Comparison between phenotypic and genotypic methods in biofilm formation in MRSA isolates based on zinc oxide quantum dots

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Abstract

Background: The MRSA bacteria are known for their high resistance to many drugs. One of the main virulence factors that support the survival of MRSA and its resistance to most antibiotics is the formation of biofilms.

Objective of study: This study aims to investigate the effect of nanomaterial zinc oxide quantum dots (ZnO-QDs) on resistant bacteria after understanding their antimicrobial resistance patterns, from MRSA to a newer antibiotic, in order to identify any extensively drug-resistant (XDR) isolates..

Materials and Methods: From 100 clinical samples, 20 isolates were identified as MRSA isolates Sensitivity testing was conducted on all isolates using the VITEK2 system. These isolates were classified into three categories: MDR, XDR, and to non-MDR isolates, based on the results of their antibiotic resistance profiles. Biofilm formation ability was investigated using TCPM, TM, CRA tests, and the fnbB gene. A strong correlation was found between MDR and XDR MRSA isolates with biofilm formation and the biofilm-related gene fnbB. TCPM showed good results for detecting biofilm producers compared to CRA and TM. By using TCPM we investigate the effect of adding ZnO-QDs alone, oxacilline also alone and oxacilline with ZnO-QDs on biofilm formation

Results: By VITEK2 system. These isolates were classified into four categories: MDR, XDR, based on the results of their antibiotic resistance profiles, showing 43.64%, 51.23%, respectively, compared to non-MDR isolates that yielded 5.13% of MRSA isolates. While Only 20 isolates were identified by using TCPM, TM, CRA tests, and the fnbB gene, with 16 showing very strong biofilm formation, 3 isolates showed moderate biofilm formation, and 1 isolate exhibited weak biofilm formation, biofilm test results showed that adding ZnO-QDs alone had a better effect on inhibiting biofilm formation in MRSA isolates than the presence of oxacillin. ZnO-QDs reduced biofilm formation ability in MRSA isolates. Using these materials, we obtained 12 isolates with very weak biofilm formation, while 5 isolates showed greater resistance to these materials in biofilm formation, and 3 isolates exhibited moderate resistance to these nanomaterial's.

Keywords: MRSA; ZnO-QDs; VITEK2; MDR; XDR; fnbB gene

INTRODUCTION

Methicillin-resistant Staphylococcus aureus is a common cause of contagion in hospitals and communities. It is a big health problem worldwide as it is not affected by many common antibiotics, such as methicillin, that leads to standard treatments not effective. This germ is well-known for being able to form biofilms, which are groups of microbes circumfluent by a protective layer. Biofilms act a big role in making MRSA (a type of bacteria) more hurtful, serving it to remain and repel the body's defenses and antibiotics. This is especially serious in long-lasting infections and those related to medical devices such as catheters and artificial joints. [1-3]. Infections raised by MRSA that are related to biofilms are difficult to cure. To weed out them, we want new methods that can prohibit biofilms from forming or get rid of the biofilms that are already there. [4-5]. the creation of biofilm in MRSA is controlled by complicated genetic systems that manage how it makes sticky molecules, sugary substances, and other parts of the biofilm. Important

genes that assist control biofilm formation include the ica operon, which administer the creation of a sticky substance called polysaccharide intercellular adhesion (PIA), and the sarA gene. The sarA gene prefer how biofilms grow by changing how proteins that help cells stick together and make up the biofilm are expressed. [6-7]. In addition, quorum sensing, planned by the agr system, is very important for managing how biofilms develop and modify. [8]. these biological processes assist MRSA produce biofilms in various places, like body tissues and medical devices. The biofilm appears like a shield, defending the bacteria from treatment and the body's immune system. [9]. recently, nanotechnology has become a sensational way to aid fight bacterial infections, including those rise by MRSA that shape biofilms. Zinc oxide (ZnO) nanoparticles, especially as quantum dots (ZnO QDs), are earning a lot of interest as they able kill bacteria. They do this by making reactive oxygen species (ROS) that has the ability damage bacterial cell membranes and deactivate their functions. [10-13]. ZnO QDs able to hold the growth of bacteria, block the formation of biofilms, and even dispose of existing biofilms in different types of bacteria, including MRSA . [14-16]. These features make ZnO QDs a good option for making new antimicrobial treatments to struggle MRSA biofilms, which can lead to problems made by antibiotic resistance. However, even though they offer promise in killing germs, we don't fully understand how ZnO QDs impact the growth of MRSA biofilms, especially when contrasted to usual ways of studying biofilms. We can research how biofilms are formed by looking at their physical features, such as their size and structure. Common ways to study how organisms grow include the crystal violet test, which measures the amount of biofilm, and tissue culture plate (TCP) tests, which check how biofilm forms on solid surfaces. [17-18]. these methods are often used, but they don't support us understand the genetic reasons behind how biofilms form. In contrast, genotypic methods look at how genes related to biofilm formation are expressed. These methods, like PCR and qPCR, assist us survey important genes that act a role in biofilm formation, including icaA, icaD, sarA, and agr. [17-21]. this study looks at how MRSA bacteria react to zinc oxide quantum dots (ZnO QDs) in their genes. By doing this, we able to understand how ZnO QDs influence the genes that control biofilm formation, which is a protective layer of bacteria. The goal is to compare how well MRSA forms biofilms when managed with ZnO QDs, using different methods to research both their physical traits and genetic traits. This research will contrast the outcomes of tests used to study bacteria (like crystal violet and TCP tests) with the activity of genes regarding to biofilms. This will provide us better understand how ZnO QDs impact the growth of biofilms in MRSA bacteria. The results of this study could assist make new ways to block and manage MRSA -related infections that make biofilms. This might lower the need for regular antibiotics and tackle the increasing issue of germs that are resistant to treatment. [22-23]. besides looking at how ZnO QDs directly affect biofilm formation, this study will also scout how nanomaterials might assist shatter biofilms more generally. Understanding how zinc oxide quantum dots (ZnO QDs) impact MRSA biofilms at a small scale will assist researchers get more about using tiny materials in fighting infections associated with biofilms. [24-28]. this research might provide new treatments that able to offensive biofilms in ways that regular antibiotics can't. This would be an important tool in the battle against bacterial infections that are resistant to drugs. [29-30].

MATERIALS AND METHODS

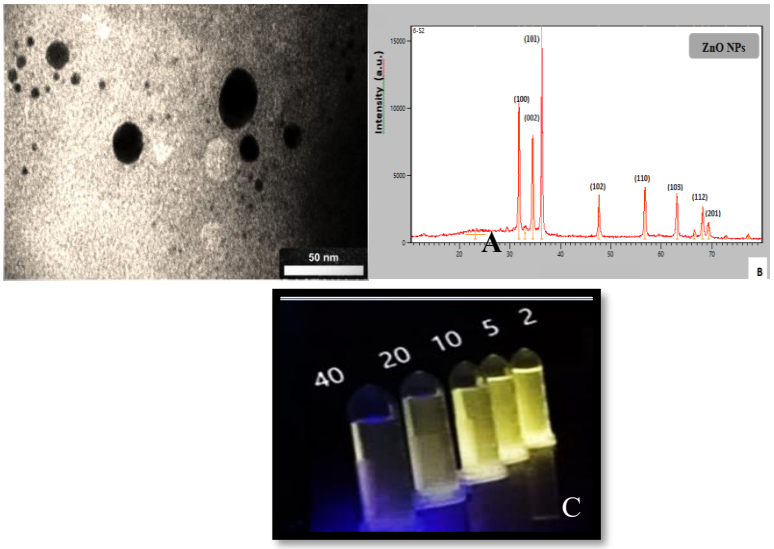
SAMPLE PREPARATION

Bacterial Isolates: In this study,100 clinical samples, 20 isolates were identified as MRSA of methicillin-resistant Staphylococcus aureus (MRSA) isolated from characteristic wound samples of burn patients at Al-Kadhimiya Teaching Hospital from December 2021 to April 2022 were examined. Samples were retrieved from the wound areas with the highest degree of burns using sterile swab sticks and surgical blade edges. Antibiotic sensitivity was determined by VITEK2 system .To further investigate the extent of antibiotic resistance and identify precise phenotypic profiles of bacterial resistance mechanisms for each isolate tested, an automated microbial identification system (VITEK 2) was used, which provides highly accurate and reproducible results (>98% accuracy and precision).These isolates were classified into three categories: MDR, XDR, and compared to non-MDR isolates [31].

Zinc Oxide Quantum Dots (ZnO QDs):

ZnO QDs will be synthesized and characterized using methods such as TEM (Transmission Electron Microscopy) for size distribution and X-ray diffraction (XRD) for crystal structure confirmation. Different concentrations of ZnO QDs will be tested to assess their impact on biofilm formation [32]. The surface morphology and size of the quantum nanoparticles produced were evaluated through TEM analysis. The TEM images show a spherical structure with an average size of less than 10 nanometers for quantum dots. The average sizes of the ZnO-QDs were calculated to be

9.52±3.05 respectively [33] [34] [35]. As shown in Fig. 1.



*Fige.1. **A**: the TEM image of ZnO-QDs scale 50 nm. **B**: X-ray diffraction pattern of ZnO QDs **C**: The synthesized ZnO-QD solution showed a yellow color under UV irradiation. By diluting the sample, the absorption peak at 360 nm and the light emission intensity decreased*

Phenotypic and Genotypic Assessment of Biofilm Formation

To evaluate the biofilm inhibition properties of the produced nanoparticles, the biofilm formation potential in 20 selected isolates was first assessed using the TM method, growth on Congo Red agar, TCPM and the detection of the fnbB gene.

Phenotypic Assessment of Biofilm Formation: using TM, CRA ,TCPM Tests

For biofilm formation determination by the TM method, a full bacterial loop from a 24-hour bacterial culture in BHI medium containing 1% glucose was inoculated and incubated for 24 hours at 37°C. Afterward, the contents in the tubes were discarded, and the remaining materials were washed with phosphate-buffered saline (PBS). The tubes were then dried in the laboratory environment and stained with 0.1% crystal violet for 20 minutes. The tubes were washed several times with distilled water. Biofilm formation was observed as a purple-stained layer on the inner surface of the tube, and the biofilm formation was categorized as strong, medium, or absent. The Congo Red medium consisted of BHI (37 g/L), sucrose (5 g/L), agar (10 g/L), and Congo Red dye (0.8 g/L). The Congo Red dye was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes. It was then added to the autoclaved BHI medium containing sucrose at 55°C. The plates were inoculated with the test organism and incubated aerobically at 37°C for 24 hours. Black colonies with a dry crystalline consistency indicated biofilm-producing MRSA isolates, while smooth red colonies indicated isolates lacking biofilm formation capacity [36].

Genotypic Assessment of Biofilm Formation: using the fnbB gene.

To detect the fnbB biofilm-producing gene in MRSA isolates, the zymoresearch MiniPrep Kit was used. The quality of the extracted DNA was checked on an agarose gel for all isolates. The DNA concentration was adjusted to approximately 100 ng/µL, and the A260/A280 ratio was evaluated using a NanoDrop-2000 spectrophotometer. A ratio between 1.8 and 2.0 is considered acceptable for DNA purity. The 20 isolates were screened for the fnbB gene using PCR with specific primers. The primers and PCR protocols used for detecting biofilm-related genes are listed in Tables 4-2 to 6-2. For all genes tested, a final PCR reaction volume of 25 µL was prepared, containing 0.7 µL of each primer, 12 µL of master mix, and 10.6 µL of sterile distilled water. Finally, 1 µL of DNA template was used for the PCR reaction [37]. Table.1,2,3.

Table 1.DNA Template Reaction Mix Producing Strain Screening

Volume (µL)	*Reagents*
12	*Master mix*

Table 2. PCR Test Conditions

Table 3. Primers for Biofilm-

Volume (µL)	Reagents
0.7	Forward primer
0.7	Reverse primer
1	DNA template
10.6	Distilled water

Step	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation	95°C	60 seconds
Annealing	55°C	50 seconds
Extension	72°C	60 min
Final extension	72°C	7 min

Gene	Primer	Sequence	Amplicon Size (bp)
fnbB	Forward	GGAGAAGGAATTAAGGCG	813
	Reverse	GCCGTCGCCTTGAGCGT	

2.5. BIOFILM INHIBITION ACTIVITY

Biofilm inhibition activity of the produced nanoparticles was assessed using the Tissue Culture Plate Method (TCPM). In 96-well microplates, 100 µL of BHI medium with 1% glucose was added to each well. Then, 100 µL of the ZnO nanoparticle solution (25.6 µg in 100 µL distilled water) was added to the first well. Afterward, a dilution process was performed up to 10 steps. After the dilution process, 10 µL of bacterial suspension with a 0.5 McFarland standard concentration was added to the wells, and the plates were incubated for 24 hours at 37°C. After incubation, each well was washed three times with PBS (pH: 7.2) and dried for 24 hours at room temperature. The samples were then stained with 0.1% crystal violet for 20 minutes, washed again with distilled water, and finally treated with 110 µL of 30% acetic acid. After 20 minutes, the absorbance was measured at 630 nm using an ELISA reader [38].

2.5.1 Biofilm inhibition tests

Biofilm inhibition tests for ZnO nanoparticles, oxacillin antibiotic, and ZnO nanoparticles + oxacillin were performed within a concentration range of 0.05–6.25 mg/mL, with three repetitions. Positive control groups (bacteria and culture medium) and negative control groups (absence of

Bacteria but containing the antibacterial agent and culture medium) were also tested to compare the results and verify the test.

The percentage of biofilm inhibition and residual biofilm was calculated using the following equation:

Inhibition percentage (%) = [(Positive control absorbance - Sample absorbance) / (Positive control absorbance)] × 100

Residual biofilm percentage (%) = 100 - Inhibition percentage

3. RESULTS

3.1. ANTIBIOTICS RESISTANCE

In this study, a total of 20 isolates of Staphylococcus aureus were studied, which were obtained from burn patients who were hospitalized at the specialized burns reference hospital in Al- Kadhimiya Teaching Hospital. By VITEK2 system these isolates were classified into three categories: MDR, XDR,based on the results of their antibiotic resistance profiles, showing 43.64%, 51.23%, respectively, compared to non-MDR isolates that yielded 5.13% of MRSA isolates. As shown in Fig. 2.

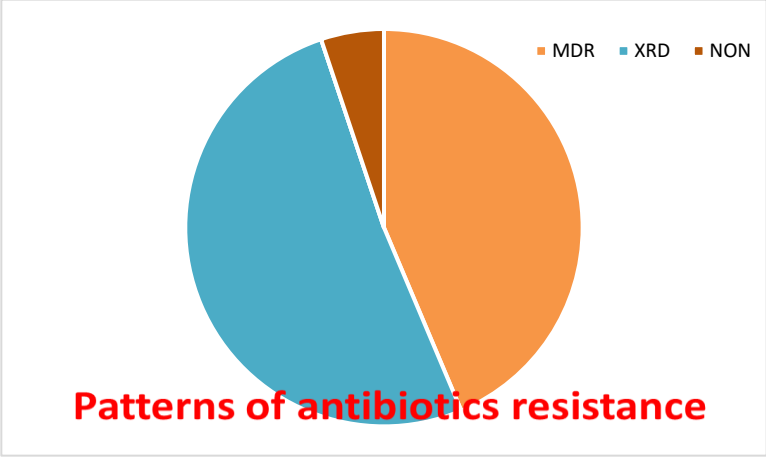


Fig.2.Classes of antibiotic resistance patterns of MRSA isolates, Multi drug resistance (MDR), Extensive drug resistance (XDR) Drug resistance and non-MDR isolates.

3.2. BIOFILM RESISTANCE

20 isolates were identified by using TCPM, TM, CRA tests, and the fnbB gene, with 16 showing very strong biofilm formation, 3 isolates showed moderate biofilm formation, and 1 isolate exhibited weak biofilm formation As shown in Fig. 3,4,5,6,7.and Table.4.

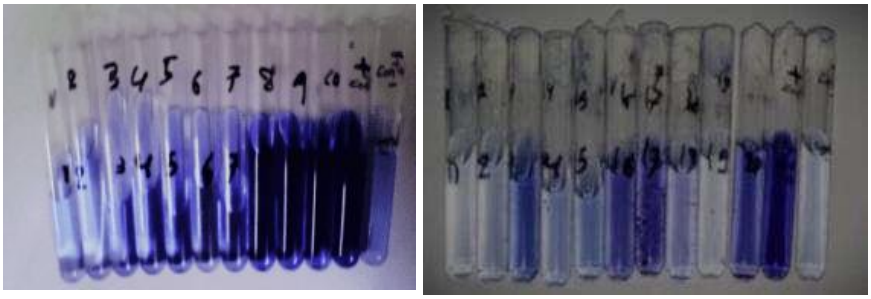


Fig.3.TM test results for 20 MRSA isolates

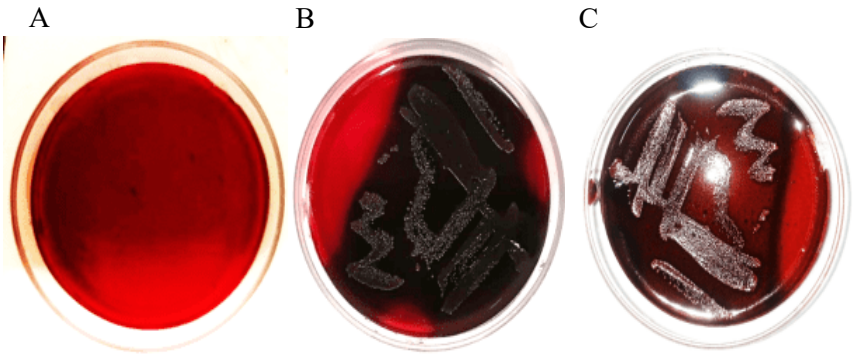


Fig.4. Congo Red agar (A), *MRSA* biofilm-producing isolate (B), and non-biofilm-producing isolate (C).

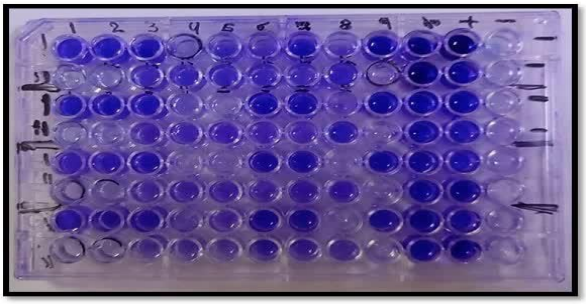


Fig.5.Results of the TCPM biofilm inhibition test

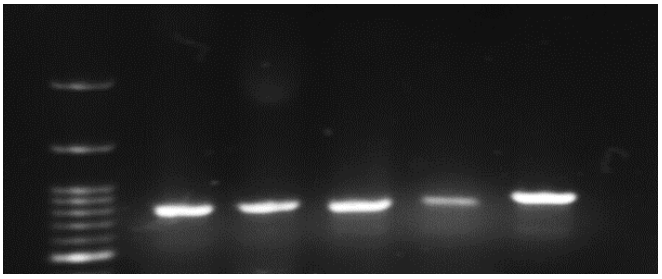


Fig.6.Presence of the fnbB gene in the biofilm-forming strain

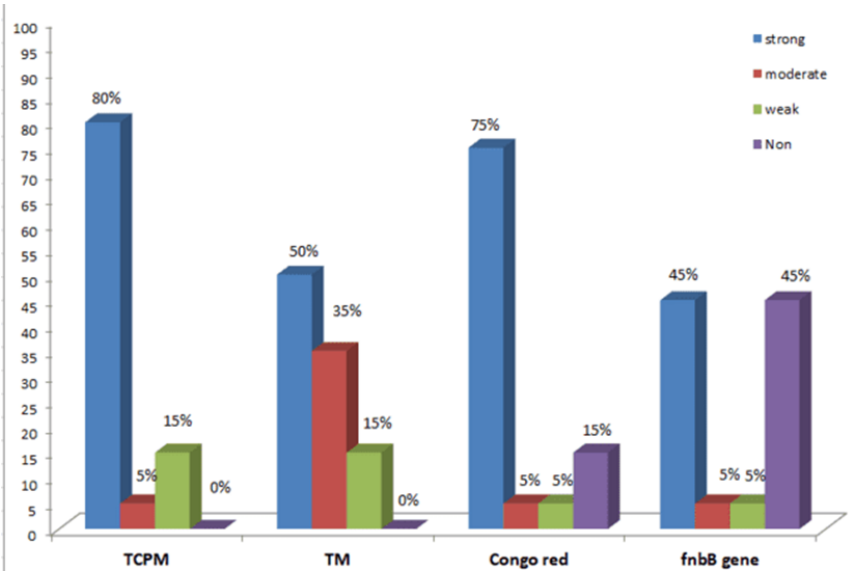


Fig.7. Comparison of biofilm formation capacity in 20 isolates using 4 different methods.

Table.4.the absorbance of 20 *MRSA* isolates at 630nm during biofilm formation studies is shown in the table. White, yellow and green cells indicate strong, medium and weak biofilm, respectively. Anti-biofilm Activity

3.3. ANTI-BIOFILM ACTIVITY

The anti-biofilm activity of ZnO-QDs, oxacillin, and ZnO-QDs/oxacillin was evaluated by measuring the absorbance at 630 nm. Absorbance values of >0.4, >0.3, and <0.2 were indicative of strong, moderate, and weak biofilms, respectively. Out of 20 MRSA isolates, 16 displayed strong biofilms, one showed a moderate biofilm, and three exhibited weak biofilms. Seventeen isolates, including those with strong and moderate biofilm production, were used to assess the anti-biofilm effect. Table 1 shows the absorbance of 20 MRSA isolates at the 630 nm wavelength during the biofilm formation assay. White, yellow, and green cells represent strong, moderate, and weak biofilms, respectively. After treating the isolates with ZnO-QDs, oxacillin, and ZnO-QDs/oxacillin, all isolates lost their biofilm-forming ability at the concentrations tested. Only at a concentration of 0.05 µg/mL ZnO-QDs was there a very small percentage of biofilm observed after treatment (Table 3-4). Dark cells in the table represent weak and very weak biofilms.biofilm test results showed that adding ZnO-QDs alone had a better effect on inhibiting biofilm formation in MRSA isolates than the presence of oxacillin. ZnO-QDs reduced biofilm formation ability in MRSA isolates. Using these materials, we obtained 12 isolates with very weak biofilm formation, while 5 isolates showed greater resistance to these materials in biofilm formation, and 3 isolates exhibited moderate resistance to these nanomaterial's. As shown in Fig .8.

10	9	8	7	6	5	4	3	2	1	MRSA
+ 0.532	+ 2.731	+ 0.451	+ 2.166	+ 2.166	+ 0.453	+ 0.302	+ 2.302	+ 1.300	+ 1.351	Abs.
20	**19**	**18**	**17**	**16**	**15**	**14**	**13**	**12**	**11**	MRSA
+ 1.138	+ 0.283	+ 0.553	+ 0.739	+ 0.540	+ 0.539	+ 0.646	+ 0.532	+ 0.124	+ 0.168	Abs.

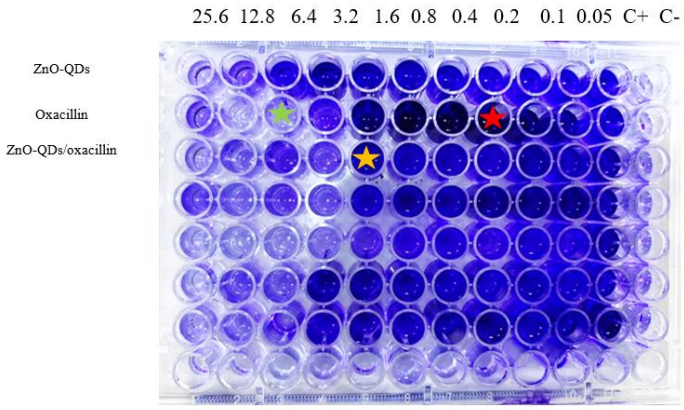


Fig .8.The results of the biofilm inhibition test in the microplate of 96 houses. Green, orange and red stars indicate weak, medium and strong biofilm, respectively.

3.4. Correlations: Correlation analysis between phenotypic biofilm formation and genotypic expression of biofilm-related genes, discussing the validity of phenotypic methods in predicting molecular changes.

4. DISCUSSION

Nanotechnology, due to its high efficiency and therapeutic index against microbes, is a promising therapeutic strategy. Nanoparticles offer a suitable alternative for managing most bacterial infections, particularly those involving drug-resistant organisms. Nanoparticles can be used alone or in combination with antibiotics, which produces excellent synergistic effects. Nanomaterials have at least one dimension in the nanometer range (1-100 nm), giving them unique physical and chemical properties compared to bulk materials. Among the wide range of nanomaterials, nanoparticles have received particular attention. They possess several characteristics that make them useful as drug carriers for fighting pathogens as well as markers for identifying pathogenic microorganisms. These properties include increased solubility and stability, ease of synthesis, and biocompatibility with target agents, which can be controlled by stimuli such as light, pH, and heat. Their distinct performance in drug delivery is achieved through their very small size and large surface-to-volume ratio. This provides a key competitive advantage over conventional treatments in managing infections caused by intracellular pathogens and antibiotic-resistant strains.

The results will be interpreted in the context of current knowledge about biofilm formation in MRSA and the impact of ZnO QDs. This section discusses how phenotypic assays correlate with genotypic data and provide insights into the molecular mechanisms underlying biofilm inhibition by ZnO QDs. The potential for using ZnO QDs as an alternative or adjunct to conventional antibiotics in preventing MRSA biofilm-related infections will be explored .This study compares phenotypic and genotypic methods for assessing biofilm formation in MRSA isolates exposed to ZnO QDs. The findings support the use of ZnO QDs as an effective biofilm inhibitor and highlight the importance of combining both phenotypic and genotypic approaches for a comprehensive understanding of biofilm formation mechanisms. Future research directions may include further optimization of ZnO QD formulations and exploring their application in clinical settings.

5. CONCLUSION

The susceptibility of methicillin-resistant Staphylococcus aureus isolates was evaluated using the VITEK2 system and the AST card according to CLSI guidelines. The results showed a strong correlation between phenotypic resistance in MRSA isolates, particularly extensively drug-resistant (XDR) and multidrug-resistant (MDR) isolates, with biofilm formation and the presence of the biofilm-related fnbB gene. The current study provides updated data on the resistance and genes involved in biofilm production of MRSA isolated from burn patients at Al-Kadhimiya Teaching Hospital in Iraq. The biofilm test results indicated that the addition of ZnO-QD reduced the biofilm formation ability of MRSA isolates. Using ZnO-QD , 12 isolates were very weak in biofilm production, while 5 isolates showed greater resistance to these materials in biofilm production. In anti-biofilm activity, ZnO-QD was more effective than oxacillin and ZnO-QD/oxacillin. The resistance of some MRSA isolates to oxacillin was associated with an increase in bacterial population, glycocalyx secretion, bacterial binding, and the formation of a protective layer that prevents further penetration of oxacillin. Furthermore, when ZnO-QDs were combined with oxacillin, the concentration of ZnO-QD was reduced, in contrast a higher concentration of ZnO-QD alone, leading to the greatest activity against bacterial biofilms. Investigations into the ability of ZnO-QD nanoparticles to bind to ITS gene fragments showed that nanoparticles functionalized with the amine group of the APTS compound near macA have the ability to bind to DNA and can be used as a marker to identify ITS gene fragments.

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