Xu (2004) were used against various pathogens. Current study revealed that antibacterial activity results of ZnO were 1.042±0.361 mg/mL which is close to the study of Siddiqi and Husen (2018) who found 1.8±0.1mg/mL. In current study antibacterial activity of MgO remained close to the findings of Nguyen *et al*. (2018) who reported 1.68±0.22 mg/mL. According to the current study findings of Fe_2O_3 were close to the study of Moradlou *et al*. (2019) who found in their study 0.4 ± 0.1mgL of Fe^{3+} after 24h of incubation.

Conclusion: The study concluded decreasing trend of antibiotics' efficacy to treat *S. agalactiae* and *K. pneumoniae* infection. Metallic oxide nanoparticles (MgO, ZnO, and Fe_2O_3) on the other hands showed significant antibacterial potential against both of bacteria. Iron oxide nanoparticles among other showed higher antibacterial potential followed by ZnO and MgO. Significant antibacterial activity of nanoparticles at early hours indicated their best utilization in case of bacterial outbreak. Study thus proposed that further *in-vivo* and field trials to be conducted for standardization of safety, efficacy, and stability parameters.

Conflict of interest: Authors declare no conflict of interest

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