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Antibacterial Activity of Zinc Oxide Nanoparticles Against Some Multidrug-Resistant Strains of *Escherichia coli* and *Staphylococcus aureus*

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ABSTRACT

Mastitis is the most common disease affecting dairy farms, causing inflammation of the mammary glands and reducing milk quality and production. Incorrect use of antibiotics leads to serious problems in veterinary medicine due to the emergence of multidrug resistant bacteria. The antibiotic resistant bacteria are considered the big problems worldwide. This study aims to isolate pathogenic bacteria from raw milk samples taken from subclinical mastitic cows. In addition, estimation of the minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of Zinc oxide (ZnO) nanoparticles was also done against *Staphylococcus aureus* and *Escherichia coli* isolated from subclinical mastitic cows. Out of 28 milk samples, 85.7% samples showed positive bacterial isolation while 33.3% samples had single isolates while, 66.7% showed mixed isolates and 4 samples showed negative bacterial isolation. The obtained results showed that the resistance ratio of *S. aureus* and *E. coli* to different antibiotics using the disc diffusion technique was highest for Amoxicillin, then Tetracycline, and finally Nalidixic acid. Results of the MIC of ZnO nanoparticles against isolated *E. coli* and *S. aureus* were 31.3µg/mL and 7.8µg/mL, respectively. While the MBC was 62.5µg/mL for *E. coli* and 15.6µg/mL for *S. aureus*. It was found that ZnO nanoparticles could be used instead of common antibiotics to treat subclinical mastitis in cows caused by *E. coli* and *S. aureus* and to overcome the problems of multidrug resistant bacteria.

Key words: ZnO nanoparticles, Mastitis, Antibacterial activity, *E. coli*, *S. aureus*.

INTRODUCTION

Mastitis is a serious disease characterized by mammary gland inflammation that is caused by either physical trauma or bacterial infection (Du et al. 2022). Subclinical mastitis is a disease widely spread among dairy farms and it is responsible for great economic losses due to the decreased milk production and reduced milk quality due to antibiotic residues (Elbayoumy et al. 2020). In addition, mastitis affects human health as a result of consuming contaminated milk with pathogenic bacteria and their toxins. The causative agents include gram negative and gram positive bacteria and can be either environmental caused by pathogens present in environment such as *Streptococcus uberis*, *E. coli*, *Enterococcus spp.*, and *Klebsiella spp.* or contagious as *Streptococcus agalactiae*, *S. aureus*, *Mycoplasma spp* and *Corynebacterium bovis*, due to infectious pathogens that spread from one cow to another and that mainly occur during the milking process (Abebe et al. 2016; Cheng and Han 2020). The disease occurs in three forms: Subclinical, Clinical and Chronic mastitis. Subclinical mastitis is the most famous form and leads to decreases in the produced milk without the appearance of clinical symptoms or milk abnormalities (Pascu et al. 2022). The usual treatment of mastitis by antibiotics such as Cloxacillin, Penicillin, Streptomycin, Tetracycline and Ampicillin is considered very expensive to some extent and also extensive use of antibiotics in the treatment of mastitis in cows increases the risk of transmission of antibiotic resistance to humans (Tenover 2006; Dijkhuizen and Schepers 2017; Pascu et al. 2022). The continuous emergence of antibiotics resistant bacteria has directed the scientists to use the nanoparticles (NPs) as antibacterial agents based on their unique chemical and physical properties and its effectiveness in the treatment of most bacterial infections including the

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drug resistant bacteria (Slavin et al. 2017; Su et al. 2020). Some NPs have been approved as antibacterial agents including zinc, silver and gold, each with different activities and properties. The size of nanoparticles used to treat infections is less than 100 nanometers (Rajendran et al. 2013; Siddique et al. 2013; Marwah et al. 2017). ZnO nanoparticles are the most widely used nanoparticles in biological applications due to their low toxicity and excellent biocompatibility. ZnO nanoparticles are of great importance in medicine, particularly in the area of antibacterial and anticancer due to their strong ability to stimulate excess reactive oxygen species (ROS), release zinc oxygen and induce apoptosis (Jiang and Cai 2018). Recently, scientists reported that the use of ZnO nanoparticles in the treatment and prevention of mastitis depends on its great ability to enhance the immune response in dairy cows and increase their milk yield. So the ZnO nanoparticles could be added as food additives to animals due to their properties as antibacterial and immune enhancers (Beyth et al. 2015; Hamilton and Wenlock 2016; Hill and Li 2017).

This study aims to isolate pathogenic bacteria from raw milk samples obtained from subclinical mastitic cows. In addition to examine the antibacterial effects of ZnO nanoparticles against *S. aureus* and *E. coli*.

MATERIALS AND METHODS

Ethical Approval

All procedures performed according to Egyptian ethical standards of National Research Committee.

Sample Collection

Samples were selected randomly from different bovine farms located in New Valley governorate; the age of cows ranged from 5 to 7 years old. Visual inspection and physical examination were done to evaluate the health condition of cows. Also, milk was examined by California mastitis test to exclude any cow that showed abnormal milk secretion or health defects.

A total of 28 milk samples were obtained from lactating healthy cows with normal milk secretions, 10 mL of milk samples were collected and placed in sterile tubes and put in an ice bag then transported immediately to the laboratory for bacteriological examination. All culture media were obtained from Oxoid, Ltd., Basingstoke, UK.

Bacteriological Isolation and Identification

About 0.01mL from each milk sample was cultivated on different bacteriological media (MacConkey agar, 5% sheep blood agar, Edward's agar medium, and mannitol salt agar) then they were incubated at 37°C for 48hours. The suspected colonies were tested for morphological characteristics including the colony size, shape, color, pigment production, colony texture (smooth or rough), type of hemolysis onto blood agar and metabolic activity onto MacConkey agar (lactose fermenter or non-lactose fermenter). Bacterial smears were made from the colonies and stained with gram stain then examined microscopically. The biochemical identification was examined according to Quinn et al. (2002).

Antibacterial Sensitivity Test

The susceptibility of the most common bacteria (*E. coli, S. aureus*) to different antibiotics was done by disk diffusion method according to Gloria et al. (2003) using commercial disks (Hi Media Laboratories Pvt. Ltd., India), to determine the susceptibility of the two well defined isolates (*E. coli* and *S. aureus*) by using Amoxicillin (1µg), Cefotaxime (30µg), Tetracycline (30µg) and Nalidixic acid (5µg).

Laboratory Preparation of Zinc Oxide Nanoparticles

Briefly, added 1M NaOH solution to 500mL Zinc sulfate heptahydrate at a rate of 0.2mL/min with continuous stirring until the pH became strongly alkaline (pH 12) (Rajendran et al. 2013; Siddique et al. 2013). The obtained product was purified by washing with sterile distilled water and centrifugation at 18000 x g for 20min. The final product was then washed again with ethanol. Finally, sonochemical treatment (Liu et al. 2009; Rajendran et al. 2013) and drying were carried out to obtain white powder of ZnO nanoparticles (Fig. 1) with average size of 30 nm. By using an electron microscope, the ZnO nanoparticles appeared spherical with some aggregation (Fig. 2). The ZnO nanoparticles stock solution was prepared by adding distilled water to reach a final concentration of 1000µg/mL, then the stock solution stored at 4°C until used.

Evaluation of ZnO Nanoparticles Antibacterial Effects against *S. aureus* and *E. coli* Isolates

The minimal inhibitory concentration (MIC) of ZnO nanoparticles against *E. coli* and *S. aureus* isolates was determined by using the method of microdilution (Andrews 2001). The bacterial isolates were inoculated in Muller Hinton broth at 37°C for 24 hours. The turbidity of obtained cultures was adjusted to 0.5 Mcfarland (1.5×10^8 CFU/mL), 50µL from diluted cultures were poured into 12 wells of 96 well microtiter plate. Then 50µL of Zinc oxide nanoparticles stock solution was added to the first well followed by two-fold serial dilution to obtain different ZnO nanoparticles concentrations (1000, 500, 250, 125, 62.5, 31.3, 15. 6, 7.8, 3.9, 1.95, 0.98, and 0.49µg/mL). Then the 96 well plate was incubated at 37°C for 24hours, visual examination of the incubated plate was done by turbidity detection and changes observation. A control positive well was having the tested culture and a negative control one that containing only sterile broth medium. The MIC is known as the least ZnO nanoparticles concentration that inhibited bacterial growth after 24 hours of incubation.

50µl from all wells that showed no visible growth or turbidity was cultivated on Muller Hinton agar and incubated at 37°C for 24 hours. The MBC (Minimal bactericidal concentration) is known as the least ZnO nanoparticles concentration that can prevent bacterial growth.

Statistical Analysis

Statistical analysis of the resulted data was performed using Minitab Statistics for Windows®, release14. All data were subjected to descriptive analysis at quality assurance Statistical analysis unit (VACSERA), promoting mean+ SD, all results were considered significant at $P<0.01$.

Fig. 1: Zinc oxide nanoparticles purified powder, solid, white, odorless powder and molecular weight: 81.38g/mol.

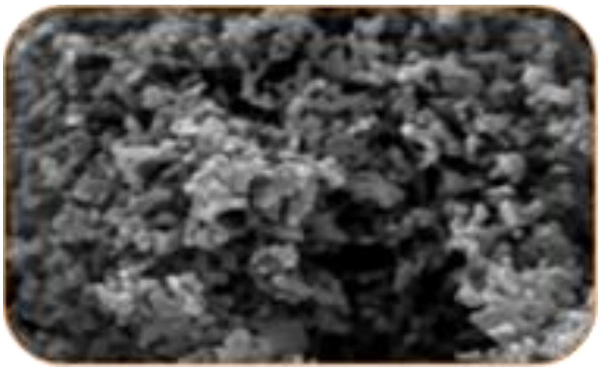

Fig. 2: Zinc oxide nanoparticles by electron microscope.

Table 1: Bacterial isolates of milk samples obtained from apparently healthy dairy cows

Bacterial isolates	Positive isolation		
	Single	Mixed	Total
E. coli	4 (16.7)	9(37.5)	13(54.2)
S. aureus	3(12.5)	5(20.8)	8(33.3)
Streptococcus spp.	1(4.2)	2(8.3)	3(12.5)
Total	8(33.3)	16 (66.7)	24(100)

Values in parenthesis indicate percentage.

Table 2: Biochemical identification of *E. coli* and *S. aureus* isolates obtained from milk samples of apparently healthy cows

Biochemical test	*E. coli*	*S. aureus*
Catalase	+	+
Methyl red	+	-
Indole test	+	-
Voges Proskauer test	-	-
Simmon's citrate test	-	-
Coagulase	-	+
Acetone	-	+
Oxidase	-	+
D-mannitol fermentation	-	+

RESULTS

The prevalence of bacteria isolated from subclinical mastitis, out of 28 milk samples, 24 (85.7%) samples showed positive bacterial isolation. Out of 24, 8 (33.3%) samples had single isolates while 16 (66.7%) showed mixed isolates and 4 milk samples showed negative bacterial isolation (Table 1). The tested milk samples showed that *S. aureus* and *E. coli* were the most common isolates. These isolates were subjected to microbiological examination for morphological characteristics followed by biochemical identification (Table 2).

As it is clear from Table 3, the results of antibiotic resistance of the 8 isolated strains of *S. aureus* and 13 isolated stains of *E. coli* using method of disc diffusion were 8 (100%) and 13 (100%), respectively for Amoxicillin, 1(12.5%) and 3(23%), respectively for Cefotaxime, while, in case of Tetracycline the results of resistance were 6 (75%) for *S. aureus* and 12(92.3%) for *E. coli*. Finally, the resistant ratio of *S. aureus* and *E. coli* against Nalidixic acid were 2(25%) and 4(30.8%), respectively.

As cleared from Table 4 that the MIC of ZnO nanoparticles against isolated *E. coli* and *S. aureus* were 31.3 and 7.8µg/mL, respectively. While the MBC was 62.5 and 15.6µg/mL for *E. coli* and *S. aureus*, respectively. All data showed statistically significant value at $P<0.01$ which interpreted as significant effect of ZnO nanoparticles against both *E. coli* and *S. aureus*.

DISCUSSION

Bovine mastitis was one of the most common disease that affecting dairy farms in Egypt and all over the world causing great economic losses due to a marked reduction in milk production (He et al. 2020). The antibiotics are usually used for treatment of mastitis in bovine, these methods for disease control have a lot of disadvantages including emergence of antibiotic resistant bacteria, antibiotics residues in milk and low cure rate (Hussein 2008; Wanjala et al. 2020). *S. aureus* and *E. coli* were found to have significant resistance to different antibacterial agents (Jahan et al. 2015). ZnO nanoparticles have been evaluated for development of new generation of nanoantibiotics against pathogenic bacteria to overcome the problem of multidrug resistant (Makabenta et al. 2021).

In the present study, pathogenic bacteria isolated from subclinical mastitic cows, out of 28 milk samples, 24 samples showed positive bacterial isolation with prevalence of 85.7%. The obtained ratio was lower than that obtained by Abed et al. (2021), 90% in Egypt and more than that obtained by Al-harbi et al. (2021) 55% in Australia. At the same time, the prevalence recorded in the present study is close to that given by Reta et al. (2016), 84.3% in Ethiopia. The high prevalence of subclinical mastitis might be due to bad hygienic condition, milking machines contamination, poor housing and history of mastitis (Abed et al. 2021).

Out of 24 milk samples, 8 samples (33.3%) had single isolates while 16 (66.7%) showed mixed isolates and 4 milk samples showed negative bacterial isolation (Table 1). The results of mixed infections seem to be more than that in single infection in subclinical mastitis. The obtained results were in agreement with that obtained by Abed et al. (2021), who found that the co infections (64%) were higher than in single infection (26%). Moreover, our results disagreed with those of Zeinhom et al. (2013), who recorded the higher prevalence of single infections (51.6%) than mixed infection (16.1%). The *E. coli* infection was the most prevalent (54.2%) followed by *S. aureus* (33.3%) and *Streptococcus* (12.5%). These findings agree with the previous studies (Ahmed et al. 2018; Abd El-Tawab et al. 2019; Mohammed et. al. 2020; Abed et al. 2021). The biochemical identification was performed on suspected colonies of *E. coli* and *S. aureus* as demonstrated in Table 2.

Table 3: Antimicrobial resistance findings for isolated *S. aureus* and *E. coli*

Bacterial isolates	Antibiotics															
	Amoxicillin				Cefotaxime				Tetracycline				Nalidixic acid			
	R		S		R		S		R		S		R		S	
	No	%	No	%	No	%	No	%	No	%	No	%	No	%	No	%
S. aureus (8)	8	100	0	0	1	12.5	7	87.5	6	75	2	25	2	25	6	75
E. coli (13)	13	100	0	0	3	23	10	77	12	92.3	1	7.7	4	30.8	9	69.2

R: Resistant; S: Susceptible; No: Number

Table 4: The MIC and MBC of ZnO nanoparticles against *S. aureus* and *E. coli* isolated from raw milk samples obtained from subclinical mastitic cows

Bacterial isolates	MIC(µg/mL)	MBC(µg/mL)
E. coli	31.3	62.5
S. aureus	7.8	15.6

MIC: Minimum Inhibitory Concentration MBC: Minimum Bactericidal Concentration

It is clear from Table 3 that the *S. aureus* and *E. coli* are highly resistant to amoxicillin followed by tetracycline and nalidixic acid. The novel methods such as bacteriophage therapy, nanomedicines and vaccination were used to overcome the problem of antibiotics resistant bacteria (Ameen et al. 2019).

Nanoparticles have been shown to be a safe and effective alternative treatment against many pathogenic bacteria without development of antibiotics resistance. (Jain et al. 2009; Alekish et al. 2018). ZnO nanoparticles have achieved more attention due to its unique chemical, optical and electrical properties. Also, ZnO nanoparticles have wide spectrum of antibacterial activities depending on concentration, size, and stability of nanoparticles in the growth medium (Baxter and Aydil 2005; Raghupathi et al. 2011).

The antimicrobial effects of ZnO nanoparticles were evaluated by measuring the MIC and MBC against drug resistant *E. coli* and *S. aureus* strains isolated from subclinical mastitic cows. As demonstrated in Table 4, the MIC of ZnO nanoparticles against isolated *E. coli* and *S. aureus* were 31.3 and 7.8µg/mL, respectively. While the MBC was 62.5 and 15.6µg/mL for *E. coli* and *S. aureus*, respectively. These results are close to the previously reported findings (Ibrahem et al. 2017; Alekish et al. 2018). In addition, our results were in agreement with previous studies done by Karvani and Chehrazi (2011), Alekish et al. (2018) and Walaa et al. (2021), who showed that the gram positive are more sensitive than gram negative bacteria to ZnO nanoparticles. Moreover, the obtained results disagree with previous study of Slavin et al. (2017) who showed that the ZnO nanoparticles have a great antibacterial activity on gram negative bacteria (*K. pneumonia* and *E. coli*) than gram positive bacteria (*S. aureus*). The dissimilarity in the cell wall structure between the gram negative and gram-positive bacteria may be responsible for this phenomenon (Slavin et al. 2017). The present study illustrated that the antibacterial activities of ZnO nanoparticles is highly dependent on its concentration. These finding agree with Palanikumar et al. (2014) who mentioned that the concentration and size of ZnO nanoparticles are the most important factors affecting the antimicrobial action of these particles.

Conclusion

Our study demonstrated the increase in the prevalence rate (85.7%) of pathogenic bacteria obtained from milk samples of subclinical mastitic cows. The presence of a high prevalence rate in our study may be due to bad hygiene, poor housing, and contamination of milking machines. The most recovered bacterial pathogens from subclinical mastitis were *E. coli* followed by *S. aureus* and *Streptococcus spp*. The *S. aureus* and *E. coli* strains were found highly resistant to Amoxicillin followed by Tetracycline then Nalidixic acid. The results of this study demonstrated that the ZnO nanoparticles have potential antibacterial activity against multidrug resistant *E. coli* and *S. aureus* isolates. Moreover, it was concluded that the antibacterial effects of ZnO nanoparticles were increased when the particle concentration increased in the media. Also, it is recommended to use ZnO nanoparticles instead of common antibiotics for the treatment of subclinical mastitis caused by *S. aureus* and *E. coli* in cows. Finally, they need further studies to evaluate the use of ZnO nanoparticles in the inactivation of *E. coli* and *S. aureus* as a step for vaccine preparation for controlling of subclinical mastitis in cows.

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Author's Contribution

All authors contributed equally.

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