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Synergistic Effect of Zinc Oxide Nanoparticles and Vancomycin on Methicillin resistant *Staphylococcus aureus*

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Abstract

The study was carried out on (120) various clinical samples collected from patients attended Al Salam and Al-Khansa Hospitals and the Public Health Laboratory in the Mosul city during 4 months (September - December 2019). Samples were cultured on Mannitol Salt Agar medium, 105 samples give a positive result (grew the bacteria). 50 isolates were fermented mannitol sugar, at a rate of 47.6%, depending on the phenotypic characteristics and production of Coagulase, 14 isolates were identified, at a rate of 13.3%, belonging to *Staphylococcus aureus* the diagnosis were confirmed using VITEK system. The highest isolation rate from wounds was 57%, then abscesses 21%, blood samples 14%, and urinary tract infections 7%. The sensitivity of the isolates was tested for 16 antibiotics, the isolates showed variation in their resistance to antibiotics. Most of the isolates showed high resistance at 92.8% to each of Oxacillin, Vancomycin, which were diagnosed as MRSA and VRSA. Vancomycin MICs against MRSA and VRSA ranged (2500-5000) µg /mL. MIC for nanoparticles sized (30, 20, 50-150) nm ranged (5000-10000) µg / mL for isolates that are positive coagulase. In this study the efficacy of vancomycin was improved in combination with ZnO nanoparticles. Results showed a decrease in vancomycin MICs from (2500-5000) µg / mL to (39-78.125) µg \ mL when mixed with ZnO 20 nm.

Keywords: *Coagulase, Nanoparticles, VITEK system, MRSA, VRSA.*

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I. INTRODUCTION

Staphylococcus aureus is a bacterium that lives on the skin, nose and mucous membranes of the human respiratory system, whether it is naturally occurring bacteria or invading bacteria, they cause many infections such as bacteremia, pneumonia, skin and soft tissue infections such as abscesses, boils, post-operative wound infections and toxic shock syndrome. (TSS) Toxic Shock Syndrome and Scalded Skin Syndrome (SSS) (Asanin *et al*., 2019; Reddy *et al*., 2017; Goudarzi *et al*., 2016).

Staphylococcus is found naturally in about 30% of healthy people, yet it is one of the most common pathogenic bacteria in community and hospital (Tong *et al*., 2015). Infections associated with pyogenic staphylococcus are often complicated by their ability to resist different classes of antibiotics and thus become more pathogenic (Rao *et al*., 2014).

The most important strains with multiple antibiotic resistance are Methicillin Resistant *Staphylococcus aureus*, which has become a major concern for hospital injuries, so vancomycin was chosen as the last line for the treatment of MRSA infections (Reddy *et al*., 2017). This led to the emergence of strains resistant to vancomycin (VRSA), Vancomycin Resistant *Staphylococcus aureus*, which was first diagnosed in the United States (Tortora *et al*., 2018), in addition to the emergence of strains resistant to Erythromycin (ERSA) Resistant *Staphylococcus aureus Erythromycin*, which was later diagnosed (Tille, 2017).

The increase in antibiotic resistance led to an increase in research to find alternative agents and treatments, including the use of nanoparticles, which showed a synergistic effect with antibiotics against bacteria as the rate of inhibition of bacterial growth is higher than the rate of inhibition when using antibiotics and nanoparticles both separately (Fayaz *et al*., 2010).

ZnO is one of the most famous metal oxides that have many properties, the most important of which are anti-microbiological activity and semiconductor properties, and it is safe and non-toxic (Maruthupandy *et al*., 2016). It is also used in many industries such as rubber, dyes, cosmetics, and sunscreens and food and pharmaceutical products as it acts as a

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medicinal agent in many fields (Kołodziejczak-Radzimska and Jesionowski, 2014). The mechanism of action of ZnO is the penetration of the bacterial cell wall by diffusion, which was observed by SEM and TEM microscopy images of the bacterial cells as it breaks down the biofilm and collects in the cytoplasm and interacts with biomolecules causing programmed cell death (Siddiqi *et al*., 2018).

The metal oxide nanoparticles, first destroy the bacterial cell membrane and then penetrate into it. Hydrogen peroxide (H_2O_2) is formed from zinc oxide, which acts as an antimicrobial (Yamamoto *et al*., 2004). Pati *et al*. (2014) showed that the nanoparticles of Zno, after rupturing the bacterial cell membrane, reduce the hydrophobic surface of the cell and work to reduce the reproduction of the oxidative stress genes in bacteria as well as enhance bacterial killing inside cells by stimulating or stimulating the production of (ROS). Reactive Oxygen Species and inhibit hemolysis induced by pathogen-produced hemolysin toxins (Cho *et al*., 2011).

Zinc oxide nanoparticles have selective bacterial toxicity, show fewer side effects on human cells, and are recommended for use in the food and agricultural industries (Ahmadi Shadmehri *et al*., 2019; Zhang *et al*., 2007). Nanoparticles prevent the formation of biofilms (Hajipour *et al*., 2012), ZnO inhibits the adhesion of bacteria to each other, and the smaller the size and the higher the surface area to mass ratio, the greater the prevention of biofilm formation (Slomberg *et al*., 2013). The formation of biofilms is an important mechanism, as the biofilms play an important role in the development of bacterial resistance. Scanning electron microscope (SEM) images and (TEM) Transmission Electron Microscopy has also been proven. The characteristics of ZnO nanoparticles depend on the size, shape, concentration, and exposure period of these particles (Wang *et al*., 2016).

The current study aimed to isolate and diagnosis of multiple antibiotic-resistant *Staphylococcus aureus*, especially MRSA and VRSA, from different clinical sources, and studying the genetic and phenotypic relationship between isolates. A study of the prevalence of resistance to treatments and nanoparticles among isolates .Determination of the minimum inhibitory concentration of antibiotics, zinc oxide in different sizes. To test the synergistic effect between antibiotics, nanoparticles.

II. Materials And Methods

Clinical samples (120) were collected from patients at Al Salam and Al-Khansa Hospitals and the Public Health Laboratory in the city of Mosul for the period. From September 2019 to December 2019.

Samples were obtained from urinary tract infections (10 samples), wounds (65 samples), pus (20 samples), blood (25) and for both sexes. Clinical samples were cultured on Mannitol Salt Agar and morphological and biochemical characteristics, like the catalase, oxidase and clotting enzyme tests, sensitivity to Bacitracin, and VITEC system were used to identify staphylococcal species.

Sensitivity testing was performed using Kirby-Bauer disc diffusion method (Kirby-Bauer *et al*.1966) according to Clinical Laboratory Standard Institute (CLSI, 2019) recommendations.

A. Measurement Vancomycin Minimum Inhibitory Concentration

The double dilution method was used, based on the method of Saginur *et al*., 2006 as follows: Add 1000 µl of nutrient broth to 10 test tubes. 1000µl of pre-prepared antibiotic at a concentration of 10,000 µg / mL was added to tube No. 1. The antibiotic was mixed by using a micropipette by withdrawing the culture 6-8 times to obtain a concentration of 5000 µg / mL. Transfer 1000 µl from Tube No. 1 to Tube No. 2 and mix in the same way as the previous method to obtain a concentration of 2500 µg / mL. The process was repeated up to tube No. 10, of which 1000 µl was withdrawn and discarded. Thus, the concentrations were obtained (5000, 2500, 1250, 625, 312.5, 156.25, 78.125, 39, 19.5) micrograms/mL. Add 100 microliters of bacterial suspension incubated for (18-24) hours at a concentration of 1.5 * 108 cells / mL compared to the third tube of McFarland tubes. Add to tube No. 11 the nutrient broth and bacterial suspension only as positive control. Tube No. 12 contains only the nutrient broth as negative control. The tubes were incubated at 37 °C for (18-24) hours. Examination of bacterial growth is visible and the MIC observed the lowest inhibitory concentration of the antibiotic**.**

B. Minimum Inhibitory Concentration of Zinc Nanoparticles in sizes (30, 20, 50-150) nm

The same previous method was used using a concentration of 20000 micrograms/mL previously prepared (where the required concentration of nanoparticles was dissolved in deionized water and sterilized by autoclave) to tube No. 1 and thus we obtain the concentrations (10, 000, 5000, 2500, 1250, 625, 312.5, 156.25, 78.125,39) µg/mL for nanomaterials (Saginur *et al*., 2006).

C. Study the synergistic effect of antibiotics with nanoparticles

The double dilution method was used, based on the method of Saginur *et al*., 2006 as follows :Add 1000 µl of nutrient broth to 10 test tubes. 1000 µl of the MIC concentration determined from the previous step for each antibiotic and nanoparticle was added to the first tube to study the synergistic effect of the nanoparticles with the antibiotics. The mixing was done through the use of a micropipette by withdrawing the culture for (6-8) times. Transfer 1000 µl from tube No. 1 to tube No. 2 which also contains 1000 µl of the nutrient broth and mixing in the same way as before. The process was repeated up to tube No. 10, of which 1000 µl was withdrawn and discarded. Adding 100 µl of 24-hour age, bacterial culture at a concentration of $1.5 * 10^8$ cells/mL compared to the third tube of McFarland tubes. The tubes were incubated at (37) C° for (18-24) hours. Examination of the bacterial growth is visible, and the MIC was observed to have the lowest inhibitory concentration of the antagonist and the synergistic particles.

III. RESULTS

120 samples were collected, including wound swabs, blood, pus and urinary tract infections for isolating of pyogenic staphylococcus using Mannitol Salt Agar medium, 105 of which showed growth on MSA medium at 87.5% of which 50 were fermented mannitol sugar isolates at 47.7% and 55 non-fermented isolates at 52.3%, as shown in Table 1.

Table 1. Number and percentage of isolates on mannitol salt agar

Total number of samples	Growth on an MSA	No growth	Fermented mannitol isolates	Non-fermented mannitol isolates
Total	105	15	50	55
	%87.5	%12.5	%47.6	%52.3
	120		105	

The biochemical tests shown in Table 2 are used to diagnose *S. aureus* and conformity as reported in internationally approved diagnostic systems (Winn *et al*., 2006; Collee *et al*., 1996). Fourteen of the 50 fermented mannitol isolates gave a positive result for the clotting enzyme test, at a rate of 28%.

Table 2. Biochemical tests used to diagnose *S. aureus* under the study

Oxidase	Catalase	Haemoltsin	Mannitol salt Agar	Motility Test	Glucos	Lactos	Bacitracin	Coagulase	Gram stain
-	+	+	+	-	+	+	+	+	+
Number and ratio									
50 100 %	50 100 %	13 %26	50 100 %	50 100 %	50 10 %0	50 10 %0	50 100 %	14 %28	50 10 %0

The highest isolation rate obtained was in post-operative wound swab samples, reaching 57.1%, followed by pus samples at 21.4%, then blood samples at 14.2%, and urinary tract infection samples, at 7.1% (Table 3).

Table 3. The ratios of *S. aureus*, depending on the type of specimen

No.	Sample type	The number of samples	The number of isolates	Isolation ratio
1	Wounds	65	8	57.1
2	Abscesses	20	3	21.4
3	Blood	25	2	14.2
4	Urinary tract infections	10	1	7.1
Total		120	14	100

A. *Antibiotic Sensitivity Test*

The *S. aureus* sensitivity test was performed to 16 antibiotics by using dick diffusion method on Muller-Hinton Agar according to CLSI to determine the sensitivity or resistance of bacterial isolates to antibiotics by measuring the inhibition zone comparing the results with (CLSI, 2019) as shown in Figure 1, *S. aureus* is highly resistant to many antibiotics such as Bacitracin and Cefoxitin with 100% resistance. Vancomycin and Oxacillin which is one of the methicillin group, as it resisted 13 isolates with a resistance rate of 92.8%.

Figure 1 illustrates the sensitivity of *S. aureus* for methicillin MSSA, at a rate of 7.1%.

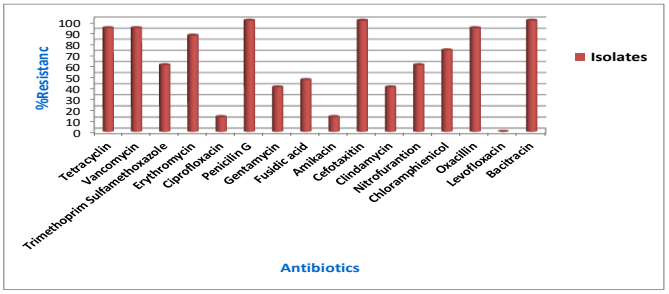

Figure 1. Percentages of resistance of *S. aureus* against a number of antibiotics.

B. *Measurement of the Minimum Inhibitory Concentration of Vancomycin*

The minimum inhibitory concentration MIC of vancomycin was determined, which gave the highest resistance against the bacterial isolates under study. Which ranged between (5000-2500) µg/mL for the Vancomycin as in the Table 4.

Table 4. Minimum inhibitory concentration of vancomycin against *S. aureus*

Isolates No.	S1	S2	S3	S4	S5	S6	S7
MIC µg /mL	5000	5000	5000	2500	5000	2500	5000
Isolates No.	S8	S9	S10	S11	S12	S13	S14
MIC µg /mL	2500	5000	2500	2500	5000	5000	5000

C. *Measurement of the Minimum Inhibitory Concentration of Zinc Nanoparticles*

The minimum inhibitory concentration MIC of zinc oxide nanoparticles (ZnO-NP) of 20, 30 and (50-150) nm, appeared between (10,000-5000) µg/mL (Table 5).

Table 5. Minimum inhibitory concentration MIC of ZnO nanoparticles against *S. aureus* MRSA and VRSA under the study

Isolates No. Nanoparticles	MIC µg /mL													
	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14
ZnO 20nm	5000	5000	10000	5000	10000	5000	10000	5000	10000	5000	10000	5000	10000	10000
ZnO 30nm	5000	10000	10000	5000	10000	10000	10000	5000	5000	5000	10000	10000	5000	10000
ZnO 50-150nm	5000	10000	5000	10000	5000	10000	10000	5000	10000	5000	10000	10000	5000	10000

D. Study of The Synergistic Effect of Zinc Nanoparticles with Vancomycin

The effect of the relationship between the vancomycin used in the study and the zinc nanoparticles on the MRSA and vancomycin VRSA-resistant *S. aureus* when mixed together using the tube method. Where the results showed that the rate of MIC for each of the antibiotics and the nanoparticles and their different sizes decreased significantly. The results showed that the MIC values of Vancomycin when combined with zinc oxide of 20nm and 30nm sizes ranged from (39- 78.125) µg/mL, and the MIC value ranged (39-156.25) µg/mL when mixed with nanostructured zinc oxide at a volume of 50 nm while the MIC value of the antibiotic alone was between (2500-5000) µg/mL (Table 6).

Table 6. Synergistic effect between vancomycin antagonist and zinc nanoparticles on *S. aureus* resistant to methicillin and vancomycin

Isolates No.	MIC of Vancomycin alone	MIC of antibiotic and nanoparticle µg / mL			MIC of nanoparticle alone µg / mL		
		Vancomycin + ZnO 20nm	Vancomycin + ZnO 30nm	Vancomycin + ZnO 50nm	ZnO 20nm	ZnO 30nm	ZnO 50nm
1	5000	78.125 78.125	78.125 156.25	156.25 156.25	5000	10000	5000
2	2500	39 78.125	39 156.25	78.125 312.5	5000	10000	10000
3	5000	78.125 156.25	78.125 156.25	78.125 78.125	10000	10000	5000
4	2500	39 78.125	39 78.125	39 156.25	5000	5000	10000
5	5000	78.125 156.25	78.125 156.25	156.25 156.25	10000	10000	5000
6	2500	39 78.125	39 156.25	78.125 312.5	5000	10000	10000
7	5000	78.125 156.25	78.125 156.25	156.25 312.5	10000	10000	10000
8	2500	39 78.125	39 78.125	78.125 156.25	5000	5000	5000
9	5000	39 78.125	78.125 78.125	156.25 312.5	10000	5000	10000
10	2500	39 78.125	39 78.125	39 78.125	5000	5000	5000
11	2500	39 156.25	39 156.25	78.125 312.5	10000	10000	10000
12	5000	78.125 78.125	78.125 156.15	78.125 156.25	5000	10000	10000
13	5000	78.125 156.25	78.125 78.125	78.125 78.125	10000	5000	5000
14	5000	78.125 156.25	78.125 156.25	78.125 156.25	10000	10000	10000

IV. DISCUSSION

A. Antibiotic Sensitivity Test

Vancomycin and Oxacillin which is one of the methicillin group, as it resisted 13 isolates with a resistance rate of 92.8%, which corresponds to many studies with a percentage of 93.42%, 93.53%, 95% (Salih *et al*., 2017; Kandala; *et al*. 2017; Mohammed and Flayyih, 2017), respectively. the sensitivity of *S. aureus* for methicillin MSSA, at a rate of 7.1%, and this result is consistent with the study Al-Maliki (2009), which was sensitive to methicillin 3.3%.

Also, the results of our study were in agreement with the study of Al-Geobory (2011), which showed the percentage of resistance to methicillin up to 90.9%, as well as the study of Al-Dahbi and Al-Mathkhury (2013) showed the percentage of resistance to methicillin up to 94.3%. While it did not agree with the study of Peck *et al*. (2009), which showed the percentage of methicillin resistance to 51.4% and methicillin sensitive 48.6%.

The MRSA strains contain the *Scc mec* gene integrate within the scc mec chromosomal *orfx* gene, which are motile genetic elements that vary in size and genetic arrangement between the various MRSA strains that contain the *mecA* or *mecC* methicillin resistance gene, which encodes PBP2a enzymes that have little or low affinity for each of the group of β-lactams, except for the fifth generation of cephalosporins such as Ceftaloridin and Ceftobiprole, or possibly *S. aureus* produces a PVL toxin that is a two-component capable of inducing holes that destroy leukocytes (Meyer *et al*., 2009) and stimulating the expression of other harmful agents (McDonald *et al*., 2005).

The mechanism of resistance may occur due to a mutation in the *mecA* gene in an increase or decrease that produces antimicrobial resistance in addition to other antibiotic resistance genes present in the cassette, leading to multiple drug resistance (Duran *et al*., 2012).

Antibiotics are related to proteins responsible for the strength and durability of the cell wall and are called penicillin binding proteins PBPs. The cause of resistance may also be the presence of the mecA gene, which reduces the antibiotic's binding to proteins responsible for cell wall strength and called penicillin binding proteins (Ekrami *et al*., 2010).

The results of this study are in agreement with the study of Talebi *et al*. (2019) where the percentage of resistance to Oxacillin were 90%. Beta-lactam antibiotics inhibit the processes of manufacturing the cell wall by interfering with the process of manufacturing the peptidoclycan layer. The cause of resistance may be due to the secretion of beta-lactamase enzymes, which may be plasmid or chromosomal, or due to the secretion of bacteriostatic inhibitory enzymes, which may also be plasmid or chromosomal, which are used to neutralize the effects of beta-lactam antibiotics by breaking the beta-lactam cycle in the penicillins and cephalosporins group (Kolář *et al*., 2010).

13 *S. aureus* show resistant to Vancomycin (VRSA) at a rate of 92.8%, and this percentage is somewhat consistent with the study conducted by the researcher Al-Khafaji in (2018), as the resistance rate was 100%.

Decreased sensitivity strains of *S. aureus* of vancomycin may led or may be associated with alteration of the bacterial target, including an increase in the thickness of the bacterial cell wall that blocks or retains Vancomycin and prevents it from reaching the target (Gonzalez-Zoom, 2003). However, a decrease in the cross-linkages of the peptidoclycan layer and a higher content of free D-alanin-D-alanin in the cell wall may increase the resistance of the strain.

Vancomycin resistance is a result of either the transmission of genes encoding resistance to the *VanA* gene carried on conjugated plasmids or transposon genes from other strains and *S. aureus* (Reipert *et al*., 2003).

The cell wall plays an important role in increasing the resistance of bacteria to antibiotics, by increasing the manufacture of amino acids required to build the cell wall such as Alanin and sugars such as N-acetyl glucosamine, which helps the cell to build a thick cell wall to show the highest level of resistance (Kuroda *et al*., 2000).

B. Measurement of the minimum inhibitory concentration of vancomycin and zinc nanoparticles

MIC results of the current study contradicted the results of several studies, including the study of the researcher Karim (2015), where the MIC values were (2500-325) µg/mL for ZnO 20 and ZnO 150-50 and nm 30 and the MIC values were between (2600-126.5) µg/mL Whereas in Abdulrahman and Nssaif (2016) the MIC values for ZnO 20 were (256) µg/mL, the MIC for ZnO 30 was (341) µg/mL and ZnO 150-50 was (160) µg/mL.

MIC results in the study of Nazoori and Kariminik (2018) were ZnO (2500 - 5000) µg /mL but for other bacterial species.

In the study of Aleaghil *et al*. (2016), the MIC values of ZnO ranged between (5000-625) µg/mL, which was close to the results of the current study.

The difference in MIC between the isolates is due to the size and concentration of the nanoparticles (Yamamoto *et al*., 2001) as well as the smaller nanoparticles will increase the surface area and thus the active groups will increase and the toxicity result of the nanoparticles increases (Aleaghil *et al*., 2016).

The high resistance to MRSA and VRSA in the current study was also proven by the study of Ansari *et al*. (2012). They found that the MIC of ZnO to MRSA could reach to 2000 µg/mL.

Several mechanisms have been proposed to explain the effectiveness of ZnO, including the production of hydrogen peroxide, which is an important factor in inhibiting bacterial growth (Yamamoto, 2001). Another mechanism is the release of zinc ions that work with H_2O_2 to break down or disrupt cell membrane lipids and proteins and thus lead to leakage of internal cellular contents. For cells and ultimately cell death, which is caused by the small size of the cell with an increase in surface area, which leads to an increase in the effectiveness of ZnO (Xie *et al*., 2011). Zinc oxide can interfere with Nor A protein, which is an advanced protein to confer resistance in bacteria and has pumping efficacy that leads to pumping Or the escape of hydrophilic fluoroquinolones from the cell, another illustration is that ZnO can interfere with the Omf protein, which is a membrane protein responsible for the permeability of quinolones into the cell (Banoee *et al*., 2010).

The mechanism by which nanoparticles interact in general with bacterial cells is that microorganisms carry a negative charge while metal oxides carry a positive charge, which creates an electromagnetic attraction between the bacteria and the surface of the minute and that the nanoparticles release ions that interact with the thiol group (-SH) Of nutrient transport proteins that emerge from the bacterial cell membrane, which reduces membrane permeability and thus cell death (Zhang and Chen, 2009).

V. Conclusion

The highest percentage of bacterial isolation from wounds. Vancomycin gave an synergistic effect with zinc oxide nanoparticle in the three sizes, but the highest effect with 20 nm zinc oxide nanoparticle.

Conflict Of Interest

The authors declare no conflict of interest

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