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ORIGINAL ARTICLE

The Effect of rsbA Gene Expression by TiO2 Nanoparticles in Clinical Isolates of *Proteus mirabilis*

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ABSTRACT

Background: *Urinary tract infections (UTIs) are primarily caused by Proteus mirabilis, especially in patients who have catheterization or structural urinary abnormalities. The rsbA gene controls the organism's robust swarming motility, which enhances virulence and infection persistence. Alternative antimicrobial methods are required because clinical P. mirabilis isolates have become more resistant due to antibiotic misuse.* ***Methodology:*** *The antibacterial and anti-swarming properties of titanium dioxide nanoparticles (TiO_2 NPs) were examined in this work, along with their impact on the expression of the rsbA gene in clinical isolates sourced from hospitals in Baghdad.* ***Results:*** *Using the VITEK 2 system, biochemical testing, and cultural traits, 35 isolates from 57 urine samples were determined to be P. mirabilis. Testing for antibiotic susceptibility showed high rates of resistance, especially to ceftriaxone (60%) in particular.15 mg/ml was found to be the TiO_2 NPs' minimum inhibitory concentration (MIC). Swarming motility was considerably decreased in all tested isolates at sub-MIC concentrations. Following treatment with TiO_2 NPs, quantitative real-time PCR analysis showed a down-regulation of rsbA expression, suggesting interference with the regulation of motility and virulence.* ***Conclusion:*** *These results imply that TiO_2 nanoparticles, which target swarming behavior and virulence gene expression, may be a promising supplementary antimicrobial treatment to control multidrug-resistant P. mirabilis.*

INTRODUCTION

Members of the *Enterobacteriaceae* family, *Proteus* species are rod-shaped, Gram-negative bacteria that are frequently found in a wide range of settings, such as soil, water, and sewage, but particularly as commensals of the gut microbiota of humans and animals[1]. The ability of microorganisms of the genus Proteus to swarm on solid surfaces was originally described by German microbiologist Gustav Hauser in 1885. The character *Proteus* from Homer's "Odyssey," who possessed the capacity for infinite alteration and shape modification, inspired the name *Proteus*. *Proteus vulgaris* and *Proteus mirabilis* are the two species of the genus that Hauser described[2].

Numerous aerobic and anaerobic species make up the human digestive tract. There are a few notable outliers, however commensals make up the majority of the organisms. They play a part as pathogens outside the intestine, which are the most common causes of nosocomial infections, wound infections, urinary tract infections, and other diseases. American Centers for Nature and Opportunistic Human Pathogens claim that... Along with *E. coli* and *Klebsiella* species, of which *E. coli* is the most common resident, it can be detected in the intestine. Additionally, it was discovered that *P. vulgaris* infections are also common and that *E. coli* accounts for about 90% of UTIs[3]. Additionally, it was shown that *Proteus*, after *E. coli*, ranks third in the United States for causing prostatitis, pyelonephritis, and uncomplicated cystitis,

However, it has also been reported that, following *E. coli*, *Proteus* species are also highly prevalent in urinary tract infections. Urinary tract infections (UTI) are thought to be frequently caused by *Proteus mirabilis* and *Proteus vulgaris*, which are typically linked to structural anomalies or urinary tract catheters[4].

It is more frequently linked to UTIs, which are further classified into ascending infections, in which bacteria gradually colonize the introitus, urethra, bladder, ureter, and ultimately the kidneys, and haematogenous infections, also referred to as systemic infections. *Proteus* strains and infections involving urinary catheters or structural abnormalities are more likely to cause the second form of UTI[5]. In addition to urinary tract infections, *P. mirabilis* has been identified as an opportunistic causative agent for gastroenteritis brought on by eating tainted meat or other food, as well as infections of the respiratory system and wounds, burns, skin, eyes, ears, nose, and throat[6].

Increased drug resistance and the widespread distribution of different resistance genes among clinical *P. mirabilis* isolates are the results of antibiotic misuse or non-specific use[7]. Microorganisms like bacteria and

fungi produce chemicals called antibiotics[8]. Antibiotics are compounds or antimicrobial agents, either synthetic or natural, that can kill or stop the growth of microorganisms by targeting specific bacterial targets. However, they must not harm the eukaryotic host cells that are harboring the infection[9]. For an antimicrobial agent to exert its antimicrobial action, it must be able to penetrate the bacterial cell and reach the target (access)[10] According to Kapoor.

Mutagenesis and library screens revealed a number of swarming motility regulating genes, such as *rsbA*, *ccmA*, *umoB*, *umoA*, *umoC*, *umoD*, *wosA*, *disA*, and the *Lon* protease[11-13].

The *rsbA is* the gene that regulates swarming. Its unique characteristics and the significant role of nanoparticles have made it the most inventive, cutting-edge, and well-known field of study in modern science. Nanoparticles are widely used in both materials research and medicine. Their surprising ascent to fame in recent years can be attributed to their creative solutions in a variety of scientific fields[14]. Their high surface area-to-volume ratio and distinctive physicochemical characteristics (such as color, dispersion, and thermodynamics) set them apart from bulk materials in comparison to their macro-scale counterparts[15].

Swarming inhibitors include simple fatty acids, structurally complex secondary plant metabolites, enzymes that block bacterial signals, phages that block flagellar motility, sub-inhibitory concentrations of nanoparticles, sub-inhibitory concentrations of antibiotics, and amphiphilic compounds[16]. Several studies have been reported to control the swarming behavior of various bacterial species.

Nanoparticles (NPs) are a broad category of materials with a single dimension and a size of less than 100 nm[17]. NPs also contain particulate matter. The importance of these materials was acknowledged when the researchers discovered that the size of NPs affected a substance's physiochemical characteristics. Nanoparticles come in a variety of forms and configurations and can be either organic or inorganic. They can be spherical, tubular, or irregularly shaped, among other shapes and forms. Additionally, they can be separated according to whether they are agglomerated or fused aggregates[18].

Research on nanoparticles and their potential applications as antimicrobials has increased as a result of their distinct physiochemical characteristics and antimicrobial activity against microorganisms[19]. Titanium dioxide (TiO2) nanomaterials are widely recognized for their applications in a wide range of fields, including bio-sensing, drug delivery, water purification, photo-catalytic degradation of pollutants, and innovative devices, sunscreens, and photovoltaic cells[20,21].

TiO2 is one of the most often employed metal nanoparticles in the fields of microbiology and medicine. This may be due to the fact that TiO2 is regarded as a nontoxic and physiologically inert metal at the microscale, and it exhibits photocatalytic characteristics when exposed to ultraviolet light[22].

METHODOLOGY

Isolation and Identification of *Proteus spp*.

57 urine samples were collected from patients with UTIs who were receiving treatment at various hospitals in Baghdad. The samples were then streaked on blood agar and MacConkey agar and incubated aerobically for 24 hours at 37ºC, over the night. In accordance with Bergy's Manual of Systematics of Archaea and Bacteria[23], morphological traits and biochemical tests were used to identify the isolates.

According to Hamed et al[24], a number of common biochemical assays were carried out to identify bacterial isolates, e.g. indole test, urease production test, motility test and detection of swarming motility.

Preparation of different concentrations of nanoparticles

In individual tubes with 9 ml of D.W., 3g of titanium dioxide nanoparticles were dissolved; to speed up the dissolution process, 50 µl of DMSO was added to each tube[25]. The volume was then increased to 10 ml using D.W. to achieve stock solutions with a concentration of 300 mg/ml. The stock solutions were then used to create various concentrations (5, 10, 15, 20, 25, and 30 mg/ml).

Confirmation of identification of bacteria by VITEK 2 compact system:

The set of biochemical tests carried out in tandem with VITEK cards is the foundation of the VITEK 2 identification system. The biochemical tests and metabolic processes used to identify the testing bacteria are represented by the 46 wells on each card. After loading the bacterial suspension onto the VITEK cards, the VITEK software computed and recorded the results.

Antibiotic susceptibility test

Using the disk diffusion technique, the antibiotic susceptibility of *P. mirabilis* isolates that were chosen was evaluated against ceftriaxone, meropenem, amikacin, gentamicin, nitrofuraniton, ciprofloxacin and cephalothin (CLSI)[26].

Determination of TiO2 nanoparticles minimal inhibitory concentration (MIC) using agar dilution method.[27]

The manufacturing company Hongwu in China used the nanoparticles, which had a diameter of 40 nm, as an antibacterial agent. The experiment was conducted in compliance with Emami-Karvani et al.

Determining the anti-swarming effect for sub-MIC of TiO2 nanoparticles on *Proteus spp.* swarming motility.

Blood agar plates were used to test each *Proteus* isolate's (35) capacity for swarming movement and the

diameters of the swarming were measured in millimeters.

Proteus spp. isolates' capacity to swarm was reexamined with sub-inhibitory TiO2 concentrations present.

Gene expression

RNA extraction and purification

Following the manufacturer's instructions and utilizing the reagents supplied by GeneAid-South Korea, total RNA was extracted from each sample. A Nano-drop 1000 spectrophotometer was used to evaluate each sample's RNA concentration and purity.

cDNA synthesis, RT-PCR, and quantitative RT-PCR

A high-capacity cDNA reverse transcription kit was used to reverse transcribe 1 µl of total RNA into cDNA for all samples except aliquots intended for RNA-Seq. The following were the thermocycling conditions:

In a PCR tube, RT-PCRs were conducted using the 2 Power SYBR green master mix. 1µl (5 U) of Taq polymerase (NEB) was well mixed with 2µl (200 µM) of deoxynucleoside triphosphates (dNTPs), 1µl (200 nM) of each primer, 1µl of ThermoPol buffer, and 2 µl (25 ng) of cDNA as template in 50µl of reaction solution for RT-PCRs.

The following protocol was used to conduct reactions on the qPCR: 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. To guarantee product specificity, a dissociation curve analysis was carried out for each assay (95°C for 15 s, 60°C for 1 min, 95°C for 15 s). Table 1 contains a list of all primer sequences. Using the relative method formula, the amount of gene expression was determined, using *rpoA* as the housekeeping gene.

Table 1: Primers sequences

Primer	Sequences ´5 to ´3
*rpoA*F	GCGTGTTATAGCCCAGTTGA
*rpoA*R	AGGCTGACGAACATCACGTA
*rsbA*F	CTATACCTACCGCACCATGT
*rsbA*R	GAAGTCCCATCCGTTGATAC

RESULTS

Isolation and identification of *Proteus spp.*

Urine samples from fifty-seven patients with UTIs who were treated at various hospitals in Baghdad were collected. The time frame for sample collection was March 2025–June 2025. In order to observe the colony morphology (size, shape, surface texture, color, edge and elevation, etc.), all of the specimens were immediately cultivated on MacConkey agar and blood agar using the streaking plate method. Of the 57 isolates, 35 (61.4%) were mainly identified as *Proteus spp*[28].

Antibiotic susceptibility test

It was determined that the chosen *Proteus* isolates were susceptible to the following antibiotics: amikacin, ceftriaxone, cephalothin, ciprofloxacin, gentamicin, nitrofurantoin, and meropenem as shown in Fig 1.

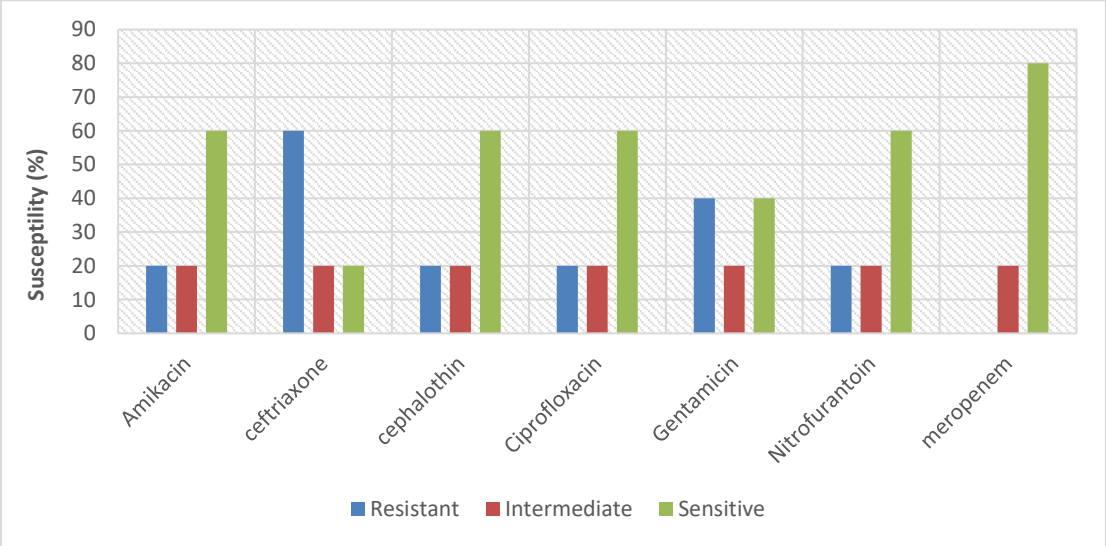

Fig. 1: Antibiotic susceptibility of *Proteus mirabilis* isolates

Proteus mirabilis isolates exhibit a similar susceptibility pattern to amikacin, cephalothin, ciprofloxacin, and nitrofurantoin, with 20, 20, and 60% exhibiting resistance, intermediate susceptibility, and sensitivity, respectively, according to the results summarized in figure 1. Ceftriaxone also had the highest percentage of resistance (60%). Additionally, 40% of the isolates of *P. mirabilis* exhibited gentamicin resistance. However, 20% of *P. mirabilis* isolates developed intermediate resistance to meropenem, making it the most effective medication. In contrast, none of the isolates were resistant to meropenem.

Proteus species exhibit a broad spectrum of resistance to several antibiotics, according to the results of the antibiotic susceptibility testing. The additional outer cytoplasmic membrane, which comprises a lipid bilayer, lipoproteins, polysaccharides, and lipopolysaccharides, may be the cause of this. Of course, antibiotic abuse and overuse may also be a contributing factor to antibiotic resistance[29].

Swarming motility assay

Their swarming behavior varied greatly, according to the results. Fig 2 demonstrates that the swarming diameters of all P. mirabilis isolates used varied greatly, ranging from 8 to 44 mm across all plates.

Determination of minimal inhibitory concentration (MIC) for TiO_2 nanoparticles

The agar dilution method was used to estimate the MIC values after testing the bacterial isolates' (*P. mirabilis*) susceptibility to TiO2 nanoparticles. For this objective, Muller Hinton agar was combined with TiO2 nanoparticles in varying increasing quantities (5, 10, 15, 20, 25, and 30 mg/ml) and then poured into Petri dishes. According to the results of this investigation, *P. mirabilis* isolates were inhibited by 15 mg/ml TiO2 nanoparticles, which was determined to be the minimum inhibitory concentration (MIC) for *P. mirabilis* isolate growth. The bacteria did not grow when treated with these nanoparticle concentrations, as shown in figure 3.

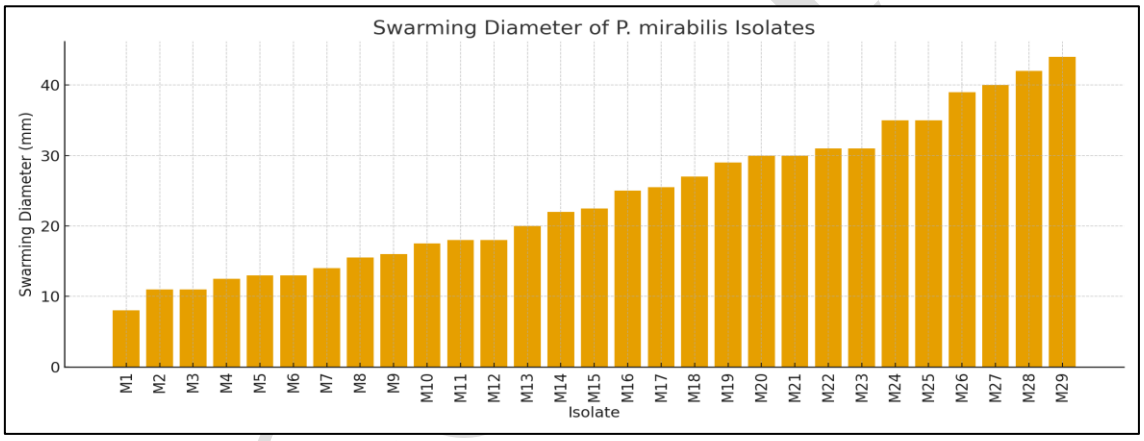

Fig. 2: Swarming diameter of *P. mirabilis* isolates

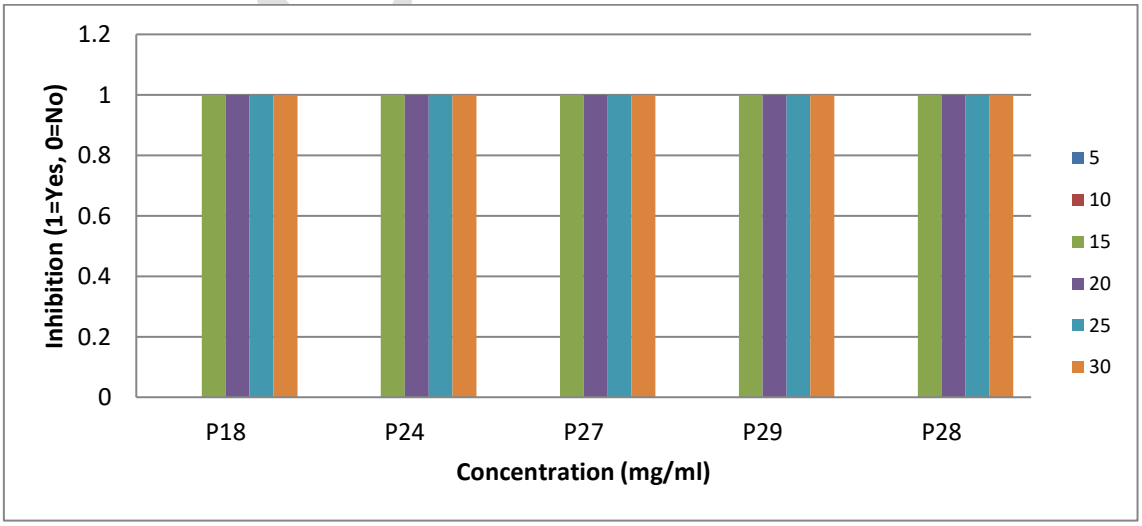

Fig. 3: MIC Values for TiO_2 Nanoparticles of *P. mirabilis* isolates

Determining the anti-swarming effect of TiO_2 nanoparticles

Five *Proteus* isolates (M 18, M 24, M 27, M 28, and M 29) were tested for their capacity to swarm when exposed to sub-inhibitory nanoparticle concentrations (10 mg/ml and 5 mg/ml for TiO2). Since three of the isolates had weak swarming ability and three had moderate ability, while the remaining four were characterized by significant swarming movement, the selection criteria for these isolates were based on the fact that their swarming behavior varies. According to the findings reported in Figure 4, all of these isolates' swarming movement was considerably reduced.

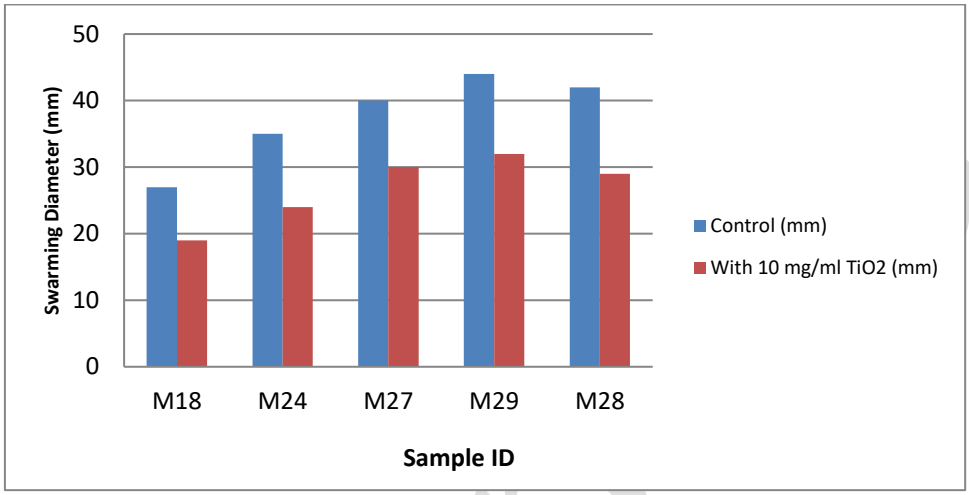

Fig. 4: The effect of sub-inhibitory concentrations of TiO2 nanoparticles on *Proteus* swarming motility

The effect of nanoparticles on *rsbA* gene expression

To determine whether or not the *rsbA*, which controls the swarming phenomenon, was impacted, the treated and untreated isolates by the sub-inhibitory concentration of TiO2 nanoparticles were genetically evaluated using real-time PCR. These findings demonstrated that the *rsbA* gene is down-regulated.

This potent effect could be linked to the antimicrobial activity, chemical makeup, and physical characteristics of nanoparticles that could disrupt various extracellular and intracellular proteins of the bacterial cells, including the flagellar protein, bacterial membrane, and cell wall, thereby preventing the bacterial activity. Additionally, oxidative stress, DNA damage, ion release, and internal bacterial damage could be the cause.

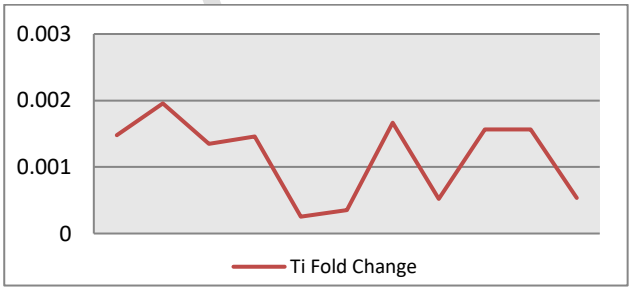

Fig. 5: Fold change value

Given that the observed fold change results clearly demonstrate the down-regulation of rsbA expression in TiO2 treatments, which is less than 1, the experimental condition's expression level is lower than the control condition's as shows in Figure 5.

DISCUSSION

The findings of the antibiotic susceptibility testing show that Proteus species have a wide range of resistance to many antibiotics. This could be due to the extra outer cytoplasmic membrane, which is made up of lipoproteins, polysaccharides, lipopolysaccharides, and a lipid bilayer. Antibiotic resistance may, of course, also result from the misuse and overuse of antibiotics[30].

Rotating flagella are what cause swarming movement across a solid surface. Since transcription factors of species-specific master regulators control the expression of the genes involved in flagellar biosynthesis, differences in swarming motility among *Proteus* isolates may result from mutations that alter master regulator activity, which can have a significant impact on swarming motility[31].

According to the current study's findings, 29 *P. mirabilis* isolates were identified. The identification of *P. mirabilis* isolates that had previously been recognized by traditional biochemical testing was confirmed using the VITEK 2 compact system. These findings were

consistent with a research by Feglo that reported 61.5% isolation of *P. mirabilis*[32].

A study by Ibrahem et al. examined biologically synthesized titanium nanoparticles and found that, after treating isolates with sub MIC (16 mg/ml) titanium nanoparticles, these TiO2 Nps significantly increased antimicrobial efficiency against multidrug-resistant bacteria that can cause recurrent urinary tract infections, including *Staphylococcus aureus*, *Klebsiella pneumonia*, *E. coli*, *Acinetobacter baumani*, and *Morganella morganii*, along with their virulence factors (biofilm formation, hemolysin, and urease)[33].

The bactericidal efficacy of Ti Nps against multiple kinds of multidrug-resistant bacteria isolated from sheep wound infections was examined in a paper published by Noomi. They stated that both Nps have an inhibitory impact on Ti nanoparticles, with inhibition zones measuring 18 and 20 mm[34].

TiO2's size, shape, and crystal structure all affect its antimicrobial properties, it is suggested that one of the most significant mechanisms for TiO2 nanoparticles (anatase forms) may be oxidative stress through the production of ROS. Then, site-specific DNA damage is caused by ROS[35].

The TiO2 nanoparticles' photocatalytic qualities enable them to effectively destroy the germs. Actually, when exposed to UV radiation, TiO2 nanoparticles generate ROS. Lipid peroxidation, which increases membrane fluidity and compromises cell integrity, was seen in conjunction with the antibacterial photo-catalytic activity[36].

The reduction in swarming movement in the presence of nanoparticles may be explained by their possible involvement in impairing the synthesis or rotation of flagella, hence reducing their activity, which is essential for Proteus species' cellular mobility. Numerous factors, such as strain variation and/or growth conditions like the bacteria's source, incubation conditions (pH, temperature, moisture, etc.), or the expression of specific related swarming genes, can contribute to the deviation in swarming exhibition for *Proteus spp*[37].

CONCLUSIONS

The current study's findings indicate that TiO2 nanoparticles exhibit potent antibacterial action against *P. mirabilis*. The swarming diameter of *P. mirabilis* is inhibited by TiO2 nanoparticles. Finally, the *rsbA* gene, which controls swarming movement, is down-regulated by the sub-inhibitory concentrations of the employed nanoparticles.

Ethical Approval Declaration

This study's procedures were in conformity with the norms of the relevant clinical research ethics committee as well as the Code of Ethics of the College of Dentistry (Mustansiriyah University). In addition, each participant gave written agreement after a brief description of the experiment.

Declaration of Conflicting Interests

The authors declare that there are no potential conflicts of interest regarding the research, authorship, or publication of this work. The study was carried out independently, with no financial or personal affiliations influencing the data collection, analysis, or interpretation.

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