Anti-quorum sensing effect of streptococcus agalatiaceae by Zinc Oxide, Copper Oxide, and Titanium Oxide nanoparticles

Haneen K. Abdul-Hamza and Ghaidaa J. Mohammed
Biology Department, Faculty of Sciences, University of Al-Qadisiyah, Iraq
Haneenkadhim29@gmail.com, ghaidaa.mohammed@qu.edu.iq

Abstract. Quorum sensing system found in many species of bacteria, including Streptococcus agalactiae that use to regulate many virulence and pathogenicity characteristics. So, this study achieved to detect the inhibitory effect of (CuO, ZnO, TiO₂) nanoparticles on quorum sensing of Streptococcus agalactiae. The bacterial isolates of Streptococcus agalactiae were obtained from vaginal and rectal swabs from pregnant women who visited maternity and children hospital in Al-Diwaniyah city from 7/2018 to 4/2019. Three metal oxides nanoparticles (CuO, ZnO, TiO) were used which was examined using a scanning electron microscope and then three different concentrations were prepared (100, 250, 500) µg/ml to study its effectiveness against quorum sensing. The nanoparticles showed their effectiveness in inhibiting the quorum sensing of Streptococcus agalactiae through the formation of a clear inhibition zone around the wells. Copper oxide nanoparticles showed high efficiency in inhibition of the quorum sensing with a diameter of (35) mm. Also, zinc oxide nanoparticles showed efficacy against the quorum sensing with diameter (27) mm, while the less inhibition effect appeared with the use of titanium oxide, where the diameter of inhibition was (20) mm. The results of the study showed the efficiency of nano-oxides (TiO₂, ZnO, CuO) in inhibiting the quorum sensing of S. agalactiae bacteria, and copper oxide was the most efficient among them. So, there is need to further studies to detect the effect of nanoparticles on quorum sensing and virulence factors of other bacteria.

Keywords: Streptococcus agalactiae, Quorum sensing, Nanoparticles oxides

1. Introduction

Quorum Sensing is considered the best metabolic and behavioral system for bacteria, which is a way of achieving cellular communication by exchanging chemical signals in bacterial groups to adjust bacterial patterns according to the density of bacterial cells and plays an important role in the production of virulence factors (1) (2). It is a response and alert system in which the expression of several bacterial genes is regulated in a cell
density-dependent manner by means of small signaling molecules known as autostimuli used by Gram-positive and negative bacteria. The quorum sensor system that uses N-acyl lactone molecules in Cram-negative bacteria is the best understood system. A quorum sensor system regulates the gene expression of many genes, including those responsible for producing virulence factors, formation of exogenous enzymes, antibiotics, and bacterial properties.\(^{(3)}\)

Many bacteria, including *Streptococcus*, are known for their ability to regulate many physiological processes through a mechanism called a quorum. In bacteria positive for the gram stain, the quorum system is generally composed of three components, a peptide signal and a two-unit regulatory system (also called a quorum system). Two-unit signal transduction (TCSTS) that contains membrane sensors of histidine amino acid and a cellular internal response regulator. The sensor system was found in positive bacteria to regulate a number of physiological activities, including the possibility of evolution during the binding of signaling molecules in the quorum system for new bacterial cell surface receptors. It helps in the reproduction of the gene within the single species of bacteria as well as the different species.\(^{(4)}\)

Nanomaterials have become a promising and effective candidate that could replace traditional materials in all areas of science and technology. Due to the small size of nanomaterials that have a greater surface to volume ratio and an increasing number of active atoms on their outer surfaces\(^{(5)}\). Some metallic nanomaterials have been approved as bactericidal agents, among them silver, gold and zinc, as each has different properties and spectral activities\(^{(6)}\)\(^{(7)}\). So, the objective of this study was to detect the inhibitory effect of (CuO, ZnO, TiO\textsubscript{2}) nanoparticles on quorum sensing of *Streptococcus agalactiae* isolated from pregnant women.

**2. Methodology**

**2.1. Collection of Bacterial Isolates:**

In this study *S.agalactiae* was isolated from 850 clinical samples including: (425) vaginal swabs, (425) rectal swabs that collected from pregnant women at (37-35) weeks of pregnancy who admitted the maternity and children hospital in Al-Diwaniyah city through the period (7/2018 to 4/2019) and identified by studying the morphological characteristics of colonies on culture, microscopic characteristics of bacterial cells
traditional biochemical methods, and confirmed the identification by API-20 Strep and by using Vitek 2 system.

2.2. Nonomaterials preparation

A scanning electron microscope is used to study the size and properties of the nanomaterial, the nanoparticles (ZnO, TiO, CuO) NPs were examined with the electron microscope unit. The nanoscale samples were prepared by grinding them or exposing them to sound waves, making a colloidal suspension from the nanomaterial, and adding a drop of suspensions to the fixing mold. Then it was dried and examined with a scanning electron microscope, where the imaging was done on voltage 15-12.5 KV, with a sample size of 5mm, working distance 10-5mm, and under different magnification forces (8). The tests were conducted in Tehran / Garage / Al-Ghazy Center. After that three concentrations (100, 250, 500) μg/ml were prepared from each nanoparticles to perform a quorum sensing suppression test for *Streptococcus agalactiae* isolates.

2.3. Quorum sensing inhibition assay

2.3.1. Extraction of Natural C6-AHL from *Chromobacterium violaceum*

*Chromobacterium violaceum* 12472 that provided from (ATCC/USA) was grown in 200 ml of Brain heart infusion broth and incubated overnight at 28 °C, then the culture was discarded using a centrifuge at 8000 cycles for 1 hour, the supernatant was filtered using filter paper(0.22 mm) and the filtrate was mixed with acidified ethyl acetate (acidified ethyl acetate / supernatant, 7/3, V / V). The final concentration was dried at 40°C, after which it was dissolved with acetonitrile and the extract was stored at 4°C until use in tests for quorum inhibition of *S.agalactiae* using nano-oxides (9).

2.3.2. Test the anti-quorum sensing activity of Nanoparticles using *Chromobacterium violaceum*

The effectiveness of the anti-quorum nano-oxides of *S.agalactiae* was tested using *Chromobacterium violaceum* according to what was mentioned by Khan et al. (10) by the Agar well method in the presence of appropriate quantities of natural C6-AHL where the pellet was plotted on the nutrient medium (0.1) ml of *C. violaceum* and *S.agalactiae* isolates, and the pits were filled with (100) μl (70 μl of nanomaterials (CuO, ZnO, TiO) at
different concentrations and 30 μl of C6-AHL extract) and the dishes were incubated for a period of time 18-24 hours, at 28 °C, to ensure growth inhibition around the pits.

2.3.3. Test the efficacy of S. agalactiae to form quorum sensing particles

Brain heart infusion broth was prepared with 1% aspartic acid, distributed into 6 glass flasks and inoculated with S. agalactiae and incubated at 37 °C for different periods (2,3,4,5,6,24 hours). At the end of each incubation period, 0.01% of KCN was added and after 18 hours of incubation, the medium containing bacteria is filtered using filter paper with a diameter of (0.4 mm). The filtrate is used in the quorum sensor test that is performed by mixing a drop of the filtrate and a drop of fresh bacterial growth on a glass slide and staining with a gram stain and examined under a microscope. The positive result is achieved when there is a collection of bacterial cells.\(^{11}\)

2.3.4. Chemical detection of homoserine lactone production

The production of homoserine lactones detected by separating the supernatant from the culture medium, filtered, and added to BHIB medium devoid of KCN and incubated for 24 hours, after 24 hours the filter medium is incubated with S. agalactiae again and then the Brand test performed to detect the formation of methionine or homocystein from the conversion of homoserin into homocystein.\(^{11})(^{12}\)

3. Results & Discussion

3.1. Results of Isolation and Identification of S. agalactiae

In this study, 16 isolates have been obtained from S. agalactiae bacteria, at a rate (1.88%) out of a total of 850 isolates, where 4 isolates were diagnosed from rectal swabs (0.94%), 12 isolates from vaginal swabs and vaginal smears, at a rate of (2.28%) and these results are more clarified in Table (1). The results of the current study also showed that the percentage of S. agalactiae isolation from vaginal swabs is higher than that of rectal swabs, and this is consistent with what Engelbrech\(^{13}\) stated the isolation rate (36.25%) was from vaginal swabs and (17.5%)rectal swabs as well as with Kadanali et al.\(^{14}\), where the percentage of bacteria isolation from vaginal swabs was (10.7%) and rectal swabs (4.7%), while these percentages were contrary to what was stated by Bidgani et al.\(^{15}\). Rectal swabs were higher than vaginal swabs respectively (27.7%, 30.7%). The difference in isolation rates is due to the difference in the implant media used\(^{14}\).
Table (1): The percentage of *S. agalactiae* isolates from Clinical Samples

| Isolates Sources | Total No. | No. of *S. agalactiae* isolates | Percentage% |
|------------------|-----------|---------------------------------|-------------|
| Vaginal Swabs    | 425       | 2.82                            |             |
| Rectal Swabs     | 425       | 0.99                            |             |
| Total            | 850       | 1.88                            |             |
| X²                |           | 4.062*                          |             |
| P value          |           | 0.043                           |             |

*There is a significant difference at P value 0.05*

The samples under study were diagnosed based on some phenotypic characteristics of the developing colonies, their microbiological characteristics and their biochemical tests \(^{(16)}\). The results of the phenotypic diagnosis showed that the colonies of this bacterium are small, they grow on Blood agar with completely hematopoietic, translucent with a smooth surface forming the capsule, producing small orange colonies on Granda agar and light purple colonies on the Chrome agar, as for the microscopic diagnosis, it is shown that it is spherical in the form of long chains that are positive for gram stain. Figures (1) and (2) show the acceptance of bacteria to Cram stain and the growth of bacteria on culture media respectively.

![Figure (1). The shape *S. agalactiae* cells under a microscope](image-url)
Figure (2): *S. agalactiae* colonies in culture media

The API-20 Strep bacterial diagnostic system (Figure 3) and the Vitek system 2 (Figure 4) were used to confirm the yield of isolates to *S. agalactiae*. The API-20 is one of the diagnostic tests distinguished by its accuracy in diagnosis\(^{(17)}\).

Figure (3): API-20 Strep strip used to diagnose *S. agalactiae*
The isolates were also diagnosed using the Vitek2 system by the GP / ID identification card for the diagnosis, and the results confirmed that the isolates were returned to *S. agalactiae*. This technique showed speed and accuracy in diagnosing isolates with a probability ratio (99%-95%). The study agreed with many previous studies that used the Vitek-2 system in diagnostics such as study by Engelbrecht (13) and Tazi et al. (18).

![Identification Information](image)

![Susceptibility Information](image)

**Figure (4):** The results of biochemical diagnosis of isolates using the Vitek 2 system include probability and an estimate of diagnostic accuracy.
3.2. Examination of nanoparticles (ZnO, CuO, TiO) using a scanning electron microscope

The results of examining the nanomaterials with a scanning electron microscope (SEM) showed the homogeneity and good distribution of the nanomaterial particles and the dominant shape of the granules was the spherical shape which is considered the best in terms of effect for all the nanomaterials, where the size of the copper oxide nanoparticles is approximately 32.3 nm (Figure 5), the size of nanostructured zinc oxide ranges from (30.8-21.1) nm (Figure 6). As for the size of nanoparticles of titanium oxide, it was (45.06) nm (Figure 7).

Figure (5): Image of copper oxide granules with a scanning electron microscope
Figure (6): Image of zinc oxide nanoparticles with a scanning electron microscope

Figure (7): Image of a scanning electron microscope of titanium dioxide nanoparticles
3.3. Results of the quorum sensing inhibition by using nanoparticles

*Chromobacterium violaceum*, commonly used as a biosensor for quorum sensor activity, is a Gram-negative bacterium that is found in water and soil in tropical and subtropical regions and produces antibacterial dyes as a result of quorum sensing by using endogenous stimuli AHL and N-hexanayl-L-homoserin\(^{(19)}\). Therefore, it has been used in studies to investigate quorum sensing formation in *S. agalactiae*.

The test of the ability of *S. agalactiae* bacteria to form the quorum particles and whether the nanomaterials had an effect on its composition was conducted with the standard biomarker *Chromobacterium violaceum* 12472 by the method of well diffusion. Where the well filled with 70μl of different concentrations (100, 250, 500) μg/ml of nanoparticles (CuO, ZnO, TiO\(_2\)) and 30 μl of C6-AHL particles. The inhibitory activity of the nanomaterials was evaluated by measuring the diameter of the halos that formed due to growth inhibition. If the inhibition diameter is less than 8 mm, it is insensitive, between 9 -14 mm is sensitive and very sensitive when the inhibition diameter is greater than 20 mm. \(^{(20)}\)\(^{(21)}\)

The results of the study showed the ability of *S. agalactiae* bacteria to form QS, as well as the nonmaterials showed their effectiveness in inhibiting the quorum sensing by forming a halo or a growth inhibition region around the holes where the copper oxide nanoparticles showed a high effectiveness in inhibiting the quorum sensing, as the inhibition diameter around the wells reached 35 mm as well as Nano zinc oxide showed efficacy against quorum sensing, as the inhibition diameter reached about 27 mm and the least inhibitory effect was by titanium oxide, where the inhibition diameter was about 20 mm, as shown in figure (8).
Figure (8): Inhibition of quorum sensing of *S. agalactiae* by using nanoparticles (CuO, ZnO, TiO2). C: represent Control

4. Conclusion

The results of the study showed the efficiency of nano-oxides (TiO2, ZnO, CuO) in inhibiting the quroum sensing of *S. agalactiae* bacteria, and copper oxide was the most efficient among them. So, there is need to further studies to detect the effect of these nanoparticles and other nanoparticles on quorum sensing and virulence factors of other bacteria.

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