Effect of Zinc Oxide Nanoparticles on *Staphylococcus Aureus* Isolated From Cows' Mastitic Milk

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A B S T R A C T

The current spreading of nanomaterial applications supports the search for further possible functions of theses diminutive particles. The antibacterial potentiality of zinc oxide (ZnO) nanoparticles (NPs) against *S. aureus* causing mastitis was evaluated using qualitative and quantitative assays. These tests were performed in nutrient broth and nutrient agar following standard methods. In addition, the effect of different concentrations of ZnO nanoparticles on the growth of *S. aureus* was measured. MIC was determined using six different concentrations of ZnO nanoparticles including 16, 8, 4, 2, 1 and 0.5 mg/ml. The MIC value *S. aureus* was 0.5 mg/ml. The results showed that ZnO nanoparticles have antibacterial inhibition zone of 29 mm at the concentration of 10 mg/ml against *S. aureus*., and the antibacterial activity of ZnO nanoparticles increased with increasing powder concentration in vitro.

Key words: ZnO nanoparticle, ***Staphylococcus aureus***, Minimum Inhibitory Concentration (MIC).

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1. INTRODUCTION

Recently, nanotechnology has become increasingly important in the biomedical and pharmaceutical areas as alternative antimicrobial strategy due to re-emergence infectious diseases and the appearance of antibiotic-resistant strains among a variety of disease-causing bacteria pose a serious threat to public health worldwide (Desselberger, 2000).

Mastitis is an important disease affecting dairy animals resulting in high economic losses to dairy producers and dairy industry as a whole estimated by 1.7 billion dollars anally in USA (Crist *et al.*, 1997), so it is considered to be the most costly disease all over the world (Sory *et al.*, 2005).

These high losses are due to reduction in milk yield, milk becomes unfit for human consumption and treatment with costly antibiotics with cure rate 60% in field condition with a problem of milk residues (Correa and Marine, 2002) and culling of infected animals and in some cases may end by death of the animal. (Santos *et al.*, 2004). The economic impact of clinical mastitis has been estimated to be about 33–38% of the total health cost for dairy herds(Fourichon *et al.*, 2001).

Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows. Numerous studies have determined the antibacterial susceptibility patterns of bacteria isolated from mastitis worldwide (Tenhagen *et al.,* 2006).

S. aureus causes different animal pathologies. In particular, it is involved in intramammary infections in cows causing economic losses and milk-safety problems (Taverna *et al.,*2007). Regarding the public health hazardes, *S. aureus* is a commensal organism and versatile pathogen in animals and human. It produces a broad spectrum of surface components (proteins and capsular polysaccharides) and exotoxins, they have virulence factors involved in the pathogenesis of bovine mastitis as these toxins and products are injurious to the milk producing cells of the mammary gland and impair the gland's immune defense mechanisms (Taverna *et al.,* 2007).

Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. They are small Gram-positive 0.5-1.5 µm spherical bacteria that occur in microscopic clusters resembling grapes and occasionally encapsulated (more virulent) (Todar ,2005).

Nanoparticles (NPs) are one of the promising and useful antibacterial agents that could possibly be applied in therapeutics.

ZnO NPs are unique in that they are not only stable under high temperatures and pressures, but they are also generally regarded as safe (GRAS) for human beings and animals relative to organic materials (Sawai 2003; Fu *et al.,* 2005).

Zinc oxide NPs are inorganic antibacterial agents used in the pharmaceutical and medical industries. ZnO NPs have a significant potential for a wide range of biological applications, including as an antifungal and antibacterial agent for antibiotic resistant organisms and for preventing infections. Recent studies have demonstrated the antimicrobial activities of ZnO NPs to pathogenic microorganisms , including *Escherichia coli* O157:H7, *Staphylococcus aureus, Streptococcus pyogenes, Listeria monocytogenes, Salmonella Enteritidis, Salmonella Typhimurium, Bacillus cereus, Campylobacter jejuni, Botrytis cinerea* and *Penicillium expansum* (Mirhosseini and Firouzabadi, 2013).

Recent studies showed that some NPs have selective toxicity to bacteria but minimal effects on human cells (Reddy *et al.,* 2007).

The synthesized ZnO NPs showed better and comparable antimicrobial activities concerning the activities of synthetic drugs (Senthilkumar and Sivakumar, 2018).

Thus, the present study was planned to study the effect of zinc oxide nanoparticle on *S. aureus* isolated from mastitic milk of cows.

2. MATERIALS AND METHODS

2.1 Collection of milk samples (A.P.H.A.,1992):

Two hundred mastitic milk samples were collected from clinical mastitic cows in private farms in Menufia governorate. The samples were transferred in ice box directly with an hour to the laboratory with a minimum delay to be bacteriologically examined.

2.2 Isolation and identification of staphylococci: (APHA, 1992):

Loopfull from deposits of centrifuged milk were inoculated into the surface of mannitol salt agar and Baired-Parker medium. The plates were incubated for 24-48 hours at 37°c The suspected colonies were purified onto slope of nutrient agar and identified morphologically by Staining with Gram stain and biochemically by (MacFaddin, 1976).

2. Antibiotic susceptibility test (Feingold and Martin 1982): *S. aureus*

Isolates were subjected to the sensitivity test against different antibiotics, using the disc and agar diffusion method.

4. Genotypic detection of *S. aureus* genes using polymerase chain reaction (PCR) (Sambrook *et al.,* 1989)

PCR using three sets of primers was used for genotypic detection of multidrug resistance *S. aureus* strains. These genes were *mecA, tetK and aac(6')aph (2'')*. *MRSA* gene, Tetracycline Resistant *S. aureus* gene and Gentamycin Resistant *S. aureus* gene respectively as shown in table(1).

*5.*Synthesis of ZnO Nanoparticles (Zhanfeng Zheng, 2009):

5.1 Preparation of Zinc acetate solution:

Five gm of Zinc acetate Zn $(CH3COO)^2$ was dissolved in 500 ml of boiling ethanol under vigorous stirring for 15 minutes (the boiling time is about 2 minutes). The solution was cooled in an ice bath for 15 minutes. During that time; 2.90gm of lithium hydroxide (LiOH.H2O) was dissolved in 600 ml ethanol under gentle stirring. The two solutions were mixed together for 30 minutes. The new solution was covered and left for 24 hours to get a white precipitate in the gel form. This precipitated gel was washed 5 times by using distilled water under stirring for 15 minutes each time. The solution was covered and left for 24 hours to get the desired zinc hydroxide gel which can be used for making nanoparticles ZnO powder after releasing of the distilled water.

5.2 Preparation of nanoparticles ZnO powder:

The previously prepared Zinc hydroxide gel and FTO glass were used for preparing nanoparticles ZnO powder using the following ; the Zinc hydroxide gel was placed in the furnace at a temperature 70˚C for 6 hours.

The temperature was gradually increased to 450˚C (sintering temperature) with a heating rate of 10˚C/min and was kept in 450˚C for 30 minutes. Finally, the furnace was turned off and samples were removed when the temperature reached 80˚C.Finally, the obtained ZnO powder was used.

6.Antimicrobial Testing Assay of the prepared Zno nanoparticles:

Two different assays (qualitative and quantitative) were carried out to evaluate the antimicrobial activity of ZnO against examined bacterial strains. Bacterial cultures were kept in dark throughout the assays to avoid the possible effect of light on the antibacterial activity.

6.1 Antibacterial activity assay

In order to examine the antibacterial activity of the ZnO nanoparticles on *S. aureus*, ZnO nanoparticles were suspended in sterile normal saline and constantly stirring until a uniform colloidal suspension was formed to yield a powder concentration of 1000 mg/ml. To assess toxicity range of ZnO nanoparticles against *S. aureus*, an appropriate volume of test bacteria were inoculated in nutrient broth medium supplemented with serially diluted ZnO nanoparticles. After these experiments, the best range was proposed from 0.5 to 16 mg/ml of nanoparticle-free medium and bacteria-free medium were used as control positive and control negative respectively.

6.2 Paper Disc Diffusion Assay

Sterile paper discs were placed on the surface of suitable media plates, freshly inoculated with bacterial cells, then 10 mL from ZnO stock solution was dispensed onto the surface of each disc. Plates were then incubated for 24 h at 37c, and diameters of the growth inhibition zones were recorded in mm. Each experiment was made in triplicate and the inhibition zones are given as the mean ± standard deviation.

6.3 Determination of zone of Inhibition

0.05 and 0.1 ml was added of various concentrations of ZnO nanoparticle in discs and wells, respectively. After inoculation and cultivation of different target bacteria on top of nutrient agar, discs and wells were placed in selected area on different plates. The zone of inhibition (ZOI) was measured after 24 h incubation. The antibacterial activity of ZnO nanoparticle were compared.

Table (1): Oligonucleotide primers sequences Source: Metabion (Germany)

Gene	Primer Sequence 5'-3'	Amplified product	Reference
mecA	GTA GAA ATG ACT GAA CGT CCG ATA A CCA ATT CCA CAT TGT TTC GGT CTA	310 bp	McClure *et al*., (2006)
aac(6')aph (2'')	GAAGTACGCAGAAGAGA ACATGGCAAGCTCTAGGA	491bp	Duran *et al*.,(2012)
tetK	GTAGCGACAATAGGTAATAGT GTAGTGACAATAAACCTCCTA	360 bp	

3. RESULTS

3.1 Bacteriological examination of mastitic milk revealed the presence of 52 *S. aureus* isolates wit percentage of 26% from the examined samples.

3.1.1 Colonial appearance:

S. aureus on mannitol salt agar, they were yellow colour surrounded by yellow halo with yellow coloured medium. On Baired Parker medium, black small colonies 1mm colonies after 24 hours incubation and large 2.5 mm after48 hours incubation surrounded by an opalescent ring and a clear zone.

3.1.2 Microscopic examination:

S. aureus appears as grapes like clusters under light microscope.

3.2 Biochemical reactions:

Biochemical test	Reaction
1.Voges- Proskauer (VP)	Positive
2. catalase test	Positive
3. oxidase test	Negative
4. coagulase test	Positive
5.β- haemolysis	Positive
6. Pigment production	Positive(yellow pigment)
7.Lipase and phospholipase	Positive
8. Indol test	Negative

3.3 Antibiogram patterns of 52 *S.aureus* isolates:

For *S. aureus* isolates recovered from clinically milk samples the majority(33 isolates) were susceptible to amoxicillin +clavulinic acid ,ciprofloxacin and rifampicin with percentage of 63.4%

On the other hand moderate sensitivity about (6 isolates) was observed to cloxacillin, clindamycin ,amoxicillin ,neomycin and ampicillin with percentage of 11.5%.

S. aureus isolates recovered from milk samples(about13 isolates)were resistant to Methicilline, gentamycin and oxytetracycline with percentage of 25%.
As shown in table(2).

Table(2): Results of antibacterial sensitivity test of *S.aureus* isolates recovered mastitic cows milk samples against different antibacterial agents: n=52

Antimicrobial agent	Sensitive		Intermediate		Resistant	
	No. of *S.aureus* isolates	%	No. of *S.aureus* isolates	%	No. of *S.aureus* isolates	%
Amoxicillin	33	63.4	11	21.1	12	23.7
Ampicillin	29	55.7	16	30.7	10	19.2
Ciprofloxacin	27	51.9	10	19.2	15	28.8
Gentamycin	19	36.5	20	38.4	13	25
Clindamycin	23	44.2	19	36.5	10	19.2
Neomycin	36	69.2	2	3.84	14	26.9
Rifampicin	31	59.6	18	34.6	13	25
Methicilline	15	28.8	26	0.5	11	21.1
Oxytetracycline	25	48	15	28.8	5	9.6

% according to number of *S.aureus* isolates in which n=52

3.4 Result of PCR for some *S.aureus* isolates:

Detection of the presence of *MRSA* gene, *tetk* gene and aac(6')aph (2")*S.aureus* gene in 10 isolates of multi-drug resistant *S.aureus:*

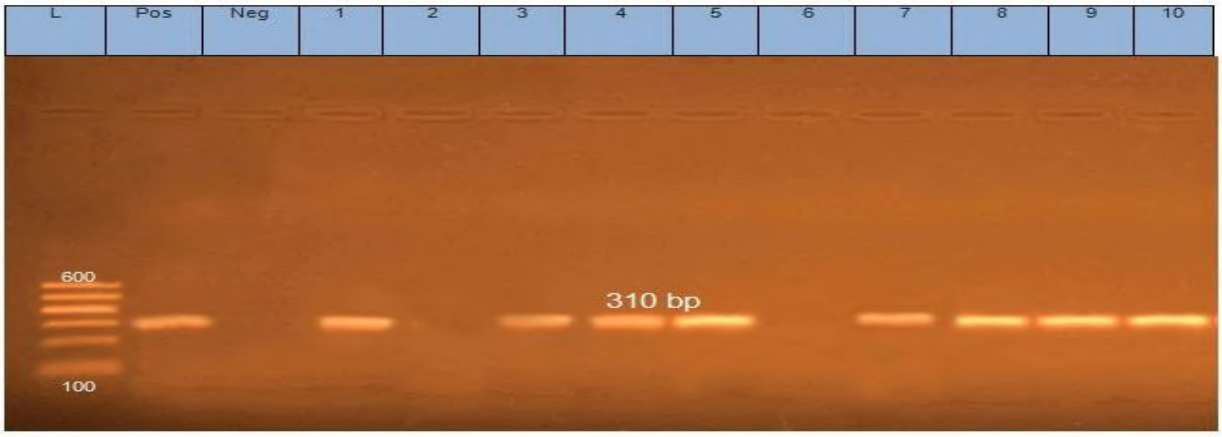
PCR test was applied on 10 *S.aureus* isolates for detection of the previous genes. These results revealed that 8 isolates of *S.aureus* were positive for *MRS*A gene with percentage of 80 % ,10 isolates of *S.aureus* were positive Gentamycin Resistant *S.aureus* gene with percentage of 100 % and 10 isolates of *S.aureus* were positive for Tetracycline Resistant *S.aureus* gene with percentage of 100 % .

It was clear that PCR test result corelated with sensitivity test . as shown in table(3).

Table(3): correlation of sensitivity test results with the occurrence of the previous genes in *S.aureus* isolates:

Sample	Results		
	*mec*A	*tet*K	*aac(6')aph (2'')*
1	+	+	+
2	-	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	-	+	+
7	+	+	+
8	+	+	+
9	+	+	+
10	+	+	+
total	8\10	10\10	10\10

Results of PCR amplification of MARSA gene as shown in Figure(3)



Figure(3) Post: positive control(at 310bp). Neg: Negative control.
Lane2,6(Negative). Lane1,3,4,5,7,8,9,10(positive)

Results of PCR amplification of tetracycline resistant gene of *S.aureus* as shown in Figure (4):

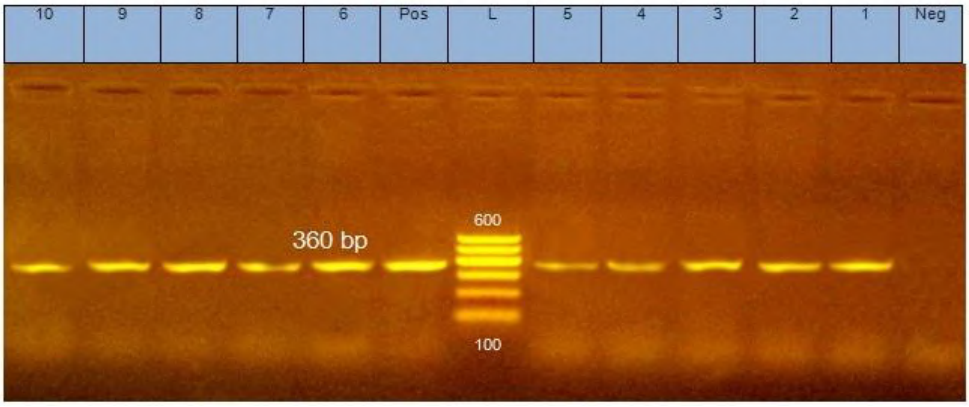
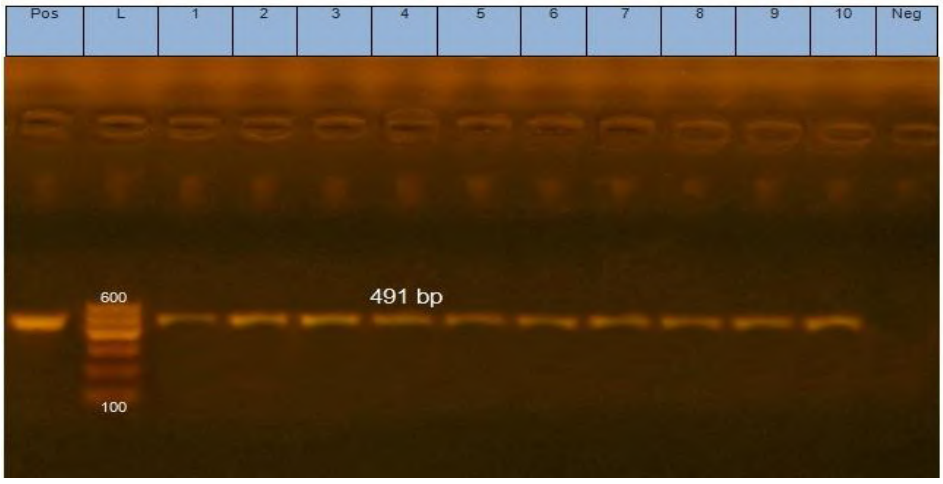


Fig (4): Post: positive control (at 360bp). Neg:Negative control.
Lane1,2,3,4,5,6,7,8,9,10(positive).

Results of PCR amplification of Gentamycin resistant gene of *S.aureus* as shown in Figure(5):



Fig(5)Post: positive control(at 491bp). Neg: Negative control.
Lane1,2,3,4,5,6,7,8,9,10(positive).

3.5 Characterization of Zno nanoparticles

The Characteristics of the prepared nanoparticles ZnO powder were investigated by carrying out the X-ray spectroscopy (XRD), while the surface morphology was characterized by using scanning electron microscopy (SEM).

X-ray diffraction (XRD) pattern of ZnO shows that the prepared ZnO powder is in hexagonal wurtzite phase with crystallite size equal to 19 nm as calculated from Scherrer formula for the major (101) diffraction peak. The values of lattice parameters a and c were found to be 3.205Å and 5.122Å, respectively.

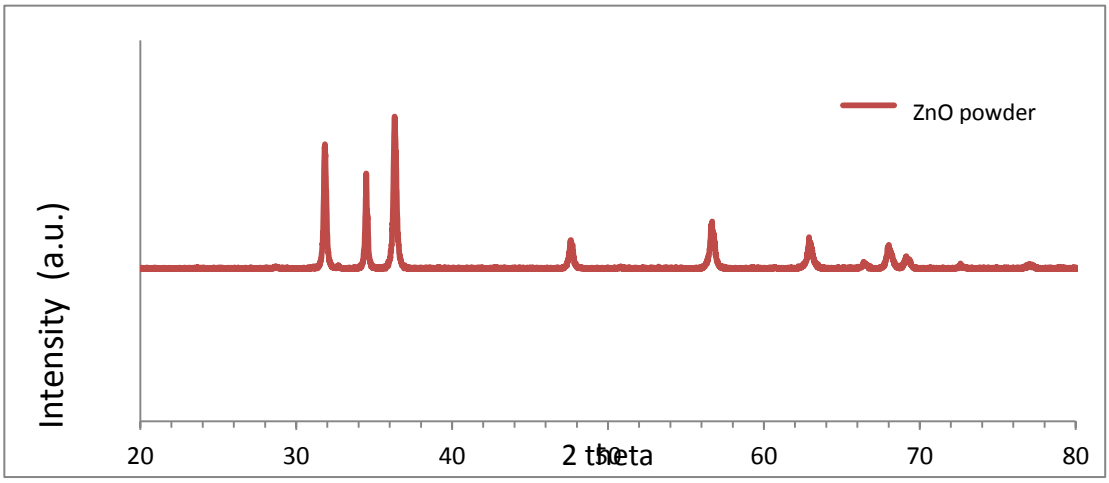


Figure (6): XRD patterns for ZnO powder charts.

The surface image for nanoparticles ZnO powder shown in figure 5. As indicated by the top view image, this ZnO image demonstrates a uniform morphology.

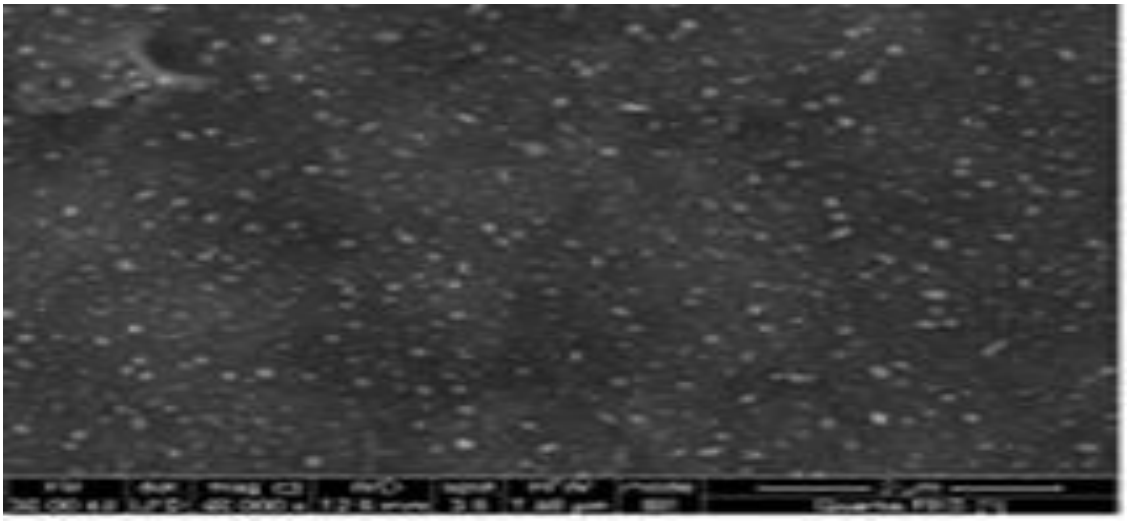


Figure 7: SEM surface image of nanoparticles ZnO powder

3.6 Effect of ZnO nanoparticles on *S.aureus* :

S.aureus isolates were examined for their susceptibility to ZnO nanoparticles. *S.aureus* were susceptible to ZnO 36 nanoparticles using either qualitative or quantitative assay.

The diameter of growth inhibition zone increased with the decrement of required MIC from ZnO for *S. aureus*.

Table(4). Zone of inhibition (ZOI) for *S. aureus*

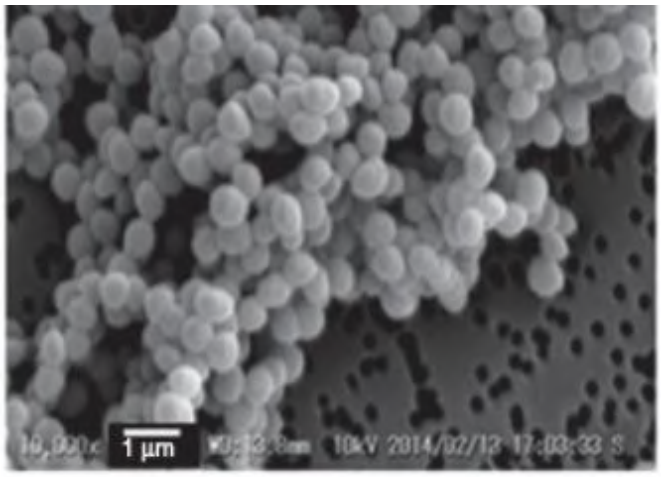
ZnO concentration in wells(mg/ml)	ZOI (mm)	ZnO concentration in discs(mg/ml)	ZOI(mm)
10	29	5	22
5	27	2.5	19
2.5	25	1.25	16
1.25	21	0.625	14
0. 625	17	0.312	12
0.312	15	0.156	10
0.156	14	0.078	9
0.078	*14	0.039	*9
0.039	0	0.0195	0
0.0195	0	0.00975	0
Control	0	control	0

* Minimum concentrations of ZnO nanoparticles at which zone of inhibition started to appear.

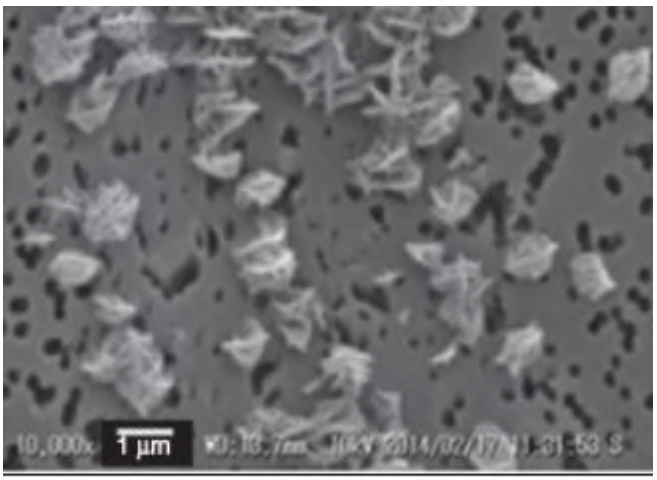
Morphological test of bacteria cells treated with ZnO NPs

The scan electron microscope (SEM) images (Figures8 and 9) of *S. aureus* incubated in respective broth medium for 10 h, with and without the presence of 20 mM of ZnO were analyzed.

As shown in Figures (8) no significant changes in bacterial morphology (e.g. size, shape, appearance, etc) were observed after ZnO NPs treatment for 10 h. Also, ZnO NPs were observed to adhere to *S.aureus* cells Figure (9). The sizes of ZnO NPs attached to bacterial cells appeared larger and clustered as compared to those shown in Figure(8).



Figure(8) *s.aureus* (control sample) nanoparticles with Zno.



Figure(9) *s.aureus* treated with Zno Untreated Under SEM.

4. DISCUSSION

Staphylococcus has the main role in the etiology of mastitis which considered an important mammary gland disease due to its association with many zoonotic diseases in which milk acts as a vehicle of infectious agents (Bramely,1996).

Resistance of mastitis pathogens to antimicrobial agents is a well-documented challenge in dairy cows. Numerous studies have determined the antibacterial susceptibility patterns of bacteria isolated from mastitis worldwide (Tenhagen et al., 2006).

S. aureus strains are known to be frequently resistant to antibiotic therapy due to their capacity to produce an exo polysaccharide barrier and because of their location within lesion, which limit the action of drugs was stated by Gu"ndogWan *et al*., (2006*).*

In this study, a total of 200 mastitic cow's milk samples were examined bacteriologically to investigate the occurrence of pathogenic *S. aureus*.

Percentage of 26 %. Similar findings were recorded by Kivaria and Noordhuizen (2007) who recorded that the incidence of *S.aureus* reached 25.7%isolated from200 mastitic milk samples, Swai *et al*.,(2005) who recorded that the incidence of *S.aureus* reached 24% and Gronlund *et al*.,(2005) who recorded that the incidence reached 27.2%. on the other hand, higher incidences were obtained by Chowdhury *et al,*. (2002) who recorded that the incidence reached 53.85%, Yavuz and Esndal (2002)mentioned that the incidence reached 53.3% and Kivaria *et al*.,(2006) who recorded that the incidence reached 82%.

Emergence of multiple drug resistant *S.aureus* including MRSA are of great concern due to difficulties in the selection of effective antibiotics to cure staphylococcal infection Seguin *et al*., (1999) .

In this study *S.aureus* isolates recovered from milk samples(about 13 isolates)were resistant to methicillin, gentamycin and oxytetracycline with percentage of 25%.Other studies show different results as sensitivity to methicillin was 80% recorded by El-jakee *et al.*,(2008).

All *S. aureus* isolated from the examined mastitic cow's milk samples were resistant to the eight different types of antibiotics. Similar findings was reported as 96.15% *S. aureus* were resistant to more than one antibiotic, it was found that there was no isolate sensitive to all antibiotic as recorded by Narmeen *et al* .,(2009).

The presence of multi drug resistant strains is alarming, because such strains considered as a serious danger to public health Zouhairi *et al.,* (2010).

Recent advances in the field of nanotechnology, particularly the ability to prepare highly ionic metal oxide nanoparticulates of any size and shape, may lead to the development of new antibacterial agents.

Preliminary studies as discussed in this report have demonstrated that ZnO metal oxide nanoparticles analysed shows a significant growth inhibition under normal laboratory conditions.

The antibacterial activity of ZnO nanoparticles was tested by the disc and well diffusion agar method). The presence of an inhibition zone clearly indicated the antibacterial effect of ZnO nanoparticles. As it was also shown in the study of Rizwan *et al.,* (2010c).

The minimum concentration of ZnO nanoparticles which inhibited the growth of bacteria was 1.5 mg/ml for *S.aureus*. This is in agreement with previously published reports on the antibacterial properties of ZnO nanoparticles which

showed that the minimum concentration at which the growth of S. aureus was inhibited was 1 mM (Reddy *et* al., 2007).

5. CONCLUSION

According to the results, it can be concluded that ZnO nanoparticles are effective antibacterial agents on *S.aureus*. The same results were confirmed in the study of Zhongbing *et a*l.,(2008) in which Gram-positive membrane disorganization was approved by scan electron microscopy of bacteria ultrathin sections.

6. REFERENCES

APHA "American Public Health Association" (1992): Compendium of methods for microbiological examination of foods. Washington, D.C.U.S.A., 105(4):100-110.

Bramely, A.J. (1996): Current concspts of bovine mastitis. National Mastitis Council, Masdison, Wisconsin.

Chowdhury, M.,Saha, G.R., Chowdhury, J. and Das, S.K. (2002): Subclinical mastitis in crossbred cows.J. of Interacademicia. 6(Special): 712-715.

Correa, M. G. and Marine, J. M. (2002): O-serogroups, *eae* gene and EAF plasmid in *Escherichia coli* isolates from cases of bovine mastitis in Brazil. Vet. Microbiol., 85 (2):125-32.

Crist, W.L., Harmon, R. J. , O' Leary, J. and McAllister, A. J. (1997): Mastitis and its control.University of Kentucky, cooperative extension service.

Desselberger U (2000): Emerging and re-emerging infectious diseases. J Infect 40: 3–15.

Duran, N., Ozer, B., Duran, G.G., Onlen, Y. and Demir, C. (2012): Antibiotic resistance genes & susceptibility patterns in staphylococci. Indian J Med Res 135, pp 389-396.

El-Jakee J., Ata Nagwa S., Bakry M., Sahar Zouelfakar A., Elgabry E. and Gad El-Said W.A. 2008. Charcteristics ofstaphylococcus aureus strains isolatedfrom human and animal sources. American Eurasian J. Agric. Environ. Sci., 4(2):221-229.

Finegold and Martin (1982): Bailey s and Scott s. Diagnostic Microbiology. 6 th Ed. The C.V. Mosby Company. St. Louis, Toronto, London.

Fourichon C., Seegers H., Beuadeau F., Verfaille L., Bareille N (2001): Health control costs in dairy farming systems in Weste Europe.Livest Prod Sci68:141– 56.

Fu G, Vary PS, Lin C-T. (2005): Anatase TiO2 nanocomposites for antimicrobial coatings. The Journal of Physical Chemistry B ,109(18):8889-98.

Gillespie E.B. (1943): The routine used of the coagulase test for staphylococci. Monthly Bulletin of the Emergency Public Health Laboratory Services, 2: 19- 22.

Gronlund,U.,HallenSandgren,C.andPerssonWaller,K. (2005): Haptoglobin and serum amyloid A in milk from dairy cows with mastitis by S. aureus . Vet.Res.,36(2):191-198.

Gu''ndog''an N., Citak S., and Turan E. (2006): Slime production ,DNAse activity and antibiotic resistannce of Staphylococcus aureus isolated from raw milk, pasteurized milk and ice cream samples .Food Control, 17:389-392.

Kivaria, F.M., Noordhhizen, J.P. and Nielen, A.M. (2006): Evaluation of the hygienic quality and associated public health

hazard of raw milk market to small holder dairy producers in the Dares-Salaam region of Tabzania.Anim. Hlth Prod., 38(3):185-195.

Kivaria, F.M. and Noordhuizen, J.P. (2007): A retrospective study of the etiology and temporal distribution of bovine clinical mastitis in smaa holder dairy herds in the Dar es Salaam region of Tanzania. Vet.J. ,173(3):617-22.

MacFaddin, J. (1976): Biochemical tests for identification of medical bacteria, ed.: The Williams & Wilkins Co., altimore.

McClure J-A, Conly JM, Lau V, Elsayed S, Louie T, Hutchins W and Zhang, K. (2006): Novel multiplex PCR assay for detection of the staphylococcal virulence marker Panton-Valentine leukocidin genes and simultaneous discrimination of methicillin-usceptible from resistant staphylococci. J Clin Microbiol, 44: 1141-114.

Mirhosseini, M. and Firouzabadi, FB. (2013): Antibacterial activity of zinc oxide nanoparticle suspensions on food borne pathogens. International Journal of Dairy Technology.

Narmeen, S.M., Jaladet, M.S. and Jubrael. (2009): Isolation and identification of staphylococcus aureus using classical and molecular methohs.The 2nd Kurdistan Conference on Biological Sciences International Journal ofMicro Biology, Genetics and Monocular Biology Research .2(2):1-8.

Oviedo-Boyso, J., Barriga-Rivera, J. G., Valdez-Alarcón, J. J., Bravo-Patiño, A, Cárabez-Trejo, A., Cajero-Juárez, M. and Baizabal-Aguirre, V. M. (2008): Internalization of *S. aureus* by bovine endothelial cells is associated with the activity state of NF-kappaB and modulated by the pro-inflammatory cytokines TNF-alpha and IL-1beta.Scand. J. Immun., 67(2):169-176.

Reddy, K.M., Feris, K., Bell, J., Wingett, D.G., Hanley, C.and Punnoose, A. (2007): Selective toxicity of zinc oxide nanoparticles to prokaryotic and eukaryotic systems. Applied Physics Letters, 90(21):213902—3.

Rizwan, W., Young- Soon, K., Amirta, M., Soon-ll Y, Hyung-Shik Sh (2010c): Formation of ZnO micro-flowers prepared via solution process and their antibacterial activity.J.Nanoscale Res. Lett.,5(10):1675-1681.

Sambrook, J., Fritscgh, E.F. and Mentiates (1989): Molecular coloning. A laboratory manual. Vol !., Cold spring Harbor Laboratotry press, New York.

Sawai, J. (2003): Quantitative evaluation of antibacterial activitiesof metallic oxide powders (ZnO, MgO and CaO) by conductimetric assay. Journal of Microbiological Methods ,54(2):177-82.

Santos, J.E., Cerri, R.L., Ballou, M.A., Higginbotham, G.E. and Kirk, J. H. (2004): Effect of timing of first clinical mastitis occurrence on lactational and reproductive performance of Holstein dairy cows. Anim. Reprod. Sci, 80 (1,2): 31-45.

Seguin, J.C., Walker, R.D., Caron J.P., Kloos ,W.E.,Gearge, C.G., Hollis, R.J., Jones, R.N. and Faller, M.A.P (1999): Methicillin-resistant S.aureus outbreak in a veterinary teaching hospital: potential human to animal transmission.J. Clin. Microbial.,37: 1459-1463.

Senthilkumar, S. R. and Sivakumar, T., (2018): Mediated synthesis of Zinc oxide (ZnO) nanoparticles and studies

on their antimicrobial activities. Int. J. Pharm. Pharm. Sci., 6(6): 461-465. 63.

Sory, H., Zerihun, A. and Abdicho, S. (2005): Dairy cattle mastitis in and around Sebeta, Ethiopia. Intern. J. Appl. Res. Vet. Med., 3 (4): 332-338.

Swai, E.S., Karimuribo, E.D., French, N.P., Ogden, N.H., Fitzpatrick, J.L., Kambarage, D. and Bryant, M.J. (2006): Risk factors, isolation and drug sensitivity of microbial organism associated with subclinical mastitis in small holder dairy farms in Tanga, Tanzania. Bull. of Anim. Hlth. And Prod., In Africa.,54(1):13-22.

Taverna, F., Negri, A., Piccinini, R., Zecconi, A., Nonnis, S., Ronchi, S. and Tedeschi, G. (2007): Characterization of cell wall associated proteins of a *S. aureus* isolated from bovine mastitis. Vet. Microbiol. ,119(2-4): 240-7.

Tenhagen, B.A., K¨oster, G.,Wallmann, J.and Heuwieser, W. (2006): Prevalence of mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Brandenburg, Germany. *J Dairy Sci* 89: 2542–51.

Todar, K. (2005): University of Wisconsin-Madison Department of Bacteriology.

Yavuz, M. K. and Esndal,O.M. (2002): Specific identification and some charcteristics of staphylococci isolated from bovine milk with mastitis Etlik Vet. Mikrobiyol.Dergisi,13(2):19-27.

Zhongbing, H. Xu. Zh., Danhong, Y., Guangfu, Y., Xiaoming, L., Yunging, K., Yadong, Yao, Di. Huang and Baoqing, H. (2008): Toxicological effect of ZnO nanoparticles based on bacteria. Langmuir, 24(8):4140-4144.

Zouhairi, O., Saleh, I., Alwan, N., Toufeili, L., Barbour, E. and Harakeh, S. (2010): Antimicrobial resistance of staphylococcus species isolated from Lebanese dairy-based products. Eastern Mediterranean Health J., 16 (12): 1221-1225.

Zhanfeng Zheng, (2009): Synthesis and Modifications of Metal Oxide Nanostructures and Their Applications. PhD thesis Queensland University.