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Original Research Article

Antimicrobial Activity and Synergistic Effect of Some Antibiotics and Nanoparticles Against Gram-Positive Bacterial Isolates Isolated from the Mouth of Dental Caries Patients

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Abstract: Tooth decay is a common disease that occurs as a result of the erosion of tooth enamel by acids produced by bacteria when they feed on sugars. This leads to the formation of cavities, and the damage may extend to the tooth nerve if treatment is neglected. Tooth decay causes varying levels of pain and sensitivity, in addition to unpleasant breath, and the condition may develop into infections and abscesses. The impact of tooth decay is not limited to oral health; it can also negatively affect overall body health. Oral infections may influence heart health, exacerbate complications for diabetic patients, and weaken the body's immune system. Furthermore, it can lead to difficulties in chewing and speaking, as well as impacting an individual's psychological well-being. "The samples included both genders across various age groups, ranging from 10 to 45 years. Appropriate microscopic, cultural, and biochemical tests were performed. Out of the total, 230 samples (92%) showed positive growth. The isolates included Gram-positive species, distributed as follows: *Strep. mutans* (120 isolates, 52.17%), *Staph. aureus* (70 isolates, 30.43%), and *Strep. pyogenes* (40 isolates, 17.40%). The susceptibility of these bacterial isolates was tested against three types of antibiotics: Metronidazole, Clindamycin, and Amoxicillin. The results indicated that *Staph. aureus* isolates exhibited resistance to all the antibiotics studied. As for the *Streptococcus* isolates, they showed varying responses to the antibiotics depending on the species studied. *Strep pyogenes* exhibited a varied response to two antibiotics, Clindamycin and Amoxicillin, with inhibition zones of 22mm and 28mm respectively, while Metronidazole showed no effect. On the other hand, the *Strep. mutans* species demonstrated a varied response toward all the tested antibiotics with better overall results. Additionally, the susceptibility of the isolates was tested against three types of synthesized nanomaterials—Zinc (Zn), Silver (Ag), and Titanium (Ti)—at concentrations of 25%, 50%, and 100% µg/ml. The results showed that Zinc and Titanium nanoparticles acted as effective antibacterial agents with varying rates. In contrast, none of the isolates showed any response to the Silver nanomaterials. Subsequently, a synergistic test was conducted by combining the nanomaterials, which yielded superior results compared to their individual use.

Keywords: Bacteria, Dental Caries, Antibiotics, Zinc Nanoparticles (ZnNPs), Titanium Nanoparticles (TiNPs), Synergistic Effect.

1-INTRODUCTION

Oral diseases, such as tooth decay and gum disease, are problems in modern and developed societies. Tooth decay is a common, chronic, non-contagious oral disease that can affect the health of both adults and children. Various factors, such as the accumulation of microorganisms and the formation of microbial plaque, contribute to the development of the disease (Karimi *et al.,* 2020). An imbalance in the microbial composition, which may result from certain local factors, including carbohydrate consumption, plaque buildup, pathogenic oral microbes, and poor oral hygiene, can lead to oral diseases (Ahmed *et al*., 2023). It is estimated that tooth decay affects between 60% and 90% of school children and nearly 100% of adults worldwide (WHO 2020). Periodontal disease is classified into four stages, with acute periodontitis ranking

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sixth among the most common health conditions between 1990 and 2010 [Kassebaum *et al*., 2014]. It is estimated to affect 10% of the world's population (Peres *et al*., 2019). Dental caries and periodontitis can progress to systemic infections, leading to infective endocarditis, diabetes, and respiratory infections such as pneumonia (Winning *et al.,* 2021). More than 390 types of bacteria live in the human mouth (Doran *et al.,* 2004). These bacteria are found attached to the surface of the tongue, tonsils, deposits on teeth and gums, and in saliva. Staphylococcus spp. is one of the most important types of bacteria that cause tooth decay in humans. Other bacterial genera such as Staphylococcus, Peptococcus, Actinomyces, and Bacteroids are also among the causes of decay (Stenud *et al*., 2001).

S. mutans bacteria are present in the oral cavity and are primarily responsible for dental caries. These bacteria adhere to the tooth surface and synthesize extracellular polysaccharides from the enzyme glucosyltransferase using sucrose, forming a biofilm on the tooth surface. The concentration of chemical signals produced by these primary colonies reaches a high level, leading to the demineralization of the teeth due to the organic acids produced. Consequently, dental decay begins, eventually resulting in cavities. Therefore, limiting or preventing the growth of S. mutans can help control dental caries (Karimi *et al*., 2020). As for S. aureus, it destroys and lyses immune cells through toxins and virulence proteins, resulting in infectious, non-neutralizing antibodies that hinder adaptive immunity. Furthermore, *S. aureus* possesses various mechanisms for biofilm formation on medical devices, necrotic bone tissue, and bone marrow, as well as within the osteocyte lacuno-canalicular networks of living bone (Zainulabdeen and Dakl, 2021). Many people resort to using antibiotics to eliminate pathogens that cause oral diseases. Similarly, some utilize brushing, interdental sticks, and dental flossing to rid the mouth of microbes, as they are responsible for numerous ailments. However, these methods do not lead to the total eradication of microbes, as they have begun to exhibit high resistance to antibiotics, particularly beta-lactam **antibiotics**. Furthermore, toothbrushes and floss cannot reach all areas within the oral cavity**.** Consequently, mouthwash has been employed as it is capable of accessing all sites within the oral cavity (Watanabe *et al*., 2015).

The present study aimed to identify the aerobic bacterial species causing dental caries and to investigate their antibiotic susceptibility, as well as the impact of certain nanomaterials. This was achieved by isolating and identifying bacteria from dental caries cases among patients visiting private clinics in Kirkuk. Additionally, the study tested the efficacy of various antibiotics against the bacteria, demonstrated their synergistic effects, and evaluated the antibacterial activity of nanoparticles on the isolated strains, as well as the synergistic potential between the antibiotics and nanoparticles.

2- MATERIALS AND METHODS

2-1 Sample Collection

A total of 250 swabs were collected from individuals of both sexes and various ages suffering from dental caries. These samples were obtained from patients visiting private dental clinics in Kirkuk city during the period from the beginning of January 2025 to the end of April 2025. The specimens were transported to the Public Health Laboratory using transport media swabs to maintain sample viability. They were then inoculated onto blood agar and incubated at 37°C for 24 hours to identify bacterial growth and the type of hemolysis produced. Preliminary identification of the isolated bacteria was conducted based on colony morphology, size, color, and hemolytic patterns on blood agar plates. Finally, pure isolates were preserved in nutrient agar stabs (or broth) in the refrigerator for subsequent morphological and biochemical testing.

2-2 Identification of Isolated Bacteria

The isolated bacteria were identified based on their morphological, cultural, and biochemical characteristics. A portion of the bacterial culture was transferred using a sterile inoculating loop onto a clean glass slide. After heat-fixation and staining with Gram stain, the slides were examined under the oil immersion lens of a light microscope to observe cell shape, size, arrangement, and Gram reaction. Furthermore, colonies grown on blood agar were identified by their morphology, size, color, and the specific type of hemolysis (Alpha, Beta, or Gamma) (Holt *et al*., 1994).

2-3 Biochemical Tests

Catalase, Oxidase, Hemoltsis, Lactose ferment.

2-4 Antibiotic Sensitivity Test

The antibiotic susceptibility test was conducted on the isolates under study using the disk diffusion method, following the modified Kirby-Bauer method as prescribed by the Clinical and Laboratory Standards Institute (CLSI, 2020). Three types of antibiotic disks were utilized on Mueller-Hinton Agar (MHA). A bacterial suspension was prepared by transferring several pure colonies into tubes containing Nutrient Broth, which were then incubated at 37°C for 18–24 hour**s**. Subsequently, the turbidity of the tubes was adjusted to match the 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). Using a sterile cotton swab, the bacteria were spread across the Mueller-Hinton agar surface, and the plates were allowed to dry for 15 minutes. The antibiotic disks were then placed on the agar surface using sterile forceps. After incubating the plates at 37°C for 24 hours, the results were evaluated by measuring the diameter of the inhibition zone and comparing it with standard reference tables to determine whether the bacteria were Resistant (R), Sensitive (S), or Intermediately Sensitive (I) to the antibiotics

2-5Antibiotic Synergistic Test

Antibiotic powders were dissolved to a concentration of three specific antibiotic combinations were prepared: (Amoxicillin + Clindamycin), (Amoxicillin + Clindamycin + Metronidazole), and (Clindamycin + Metronidazole). The efficacy of these combinations was evaluated using the agar well diffusion method on Mueller-Hinton agar. After pouring the agar into Petri dishes and creating a well in the center of the plate, the target bacteria were inoculated by spreading of the bacterial suspension across the agar surface. Subsequently, of the antibiotic combination was introduced into the well. The plates were incubated at for 24 hours, after which the diameters of the inhibition zones were measured.

2-6 Preparation of Nanoparticle Dilutions and Their Effects on Resistant Bacterial Isolates

Commercially prepared nanomaterials were obtained for this study. A bacterial suspension was prepared by inoculating bacterial colonies into Brain Heart Infusion (BHI) broth, adjusted to match the McFarland standard. Different concentrations (100, 50, and 25) were prepared as follows:

- **First Concentration (100%):** Prepared by dissolving 10,000 of the nanomaterial in distilled water, completing the volume to 1 mL.
- **Second Concentration (50%):** Prepared by mixing 500 (or 0.5 mL) from the first concentration and completing the volume to 1 mL with distilled water.
- **Third Concentration (25%):** Prepared by mixing 250 (or 0.25 mL) from the first concentration and completing the volume to 1 mL with distilled water."

2-7 Measurement of the Inhibitory Effect of Nanoparticles

The efficacy of the nanoparticles was evaluated using the agar well diffusion method on Mueller-Hinton agar. After pouring the medium into Petri dishes, a well was created in the center of each plate. The target bacteria were then inoculated by spreading of the bacterial suspension across the agar surface. Subsequently, of the diluted nanoparticle solution was added to the central well. The plates were incubated at for 24 hours, after which the diameters of the inhibition zones were measured.

3- RESULTS AND DISCUSSION

3-1 Results

The results of the bacterial isolation from 250 samples collected from individuals with dental caries revealed that 230 samples (92%) showed positive bacterial growth, while 20 samples (8%) showed no growth. The isolates consisted of various Gram-positive bacterial species. Streptococcus mutans was the most prevalent, with 120 isolates (52.17%), followed by Staphylococcus aureus with 70 isolates (30.43%). Streptococcus pyogenes accounted for 40 isolates (17.40%), as detailed in the table below

Table 1: Frequency and Percentages of Bacterial Species Isolated from Dental Caries

Bacterial Isolates	Number of Isolates	Percentages (%)
Strep.mutans	120	52.17
Staph.aureus	70	30.43
Strep.pyogenes	40	17.40
Total	230	100

3-2 Microscopical Examination

The bacterial isolates were identified based on their response to Gram staining, as well as the morphological characteristics of the colonies on culture media and their biochemical profiles. Upon Gram staining, Staphylococcus species appeared as purple, spherical cells arranged in irregular grape-like clusters. On blood agar, these bacteria exhibited beta-hemolysis (complete hemolysis), forming shiny, white, smooth, and oily colonies with a diameter of 2–3 mm, as shown in Figure (1). Additionally, Streptococci appeared as pairs, short chains, or coccobacillary forms. When cultured on blood agar, the colonies showed alpha-hemolysis (partial hemolysis), appearing as shiny, spherical, small, and convex colonies with a dark green hue. Furthermore, some bacterial colonies on blood agar appeared pink or red, indicating lactose fermentation, while others, specifically *Staph. aureus*, appeared as yellow colonies that were non-lactose fermenting.

Figure 1: *Staph. aureus* isolates incubated for 24 hours at 37°C on Blood Agar medium

Table 2: Biochemical tests used for the identification of Gram-positive bacteria

Bacterial Isolate	Lactose ferment	Hemoltsis	Oxidase	Catalase
Strep.mutans	+	Alpha hemolysis	-	-
Staph.aureus	-	Beta hemolysis	-	+
Strep.pyogenes	+	Beta hemolysis	-	-

(+) Positive result; (-) Negative result

3-3 Antibiotic Susceptibility of Bacterial Isolates

The results of the study demonstrated varying responses of the bacterial isolates to the antibiotics used, which included Amoxicillin, Clindamycin, and Metronidazole, as shown in Table (3) and Figure (2). The *Strep. mutans* isolates exhibited sensitivity to all tested antibiotics, with inhibition zones of 28 mm, 22 mm, and 25 mm for Amoxicillin, Clindamycin, and Metronidazole, respectively. These findings are in complete agreement with the results reported by (Salh *et al*., 2022).

In contrast, the *Staph. aureus* isolates showed resistance to all types of antibiotics, a result that aligns with the findings of (Gousia *et al*., 2011). Regarding the *Strep pyogenes* isolates, the test results indicated resistance to Metronidazole, while displaying sensitivity to Amoxicillin with an inhibition zone of 23 mm and to Clindamycin with an inhibition zone of 15 mm.

Figure 2: The inhibitory activity of antibiotics against *Strep. mutans*, incubated for 24 hours at 37°C.

Table 3: The inhibitory activity of antibiotics against the bacterial isolates

Bacterial Isolates	Number of Isolates	Amoxicillin	Clindomycin	Metronidazole
Strep.mutans	120	28mm	22mm	25mm
Staph.aureus	70	0mm	0mm	0mm
Strep.pyogenes	40	23mm	15mm	0mm

3-4 Synergistic Effect of Antibiotics against Gram-Positive Bacterial Isolates

The susceptibility of the isolates in our study was tested against the synergistic effects of three types of antibiotics: (A) Amoxicillin, (B) Clindamycin, and (C) Metronidazole. The results demonstrated a variation in the isolates' response to the synergistic effect, with differences in sensitivity and resistance from one isolate to another. This variation may be attributed to the individuals' prior antibiotic use, geographical location, and the site of isolation. Susceptibility was tested using the Kirby-Bauer disk diffusion method by measuring the inhibition zones around the antibiotic disks. The bacteria exhibited diverse responses to the antibiotics, as shown in Table (4) and Figure (3). *Staph aureus* showed total resistance to all synergistic combinations. In contrast, *Strep. pyogenes* and *Strep. mutans*, which were initially resistant, became sensitive following the combinations of (A+B), (A+C), and (B+C).

Table 4: The synergistic effect of antibiotics on the studied bacterial isolates

	Amoxicillin(A) + Clindamycin(B)	Amoxicillin(A) + Clindamycin(B) + Metronidazole ©	Clindamycin(B) + Metronidazole ©
Staph.aureus	0	0	0
Strep.pyogenes	29mm	32mm	25mm
Strep.mutans	25mm	28mm	30mm

Figure 3: Below illustrates the inhibitory activity of antibiotic synergy against *Strep. pyogenes* isolates, incubated for 24 hours at 37°C.

3-5 Antibacterial Activity of Nanomaterials against Bacterial Isolates

The susceptibility of the bacterial isolates under study was tested against various types of nanomaterials (Zinc, Silver, and Titanium) at different concentrations. The susceptibility was evaluated using the Kirby-Bauer method by measuring the inhibition zones for the used bacterial isolates, as detailed in Table (5) below.

Table 5: The inhibitory effect of Zinc and Titanium nanoparticles on the studied bacterial isolates

Bacteria	Zinc			Titanium		
	25%	50%	100%	25%	50%	100%
Staph.aureus	0mm	15mm	20mm	9mm	15mm	16mm
Strep.pyogenes	0mm	10mm	15mm	11mm	12mm	13mm
Strep.mutans	0mm	12mm	17mm	12mm	14mm	15mm

The results indicated that the 25% concentration of Zinc exhibited no inhibitory effect on the tested bacterial species. However, at concentrations of 50% and 100%, the inhibition zones for *Staph aureus* were 15 mm and 20 mm, respectively. The presence of these inhibition zones serves as an indicator that Zinc nanoparticles possess effective antibacterial properties.

Regarding the Streptococcus isolates, there was a broad variation in response to the Zinc nanoparticles across different species. For *S. pyogenes*, the inhibition zones were 10 mm and 15 mm at concentrations of 50% and 100%, respectively. Similarly, for *S. mutans*, the inhibition zones reached 12 mm and 17 mm at the same concentrations. The 25% concentration also failed to show any effect on the Streptococcus isolates. According to the table above, the effect of Titanium nanoparticles on the isolates under study showed that the 25% concentration produced inhibition zones of 9 mm, 11 mm, and 12 mm for *Staph. aureus*, *S pyogenes*, and *S. mutans*, respectively. At the 50% concentration, the inhibition zones for the same isolates were 15 mm, 12 mm, and 14 mm. Furthermore, the 100% concentration yielded comparable results, with inhibition zones of 16 mm, 13 mm, and 15 mm, respectively.

Notably, the bacteria showed no response to Silver, which contradicts the findings of (Yaseen *et al*., 2019), whose study reported a weak response from both Gram-positive and Gram-negative bacteria toward Silver nanoparticles.

3-6 Synergistic Effect of Nanomaterials

The susceptibility of the isolates was tested against three types of nanomaterials (Zinc, Silver, and Titanium) at concentrations of 50% and 100%. The results showed a variation in the response of the isolates to the synergistic effect of the nanomaterials, with differences in susceptibility and resistance from one isolate to another. The susceptibility of the isolates to nanomaterial synergy was tested using the Kirby-Bauer disk diffusion method by measuring the zone of inhibition. The bacteria exhibited diverse responses toward the nanomaterials, as detailed in Table (6)

Table 6: Synergistic effect of nanomaterials on a group of the studied isolates

Bacteria	(ZnO +Ag) NPs	ZnO+ TiO_2	(ZnO +Ag+ TiO_2) NPs
Staph.aureus	8mm	11mm	9mm
Strep.pyogenes	0mm	0mm	0mm
Strep.mutans	16mm	12mm	17mm

The results of the study showed a varied response among the bacterial isolates. For Staph aureus isolates, a weak response was observed toward the synergy of nanomaterials; the inhibition zones for ZnO+Ag+TiO2 NPs, ZnO+TiO2, and ZnO+Ag NPs were 9mm, 11mm, and 8mm, respectively, whereas the use of zinc nanoparticles individually was more effective. Regarding Streptococci isolates, the response to nanomaterial synergy varied by species; Strep pyogenes showed no synergistic response at all. Inco ntrast, Strep. mutans exhibited synergistic responses with inhibition zones of 16mm for ZnO+Ag NPs, 12mm for ZnO+TiO2, and 17mm for ZnO+Ag+TiO2 NPs.

4- DISCUSSION

The results in Table (1) indicated that the genus Streptococci was the most frequently isolated genus causing dental caries, specifically Strep mutans**.** This finding contradicts the study by (Jalal *et al*., 2017), which found that Staphylococcus was the most prevalent genus causing dental caries.

The catalase test results for all Gram-positive *Staph. aureus* isolates were positive, which is consistent with the findings of (Wifaq *et al*., 2017) stating that *Staph aureus* is catalase-positive. This positivity is indicated by the formation of air bubbles resulting from the conversion of into oxygen and water by the catalase enzyme. This test is essential for distinguishing them from *Streptococcus spp.*, which are negative for this test. Conversely, when the oxidase test was performed, all Gram-positive bacterial isolates yielded negative results. As for the Coagulase test**,** all Gram-positive *Staph. aureus* isolates showed a positive response. This is because these bacteria contain the coagulase enzyme, which has the ability to convert fibrinogen to fibrin**,** as noted by (Kateete *et al*. 2010). These results are illustrated in Table (2).

Regarding antibiotic results, (Deyno *et al*., 2017) tested *Strep mutans* against various types of antibiotics and observed varied results depending on the antibiotic type; it was noted that bacterial resistance to Amoxicillin reached 77%, while resistance to Gentamicin was 26%. This resistance is attributed to genetic mutations or alterations, such as the deletion or substitution of a specific gene, or gene transfer within the same or different species (Bitrus *et al.,* 2018). Furthermore, (Mustafa *et al*., 2008) stated that Metronidazole lacks the ability to inhibit aerobic bacteria.

As for the Strep. pyogenes isolates, the test results showed that the bacteria were resistant to Metronidazole, while displaying sensitivity to Amoxicillin and Clindamycin. The reason is that Clindamycin inhibits protein production at the 50S ribosomal subunit level and reduces toxin production (Olajuyigbe *et al*., 2014). The purpose of testing the synergistic effect of antibiotics, as shown in Table (4), is to achieve fast and effective treatment, increase their efficiency, and overcome the problem of antibiotic resistance (Olutumbi *et al*., 2019).

(Karvani and Chehrazi 2011) demonstrated that zinc nanoparticles (ZnO NPs) were inhibitory against *Staph aureus*. They also observed that inhibition increases as the nanoparticle size decreases, due to the increased surface area. Based on these findings, it was noted that zinc nanoparticles serve as a potent antibacterial agent against Gram-positive bacteria, as these particles produce Reactive Oxygen Species (ROS) and hydrogen peroxide on the cell surface. This leads to the destruction of cellular components such as proteins, lipids, and DNA, ultimately resulting in cell death. The most critical factors affecting the efficacy of nanoparticles are size, shape, surface area, purity, and concentration (Khan *et al*., 2019). Furthermore, the impact of zinc nanoparticles on *Strep. pyogenes* is attributed to their binding with polypeptides and glycogen present in the bacterial cell wall, leading to the destruction of the wall and subsequent cell death (Liang *et al*., 2020; Humada *et al.,* 2024).

(Albukhaty *et al.,* 2020) synthesized titanium nanoparticles at concentrations of 25%, 50%, and 100% under various temperatures, achieving inhibition of both Gram-positive and Gram-negative bacteria. These synthesized nanoparticles were highly efficient due to their unique chemical and physical properties. (Asamoah *et al*., 2020) tested the synergistic effect of zinc and silver nanoparticles against *Staph aureus*, stating that silver was the primary cause of inhibition. This contradicts our findings, as silver showed negligible effects on the isolates used in this study. Conversely, (Bednař *et al*., 2019) achieved inhibition of *Staph aureus* using an antibacterial agent synthesized from nanoparticle synergy, which was five times more effective than silver nanoparticles alone.

5- CONCLUSIONS

The isolation rate of the genus Streptococcus was higher compared to Staphylococcus, with Strep. mutans recording the highest isolation rate of 120 isolates from the oral environment of individuals with dental caries. The bacterial isolates varied in their antibiotic susceptibility, with Amoxicillin being the most effective against the studied bacterial species. Furthermore, the current study demonstrated that zinc and titanium nanomaterials had a significant antibacterial effect, whereas silver showed no effect on the bacterial species. It was also found that the synergistic effect of the nanomaterials was superior to the effect of each nanomaterial used individually.

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