

Nanomaterial Applications of Titanium Dioxide Nanoparticles as Antibacterial Agent against Multi-Drug Resistant *Streptococcus Pneumoniae*

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ABSTRACT

For four months (1st October 2019 to 31st January 2020), fifty sputum specimens collected from patients with suspected pneumonia in AL-Hilla General Teaching Hospital and Babylon Hospital for Pediatric and Gynecology in Babylon Province, Iraq. Only 10 isolates of *Streptococcus pneumoniae* were isolated and identified. Depending on the results of antibacterial susceptibility test with 16 antibiotics by disc diffusion method, *S. pneumoniae* isolates have multi-drug resistance. Full resistance (100%) against erythromycin, Penicillin G and Amoxicillin, full sensitivity (100%) for vancomycin and moxifloxacin, 90% for each Sulfamethoxazole/Trimethoprim and Tetracycline, 80% for each Azithromycin and Amoxicillin/Clavulanic Acid, 70% for Clindamycin, 50% for each Ceftriaxone and Chloramphenicol, 30% for Ciprofloxacin, and 20% for each Levofloxacin, Cefepime and Meropenem. By using agar-well diffusion method the antibacterial activity of Titanium Dioxide Nanoparticles (TiO₂NPs) against *S. pneumoniae* was determined in three concentrations: 20, 30, 40 µg/ml. TiO₂NPs exhibit significant antibacterial efficacy in all concentrations while the biggest diameter of inhibition zone was measured in the higher concentration 40 µg/ml. MIC and MBC of TiO₂ Nano-particles were evaluated by using two-fold serial dilutions method, MIC ≥ 50 µg/ml while MBC ≥ 100 µg/ml.

Keywords: TiO₂ Nanoparticles, *Streptococcus pneumoniae*, antibacterial agent.

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INTRODUCTION

The medical applications of nanotechnology included different fields such as medical applications of nanomaterial in nanomedical therapeutics, nanomedical diagnostics, nano electronic biosensors, antimicrobial nanomedicine and may be molecular nanotechnology at the future. It will hopefully allow the specialists to create more effective antibacterial therapeutics and more rapid and ultra-sensitive bacterial diagnostics and provide us further protection against pathogenic bacteria. recently, many novel nanocarriers have been provided in order to deliver the therapeutic agents by target-specific delivery. The studies reported that Nanoparticles have a broad-spectrum range of microbial agents more than antibiotics and can deal with various types of cells. Thus, nanoparticles have been candidate as an attractive alternative of antibiotics for the medical diagnosis and therapy [1-5]. On the other hand, many studies found that nanoparticles had a Biofilm inhibition activity in many bacterial species [6-8].

Recently, Antiviral nanomedicine is increasing concern as an important part of the nanomedicine, because the control of viruses (treatment or disinfection) is considered a big challenge in the health institutes. After the global spread of the COVID-19 pandemic, global health systems' suffering still in the face of this virus and the inability of common medicines to provide assistance. All attentions have been drawn to the nanoparticles and their potential role in both treatment and disinfection and many studies tried to the effectiveness of nanoparticles as diagnostic or antiviral tools against coronaviruses.

The possibilities of effectively using nanomaterials as vaccines and nanosensors in this field are also presented. many studies tried to prove the effectiveness of

nanoparticles as diagnostic or antiviral tools against coronaviruses. Many studies tried to prove the potential effectively using nanomaterial such as Nano sensors, Nano based vaccine candidates and treatments for coronaviruses. The most important conclusions of these studies can be summarized, The Nano medicine field could be successfully applied for development of the most promising candidate drugs for coronaviruses as a treatment for the symptoms or as antiviral agents (concentration dependent responses). There are some advantages for using Nano sensors for diagnosing of coronaviruses, such as enhancing sensitivity with reducing the test time. in addition, nanoparticles may be used for simple, rapid and low-cost colorimetric test for coronavirus detection. on the other hand, depending to the previous studies on the other types of coronaviruses, the nano-based vaccine has been proven to induce a more potent immune response and need more studies to provide long-term immunization [9-13].

The dominance of multi-drug resistance in the pathogenic bacteria induced the bacteriologists to search about more suitable alternatives than antibiotics. Recently, the antibacterial nanomedicine nanoparticles represented the most attractive alternative as a new drug against bacteria. There is increasing interest of the antibacterial activity of nanoparticles of ZnO and TiO₂ against many species of pathogenic bacteria [14-17]. In order to get best results for using of nanoparticles in the battle against multi-drug resistant bacteria, It is taken into account that new classes, novel mechanism of action and different targets must be provided as compared as the traditional antibiotics. A review by Yacoby and Benhar [18] presented a number of nanomedicine-oriented applications of antiseptics, disinfectants and antibacterial therapeutics, by

summarized many studies described the antibacterial activity of nanoparticles against both Gram-positive and Gram-negative bacteria. Antibacterial activity of nanoparticles could serve as a marker for their potential general toxicity for the animals or humans. The evaluation of this potential toxicity, more complex applications (preclinical or clinical) must be done, in vivo, as actual therapeutics [19].

Among several candidates, TiO₂ stands out due to its potential for use in multifaceted applications. The clinical applications of titanium and its derivatives still account for a high percentage in the field of biomedicine and nanomedicine because of their biocompatible features [20]. Titanium dioxide nanoparticles (TiO₂NPs) has wide using in the medical applications because of its specific properties such as the good stability (physical and chemical), low cost, non-toxicity, non-polluting behavior and high photo-catalytic activity under near ultrasound violate light illumination [21, 22]. Most importantly, the using of titanium dioxide as a nonspecific significant antimicrobial agent against a broad spectrum of pathogenic bacteria (even antibiotic resistant strains), fungi and viruses such as herpes simplex virus, influenza virus and zika viruse. The published studies have showed several mechanisms of antimicrobial action in the nanoparticles which make it the most appropriate promising candidate to overcome multidrug-resistant bacteria because the microorganisms are much less likely to develop resistance to metal nanoparticles compared with the common antibiotics [23-27]

Streptococcus pneumoniae is Gram-positive diplococci "pneumococci". It has many virulence factors helping of evasion from immune-system and invasion the lower respiratory tract. Although it is normal flora in upper human respiratory tract, pneumococci may be lethal pathogen by causing many severe infections, such as pneumonia, meningitis, septicemia, otitis media and bronchitis. Every year, during influenza season, pneumococci have the responsibility of higher mortality in the children worldwide because of its ability to damage the pulmonary cells causing pneumonia. in addition to its ability to cross the blood-brain barrier and infect the brain and spinal cords resulting in pneumococcal meningitis [28,29]

In Iraq, many molecular studies detected that most *S. pneumoniae* isolates have the genes responsible for the virulence factors such as *cspA* gene for capsule, *lytA* gene for autolysis, *ply* gene for pneumolysine, *PspA* for pneumococcal surface protein, *luxS* for luminescence *S*, *α-Eno* for α-enolase and *nanA* gene for neuroaminidase [30,31,32].

Although there is a vaccine, *S. pneumoniae* still represents a significant life-threatening pathogen in different ages worldwide. On the other hand, the emergence of penicillin resistant *S. pneumoniae* and dissemination in different regions and countries made it is a global significant challenge which hinders the control and treatment of pneumococcal infections in elderly and children [33,34]. In Asian countries, the studies have described the changing trends in antimicrobial resistance and serotype distribution of pneumococci and extremely high prevalence of multi-drug resistance [35,36,37].

MATERIAL AND METHODS

Specimens Collection, Bacterial Isolation and Identification

Fifty sputum specimens were collected from patients with suspected pneumonia who visited AL-Hilla General Teaching Hospital and Babylon Hospital for Pediatric and Gynecology in Babylon Province, Iraq, during the period from 1st October 2019 to 31st January 2020. The age of patients was ranging from 5 to 65 years. The sputum specimens had been inoculated on specific culture media and incubated aerobically at 37°C for 24hr. The bacterial isolates were diagnosed depending on the colonial morphology, cellular microscopic examination and biochemical tests. Confirmation of identity of *S. pneumoniae* was performed with VITEK 2 system.

Preparation of Bacterial Suspension

Bacterial suspension was prepared by suspending 2-3 pure colonies of previously identified *S. pneumoniae* in 5ml of sterile brain heart infusion broth and incubated at 37°C for 18h., it was standardized by gradually adding normal saline to compare their turbidity to McFarland standard.

Preparation of TiO₂/ Dimethyl Sulfoxide Solutions (DMSO)

Titanium dioxide nanoparticle stock solution was prepared by dissolving 10 mg of TiO₂NPs in 10 ml dimethyl sulfoxide (DMSO) yielding stock solution of 1mg/ml concentration. 1 ml of the stock solution was diluted with 10 ml of DMSO giving a solution of 100 µg/ml concentration. Further dilution was done to prepare three concentrations included 40, 30, 20 µg/ml.

Antibacterial Susceptibility test by Disc Diffusion Method

This test was performed by using the disc diffusion method on chocolate agar with 16 antibiotics [38]. An inoculum from the bacterial suspension was streaking on a chocolate agar plate. The antibiotic discs were placed on the surface of the inoculated plate at evenly spaced intervals with flamed forceps. Incubation was usually overnight with optimal time of 18h at 37°C. Antibiotic inhibition zone surrounded every disc was measured in millimeter and compared to standard criteria in CLSI [39]

Screening of Antibacterial Activity of Titanium Dioxide against *S. pneumoniae*

TiO₂NPs have been gotten from laboratory of medical physics in faculty of pharmacy, university of Babylon where it has prepared according to Aysa [40] and examined by scanning electron microscopy (SEM).

Modified agar-well diffusion method was used to determine the antibacterial activity of TiO₂NPs against *S. pneumoniae*. Chocolate agar plate was inoculated by streaking with bacterial suspension; each plate was performed in duplicates. Three concentrations of TiO₂NPs solution (20, 30, 40) µg/ml were prepared previously. Four holes with a diameter of (6) mm were punched aseptically with a sterile corn borer (No. 6) on the inoculated plate. Approximately 20 µl of TiO₂NPs solution was introduced into each hole, one concentration for each hole. In addition, 20 µl of DMSO was introduced to the fourth hole as a negative control. One-hour pre-diffusion time was allowed at 4°C, then, plates were incubated at 37°C for 18h. The diameter of inhibition zone was measured in millimeter for the duplicates and the mean was taken

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of TiO₂NPs against *S. pneumoniae*.

MIC is the lowest concentration of antibacterial agent required to inhibit bacterial multiplication. In the present

study two-fold serial dilutions method was used in order to evaluate MIC of TiO₂NPs. Six tubes of sterile brain heart infusion broth BHI (5ml) were prepared. As described in figure 1, stock solution of TiO₂NPs (200 µg/ml) and six dilutions (100, 50, 25, 12.5, 6.25, 3.125 µg/ml) were prepared with brain heart infusion broth. 0.1 ml of bacterial suspension of *S. pneumoniae* was added to each dilution. Finally, each tube containing 2.5 ml nanoparticle solution & 2.5 ml BHI &0.1 ml bacterial suspension. Control tube containing BHI was inoculated with the

bacterial suspension without treatment with TiO₂NPs solution. All tubes were incubated at 37-18h. Visually, MIC represents the first clear tube has no growth.

Minimum Bactericidal Concentration (MBC) is the least amount of antibiotic required to kill a bacterium. In the present study, MBC was determined by plating out the MIC tube and other clear tubes on chocolate agar plate as described in figure 2. The first tube has no growth on chocolate agar plate represents MBC because the bacteria has been killed firstly in this tube.

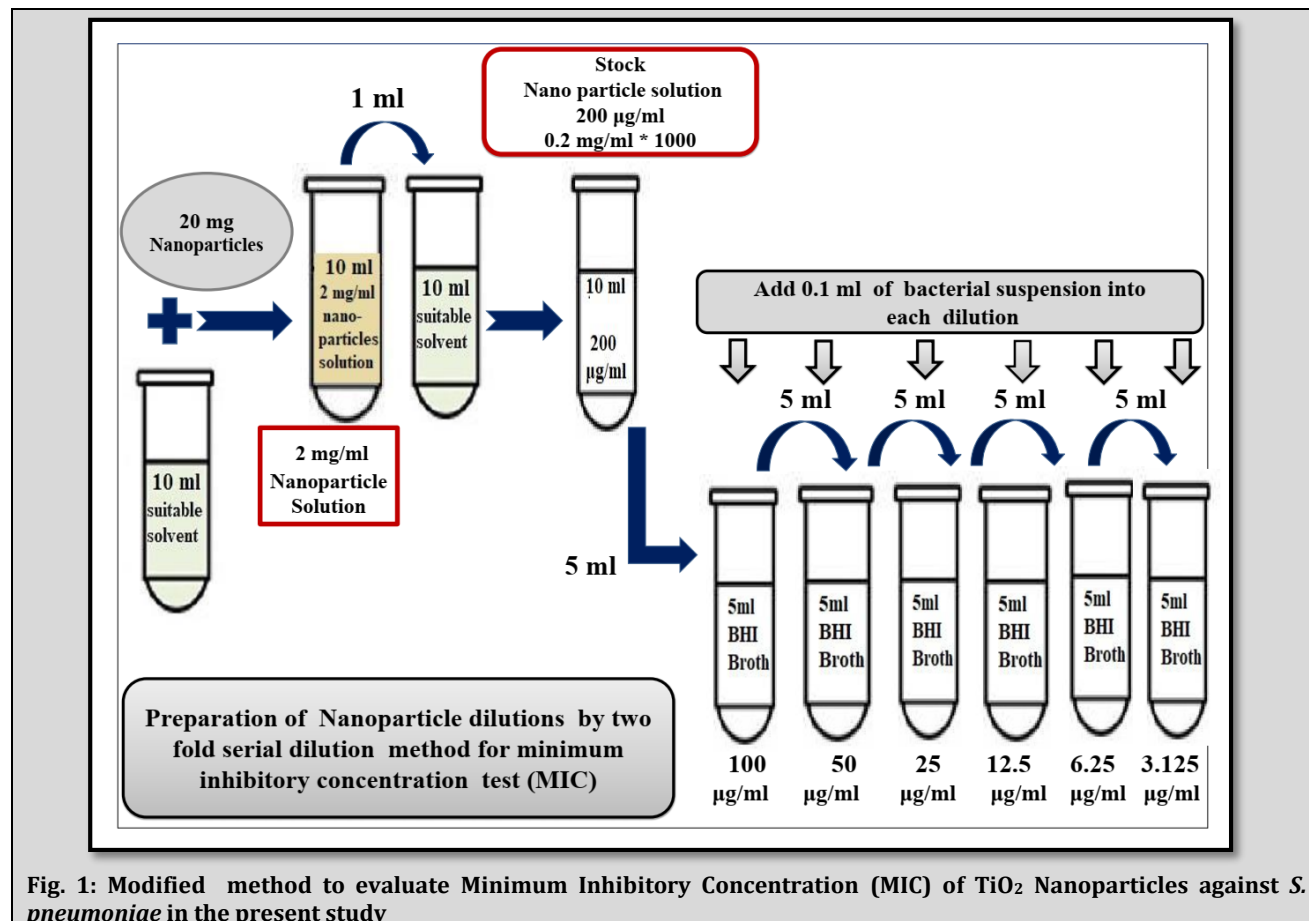


Fig. 1: Modified method to evaluate Minimum Inhibitory Concentration (MIC) of TiO₂ Nanoparticles against *S. pneumoniae* in the present study

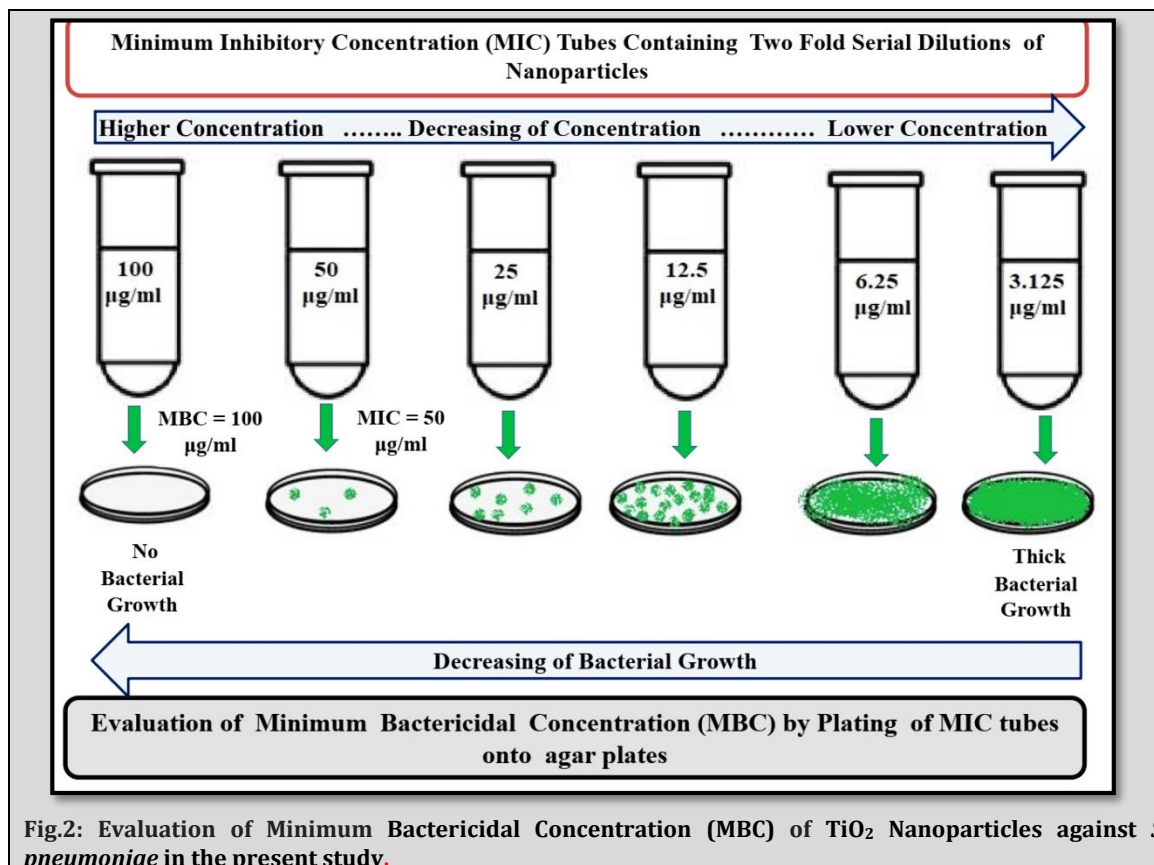


Fig.2: Evaluation of Minimum Bactericidal Concentration (MBC) of TiO₂ Nanoparticles against *S. pneumoniae* in the present study.

Table 1: Diagnostic characteristics of *S. pneumoniae* in the present study

No.	The Test	The Result
1	Gram Stain	Gram-Positive Diplococci, Lancet Shaped
2	Cells Arrangement	Diplococci
3	Hemolysis	Alpha Hemolysis, Partial Hemolysis
4	Optochin Sensitivity	Sensitive
5	Bile Salt Solubility	Soluble
6	Autolysis	Positive
7	The Capsule	Positive
8	Catalase	Negative

RESULTS

Specimens Collection, Bacterial Isolation and Identification

Among Fifty specimens of sputum, only 37 specimens have growth in the different culture media. Forty-six bacterial isolates were identified from these specimens

(mixed infections). A total of 46 isolates were isolated and identified, included different species of gram positive and gram-negative bacteria caused pneumonia. Only 10(21.7%) isolates of *S. pneumoniae* were identified depending on the results of colonial morphology, cellular microscopic examination (table 1), optochin sensitivity (figure 3), biochemical tests and confirmative VITEK 2 system.

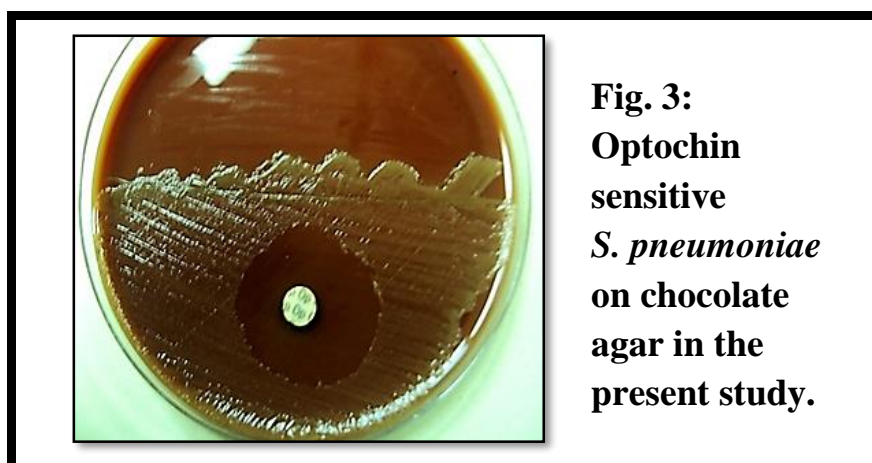


Fig. 3:
Optochin
sensitive
S. pneumoniae
on chocolate
agar in the
present study.

Scanning Electron Microscopy (SEM) examination of TiO₂NPs

Figure 4 portrays the SEM micrographs of TiO₂ NPs. SEM micrographs uncovered group appearances with crystalline natures. Be that as it may, circular structures of size under 20 nm with unpredictable surface

morphologies signified an expanded grain size because of the expansion in temperature prompting crystalline just as grain development. The stony appearance may have been because of the conglomeration of TiO₂ NPs extending between 19-23 nm in size. these findings are comparable with the results of Dai *et al* [41].

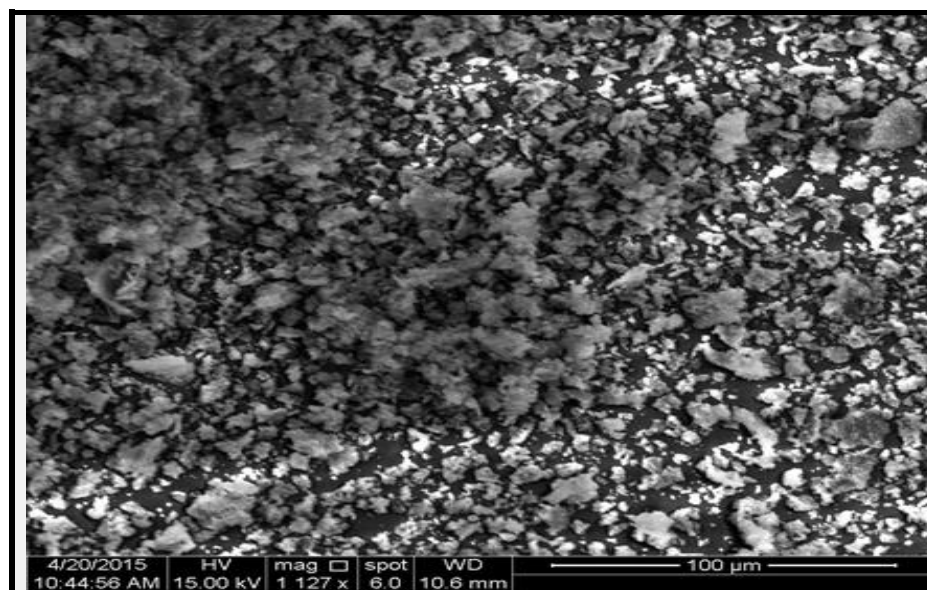


Figure 4 portrays the
Scanning Electron
Microscopy (SEM)
micrographs of TiO₂ NPs
in this study

Antibacterial Susceptibility test of *Streptococcus pneumoniae* by Disc Diffusion Method

Disc diffusion test for antibacterial susceptibility of *S. pneumoniae* against sixteen antibiotic discs was performed in the present study. The results showed that *S. pneumoniae* has clear resistance against most common antibiotics, table 2. Depending on the diameter of inhibition zone surrounded the antibiotic discs, *S. pneumoniae* has a wide variety rang of resistance against different antibiotics. It has full resistance (100%) against

erythromycin, Penicillin G and Amoxicillin. while full sensitivity (100%) was reported for vancomycin and moxifloxacin. The resistance percentage against other antibiotics was a variable as following: 90% for each Sulfamethoxazole/Trimethoprim and Tetracycline, 80% for each Azithromycin and Amoxicillin/Clavulanic Acid, 70% for Clindamycin, 50% for each Ceftriaxone and Chloramphenicol, 30% for Ciprofloxacin, and 20% for each Levofloxacin, Cefepime and Meropenem, Fig. 5.

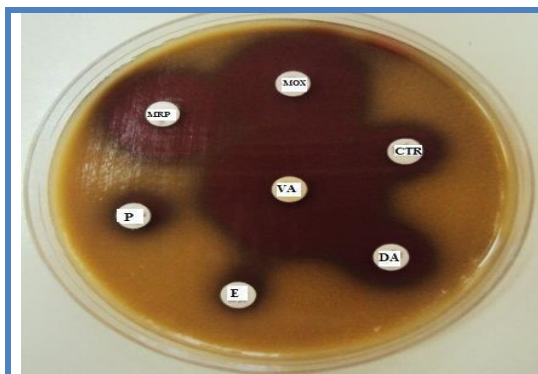


Fig. 5: *S. pneumoniae* has full resistance against Erythromycin and full sensitivity against Vancomycin while the resistance against other antibiotics was variable between them.

Table 2: The percentage of antibiotic resistance of *S. pneumoniae* in the present study

No.	Antibiotic	Resistance %
1.	Erythromycin(E)	100
2.	Azithromycin (AZ)	80
3.	Clindamycin (DA)	70
4.	PenicillinG (P)	100
5.	Amoxicillin (AMC)	100
6.	Sulfamethoxazole / Trimethoprim (S/T)	90
7.	Amoxicillin/Clavulanic Acid (AUG)	80
8.	Ceftriaxone (CTR)	50
9.	Cefepime (CPM)	20
10.	Levofloxacin (LEV)	20
11.	Ciprofloxacin (CIP)	30
12.	Moxifloxacin (MOX)	0
13.	Meropenem (MRP)	20
14.	Tetracycline (TE)	90
15.	Vancomycin (VA)	0
16.	Chloramphenicol (C)	50

Screening of Antibacterial Activity of TiO₂ Nanoparticles against *S. pneumoniae*.

Agar-well diffusion test was used to evaluate the antibacterial activity of TiO₂NPs against ten isolates of *S. pneumoniae* in the present study. As shown in table 3, TiO₂NPs exhibits significant antibacterial efficacy and the diameter of inhibition zones were influenced by the concentration of nanoparticle, Fig. 6.

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of TiO₂ Nanoparticles against *S. pneumoniae*.

In the present study, MIC and MBC of TiO₂ Nanoparticles were evaluated by using two-fold serial dilutions method with six dilutions 100, 50, 25, 12.5, 6.25, 3.125 µg/ml. As detailed in table 4 and fig.7, MIC ≥50 µg/ml for all bacterial isolates, While MBC ≥100 µg/ml.

Table 3: The diameter of inhibition zones of titanium dioxide nanoparticles TiO₂NPs against *S. pneumoniae* in the present study

Conc. Of TiO ₂ NPs (µg/ml)	The diameter of inhibition zone in millimeter										The Range of Diameter of Inhibition Zone
	Number of Isolate										
	1	2	3	4	5	6	7	8	9	10	
20	0	9	0	0	9	7	8	0	8	8	0-9
30	15	20	16	14	21	19	18	14	19	20	14-21
40	23	26	24	24	27	26	27	25	27	27	23-27
Control	0	0	0	0	0	0	0	0	0	0	0

Table 4: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of TiO₂NPs against *S. pneumoniae*.

Number of isolates	1	2	3	4	5	6	7	8	9	10
MIC	MIC ≥ 50					µg/ml				
MBC	MBC ≥ 100					µg/ml				

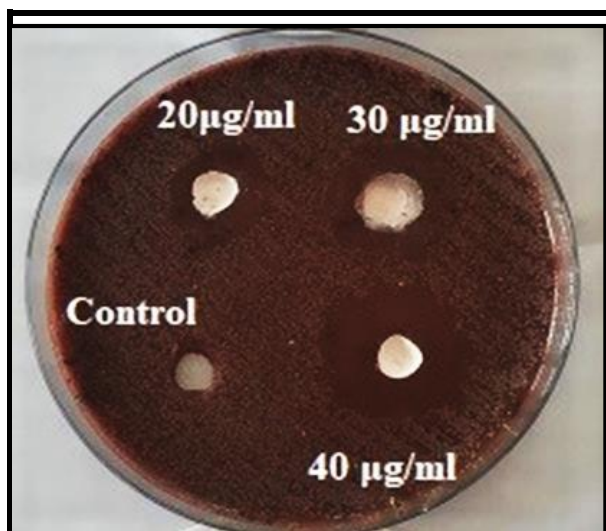


Fig. 6: Antibacterial Activity of TiO₂NPs against *S. pneumoniae* was influenced by the concentration of nanoparticle solution.

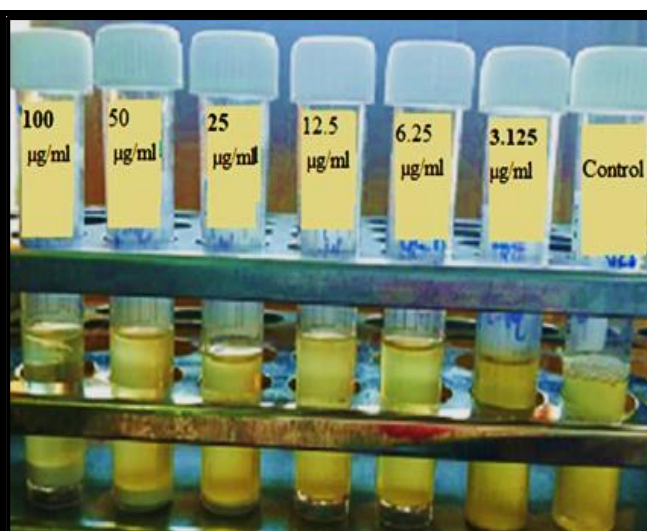


Fig. 7: Six Dilutions 100, 50, 25, 12.5, 6.25, 3.125 µg/ml were used to evaluate MIC and MBC for TiO₂NPs against *S. pneumoniae*. MIC ≥ 50 µg/ml for all bacterial isolates, While MBC ≥ 100 µg/ml.

DISCUSSION

Antibiotic Susceptibility test of *S. pneumoniae* by Disc Diffusion Method.

In the present study the results showed that, *S. pneumoniae* isolates have full resistance 100% against each erythromycin, Penicillin G and Amoxicillin. While no resistance was observed against both vancomycin and moxifloxacin, as well as a variety percentage of resistance against other studied antibiotics were reported, table 2.

In Iraq, many publications studied the prevalence of antibiotic resistance in *S. pneumoniae* and their findings introduced an evidence for the truth that prevalence of multi-drug resistance depend on the geographical area. In Najaf province, full resistance in *S. pneumoniae* against erythromycin and high resistance against azithromycin (83.8%), clindamycin (83.8%), remarkable sensitive to Vancomycin (100%) and Imipenem (100%) were reported. In addition to the molecular detection of the genes responsible for the most common mechanisms of resistance to the macrolides in erythromycin resistant *S. pneumoniae* [40,41]. In Duhok city /Iraq, all isolates of *S. pneumoniae* have full sensitivity to vancomycin and penicillin and 50% to augmentin, but ≤ 41% to clindamycin, cephalothin, gentamicin, erythromycin, cefotaxime, and Co-trimoxazole [42,43].

On the other hand, our results are in disagreement with a study by Alfayate-Miguélez *et al* [44] who pointed out that, oral amoxicillin and intravenous penicillin or ampicillin represent excellent options for the treatment of *S. pneumoniae* isolated from nasopharyngeal samples and

causes non-meningeal infections. Van Bambeke *et al* [45] suggested that, ketolides and fluoroquinolones could be considered for multi-drug resistant pneumococci treatment. The studies emphasized that more than 30% of *S. pneumoniae* are multi-drug resistant worldwide and suggested that the drug resistance genes of *S. pneumoniae* transfer by conjugative Transposon *Tn* which may play an important role in horizontal transfer and clonal dissemination of drug resistance genes in this bacteria [46,37]. In this study, we conclude that most isolates of *S. pneumoniae* developed multi-drug resistance which is often related to the availability of antibiotics out of hospitals which encourage self-medication.

Screening of Antibacterial Activity of TiO₂NPs against *S. pneumoniae*.

Recently, many types of nanoparticles were studied because of it' bacteriostatic and bactericidal efficacy which may be ascribable to the increasing surface area with decreasing size of nanoparticles. TiO₂ NPs which belong to the category of metallic nanoparticles have many applications as photosensitizing agents in the treatment of cancer as well as in photodynamic inactivation of antibiotic-resistant bacteria. The wide applications of TiO₂ and ZnO NPs are related to their low toxicity and high activity [47-52].

As detailed in table 3, TiO₂NPs exhibited significant antibacterial activity against *S. pneumoniae* in all concentrations. This finding is in accordance with that of other studies which showed that TiO₂ NPs have inhibition ability for multidrug-resistant bacteria by specific

mechanisms such as releasing of positively charge ions leads to increase the cell wall permeability; structural deformation of DNA, ribosomes, and cellular enzymes; releasing of reactive oxygen and contributing in the degradation of biomolecules resulting in bacterial cell oxidization and finally bacterial cell death. This bactericidal characteristic of TiO₂ is primarily attributed to the oxidative stress present due to the production of reactive oxygen species (ROS) containing hydroxyl radicals and generation of hydrogen peroxide (H₂O₂) [53-57].

Notably, interesting with using TiO₂NPs in food packaging and keeping was increased by researchers who showed that the packaging films coated by TiO₂ NPs have ability to prevent the bacterial contamination on the food product surfaces by inhibition of biofilm formation by bacteria and therefore reducing the risks of food poisoning [58,59]. On the other hand, TiO₂ NPs can reduce the biofilm formation in the glass surfaces in high rates [60].

In a study by Aysa [40] improved that TiO₂NPs Has antibacterial activity against gram positive and gram negative bacteria may be due to any energy stored inside the TiO₂ to maintain low bacterial levels and reduce the risk of bacteria spreading around water reservoirs and surfaces that are often touched such as phones, keyboards and iPad covers. The mechanism of action of nanoparticles as antibacterial agents include some steps: first, the attachment of nanoparticle on the surface of the bacterial cell, then spreading through the cell wall followed by the adsorption on the cytoplasmic membrane and rupturing it which causes the leakage of the bacterial cytoplasm and finally cell death[61].

As shown in table 3, the diameter of inhibition zone and subsequently the antibacterial activity has increased with increasing of TiO₂NPs concentration, the biggest diameter of inhibition zone was measured in the highest concentration 40 µg/ml, Fig.7. this result is in agreement with other studies [62,63]. Hence, the appropriate concentration of nanoparticles play an important role and help to overcome drug resistance in the pathogenic bacteria [64]. This finding is comparable with Abdulazeem *et al.* [62] who concluded that ,in the suitable concentrationTiO₂NPs have broad-spectrum antibacterial activity and can reduced the biofilm formation significantly.

Evaluation of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of TiO₂ Nanoparticles against *S. pneumoniae*.

As detailed in table 4, MIC ≥50 µg/ml for all bacterial isolates, While MBC ≥100 µg/ml, Fig. 8. These results are comparable with Abdulazeem *et al.* [62] who reported MIC of TiO₂NPs ranged from 31.25 µg/ml to 125 µg/ml and the MBC ranged from 125 µg/ml to 500 µg/ml. A study by Lavaee *et al.* [47] has demonstrated that TiO₂NPs alone have higher MIC and MBC than using them synergistically with other types of nanoparticles.

CONCLUSION

TiO₂NPs exhibited significant antibacterial activity against *S. pneumoniae* which make it the most appropriate promising candidate to overcome multidrug-resistant bacteria because the bacteria are much less likely to develop resistance to nanoparticles compared with the common antibiotics.

Ethical Approval and Consent to participate

We gain the access permission from the authority of Al-Hillah General Teaching Hospital and Babylon Hospital for Pediatric and Gynecology to collect the samples together the verbal consent from patients and their relatives to take samples from them for scientific purpose with maintaining the safety of patient and respect his privacy. We offer our sincere thanks to all patients who agreed to participate in this study and made this work possible

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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