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Effect of Chitosan Nanoparticles on Growth, Swarming Motility, and Biofilm Formation in *Proteus Mirabilis* Isolated from Urinary Tract Infections

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ABSTRACT

Proteus mirabilis is the third most prevalent cause of urinary tract infection (UTI), the goal of this study was to see how chitosan nanoparticles (CS-NPs) affected on growth, swarming motility and biofilm formation of *Proteus mirabilis*, after isolation from patient infected with urinary tract infection and identified with routine methods and confirmed using API system. Minimum inhibitory concentration (MIC) of chitosan nanoparticles (CS-NPs) against the clinical strain of *Proteus mirabilis* was identified. The sub-MIC (CS-NPS) was used to evaluate their inhibitory effect on swarming motility and forming of biofilm. The results showed that the chitosan nanoparticles caused a remarkable inhibition of the two important virulence factors swarming motility and biofilm formation as compared with the control. As well as swarming motility's mean diameter was reduced from 8.5 to 0.5 cm. The clinical strain of *Proteus mirabilis* developed into a moderate biofilm producer after treatment, with a mean percentage rate of biofilm inhibition of 58%.

Keywords: Chitosan nanoparticles, Swarming, motility, Biofilm formation, P. mirabilis.

INTRODUCTION

Proteus mirabilis is a gram-negative rod-shaped bacterium. Recently, it was placed in the Morganellaceae family (Wasfi *et al*., 2020). *P. mirabilis*, after *E. coli* and *Klebsiella pneumoniae*, is the third most prevalent cause of urinary tract infection (UTI) (Armbruster *et al*., 2018) in addition to UTIs, these pathogens can cause various diseases such as respiratory tract infection, skin inflammation and soft tissue infections including burns, wounds after surgery, etc, it has different virulence factors such as swarming motility, capsule polysaccharide, urease synthesis, and efflux pumps (Filipiak *et al*., 2020).

P. mirabilis is characterized by a special form of motility depending on the nature of the media, in a liquid medium. Proteus cells are called swimming cells because they move by employing peritrichous flagella (6–10 flagella per cell), which are 1.0–2.0 m long. When these bacteria are placed to a solid media, they undergo morphological modifications and become swarmer cells, which are longer 20–80 nm, multi-nucleated, not separated by barriers, and have a 50–500 times augmentation of flagella number. Swarmer cell populations may swarm across the surface of a solidified medium, generating swarming positions. This is referred to as a specific swarming phenomenon called as a bull-eye (Kwil *et al*., 2013). *P. mirabilis* also has the ability to produce exclusive biofilms, which are described as an aggregation of bacteria attached by a polysaccharide matrix. This frequently results in encrustation and catheter occlusion, making CAUTIs more problematic, and is indicated as crystalline biofilm (Vestby *et al*., 2020; Sanchez *et al*., 2021). *P.mirabilis* is a multidrug bacteria (MDRB), therefore treating it requires a long course of antibiotics, and the outcomes might not always be good. This is due to poor antibiotic penetration or inefficiency against bacterial resistance (Hsu *et al*., 2008; Maruthupandy *et al*., 2020). As a result of this resistance, other materials such as therapeutic nanoparticles, which are now employed to get rid of (MDRB).

Because of their excellent ability for tissue targeting, nanoscale materials (1–100 nm) are frequently employed at the moment (Savolainen *et al*., 2010; Wasfi *et al*., 2020). Chitosan is one of the most common biopolymers derived from chitin. It is made by removing the acetate moiety from chitin, which is found in the exoskeletons of arthropods, crustaceans such as prawns and crabs, insects, and fungal cell walls (Mohammed *et al*., 2017; Li *et al*., 2018). Chitosan is a polycationic amino-polysaccharide made up of D-glucosamine (GlcN) and N-acetyl glucosamine joined by a -1, 4-glycosidic link (Khan *et al*., 2019). However, chitosan is the second biggest polymer, it has been used in a variety of industries including agriculture, medicines, foods, and biomedical applications (Liaqat and Eltem, 2018). It is readily available, has unique traits such as being bio-degradable, bio-compatible, bio-renewable, non-toxic, non-allergenic, and bio-adhesive, does not have antigenic properties, and is ecologically beneficial (Shourir *et al*., 2021; Herdiana *et al*., 2022). It has antibacterial, anticancer, and sustainable biomaterial properties that are useful in the creation of health care products. As a result, it has the potential to be useful in biological applications as an antibacterial agent, either alone or in combination with other polymers. (Tao *et al*., 2021; Choudhary *et al*., 2020).

The current work seeks to evaluate the inhibitory effect of chitosan nanoparticles on two critical virulence aspects of *P. mirabilis*, biofilm formation, and swarming motility to uncover novel treatment options for resistant diseases.

MATERIALS AND METHODS

Ten urine samples were obtained from individuals with urinary tract infections (UTIs) at Mosul hospitals. All samples were collected under sterile circumstances and sent to the laboratory within 1-2 hours.

Isolation and identification of *Proteus mirabilis*

After inoculating samples in various culture medium such as nutrient agar, MacConkey agar, and blood agar, they were cultured at 37°C for 24 hours under aerobic conditions. The

colonies' appearance, morphology, and color were studied. The API 20E Kit (Bio Merieux, France) was then used to confirm the diagnosis.

Detection of the MIC of Chitosan nanoparticles.

Micro dilution method was used to find out chitosan's MIC as described by (Fattah *et al*., 2021) as follows:

1. A serial dilution of chitosan in Brain heat infusion broth was made, and then (0.1 ml) of each concentration was added to each well.

2. Bacterial suspension was prepared by adding 2-3 colonies of *P.mirabilis* to (5ml) of normal saline to obtain suspensions with final concentration equal to 0.5 macFarland tube.

Bacterial suspension was diluted at 1:20 to yield 5610 $_{CFUml}$, then 0.01 ml of bacterial suspension was applied to each well.

3. Adding broth only in some well was regarded as a negative control, by adding CS-NPs with several concentrations in other wells, while broth with bacterial suspension was considered as a positive control.

4. The microliter plate was incubated at 37°C for 24 hours.

5. After incubation 0.03 ml of resazurin stain was added which is a redox indicator, determining the MIC depending the color changing of redox indicator from blue to purple indicated the presence of viable cell. Then microliter plate was incubated overnight to detect the color change (Fattah *et al*., 2021).

Resazurin was performed at 0.015% concentration by dissolving 0.015 gm of resazurin then vortex and refiltered by (0.22 um filter paper), and stored at (4°C) as maximum for two weeks after preparation (Elshikh *et al*., 2016).

Motility assay

P.mirabilis cultures were diluted to a concentration of 0.5 macFarland tube. Swarming motility was determined at 25°C using swarming media (15g agar, 5g Nacl, 5g tryptose, 2.5g soy peptone, and 5g yeast extract/L). Plates were inoculated with 5 µl of bacterial suspension and incubated for 24 hrs. at 30°C. The swarming motility was measured in millimeters (mm). The test was trialed 3 times to extract the mean of diameter (Shah *et al*., 2019).

Inhibitory activity of Chitosan nanoparticles on swarming motility.

The inhibitory test of chitosan nanoparticles on swarming motility was described by (Fattah *et al*., 2021) with the addition of Sub –MICs.

Microtiter plate assay for quantifying biofilm development.

1. A loopful of *P.mirabilis* overnight culture was injected in 5ml of brain heart infusion broth (BHIB) with 1% of glucose addition. Then, the culture was incubated at 37°C.

2. We prepared the suspension of bacteria equal to 0.5 Macfarland tube and was diluted to 1:100 with (BHIB).

3. (0.2ml) of bacterial suspension was added to the well of polystyrene flat bottom plate.

4. Adding broth only was considered as a negative control to ensure the sterilization.

5. The microtiter plate was incubated at 37°C overnight. The contents wells were removed by gently clicking. Then, the plate was rinsed 3 times with 300 µL of regular saline.

6. Adherent bacteria were fixed by exposing them to hot air at 60 °C for 1 hour.

7. Wait 15 minutes after adding 150 µl of crystal violet stain. The surplus color was then removed, and the plate well was thoroughly cleaned.

8. Adding 150 µl of ethanol 95% to all wells.

9. After 30 minutes, the optical densities (OD) were measured with a microtiter plate reader at 630 nm.

The experiment was carried out 3 times. The OD values for the test sample and negative controls were computed, and the cut-off value was determined. To explain the results, the isolate's effectiveness in biofilm development was evaluated by comparing the measurements using the following equation described by (Badawy *et al*., 2020).

Chitosan nanoparticles inhibit biofilm development.

The inhibition impact of chitosan on biofilm development was investigated previously approach by the addition of sub-MICs (CS-NPs). The percentage rate of inhibiting biofilm creation was estimated of the equation:

Inhibition rate $=1-\frac{\text{OD treatment}}{\text{OD control}}\times 100$. (Divya *et al*., 2017)

RESULTS AND DISCUSSION

Isolation, Identification and Selection of Bacterial Strain to Study:

The *P. mirabilis* was isolated from urine sample of patient suffering from (UTI) and identified by using conventional method, it appeared pale on MacConkey agar because it is non lactose fermenter, and formed swarming phenomenon on blood agar. In microscopic identification by using gram stain, it showed red, coccobacillus cells (AL-bassam, and AL-kazaz, 2013). The identification was confirmed by API E20 kit.

Selection of one strain to complete the study depending on its swarming ability, so we select the strongest swarming strain with the diameter of swarming (8.5 mm).

Chitosan Nanoparticles' Efficacy Against *P. mirabilis*

The antibacterial activity of CS-NPs was examined against *P.mirabilis*, chitosan was tested in six different concentrations in double serial dilution (40 mg\ml to 1.25 mg\ml) to test its antibacterial activity. The result showed that the minimum concentration that inhibit the growth (MIC) is (10 mg\ml) that no change in color of resazurin occurred after incubation Fig. (1). There are several explanations for the impact of CS-NPs on bacterial activity, first of them is due to the binding between the +ve amino groups of CS-NPs and the -ve of the bacterial cell wall causing disorder of the cell. Changes in membrane permeability, adhesion to DNA, suppression of DNA replication, and failure to produce mRNA culminate in cell death. The second interpretation is that the CS-NPs act as a chelating agent that elective combines to the trace metal elements and as a result toxin production so, cell death. (Divya *et al*., 2017; Atay, 2019). Chitosan may also permeate through the bacterial membrane, disrupting DNA and RNA production (Kadhum and Zaidan, 2020). When compared to other carrier molecules, chitosan serves as a good reservoir for medication delivery and antibiotics. It has a high adhesive property due to its non-toxic nature, as well as other biological features such as anti-oxidant (Bilal *et al*., 2020; Keykhosravy *et al*., 2020; Govindan *et al*., 2022). Chitosan is safe to use and was classified by the FAD in 2001 as generally recognized as safe (GRAS) (Tao *et al*., 2021; Herdiana *et al*., 2022).

Kadhum and Zaidan, 2020 showed chitosan alginate nanoparticles caused 100% inhibition rate to *P.mirabilis* at 0.15 and 0.3 mg/ml, while the study by Hussein and Aldujaili, 2020 showed that 80mg\ ml of CS-NPs exhibited a large inhibition rate to *P.mirabilis* growth and lower inhibition rate in 20 and10 mg\ml. In other study (Fattah *et al*., 2021) their result showed effectiveness of CS- NPs against the growth of clinical strain of *Ps.aeruginosa* with MIC value (10 or 5 mg\ml) depending the type of strain isolates used in study.

Effect of CS-NPs on swarming motility

P.mirabilis is motile by swarming motility in the media containing 1.5% agar. (Little *et al*., 2018) the swarming motility is the most important virulence factors, it is playing a key role in the first stages of biofilm formation (Kilmury and Burrows, 2018). Our results showed that the active inhibition of Chitosan nanoparticles on the swarming motility of clinical isolate *P.mirabilis* which was assessed at the sub-mic cons.(5 mg\ml). The mean diameter of swarming motility was decreased from 8.5 to 0.5 cm (Table 1) so, the rate of swarming motility inhibition is 94 %. Fig. (2).

The presence of chitosan–polypyrrole nanocomposites reduced the mean diameter of the

swarming motility position of *P. aeruginosa* from 3.05(0.07) cm to 0.95(0.07) cm, according to (Khan *et al*., 2019). In addition, (Fattah *et al*., 2021) revealed that the mean diameter of swarming motility in non-treated cultures was 3.5 (1.6) cm, whereas in the presence of Chitosan nanoparticles, the mean diameter of swarming motility was decreased to 1.9(1.07) cm. In addition to the research of (Bernal- Mercado *et al*., 2022) who noticed that the rate of swarming motility decreased approximately 40-60% in the presence of nanoparticles compared to the control.

Biofilm Formation

In the current study, the *P.mirabilis* was moderate biofilm producers (+2) that it gave 2×ODc≤ OD ≤ 4× ODc value (Table 1). The method used for estimation of adherent bacterial numbers is the crystal violet method (Maruthupandy *et al*., 2020). *P. mirabilis* therapy has become extremely challenging; also, P. mirabilis biofilm production has raised the complexity of bacterial resistance, lengthened healing time, and worsened infections. As a result, multidrug-resistant (MDR) and extensively drug-resistant (EDR) bacteria were existed. *P. mirabilis* isolates have grown during the last two decades. Biofilms also shield pathogens from the human immune system (Jacobsen and Shirtliff, 2011). The MDR of *P. aeruginosa* and *K. pneumoniae* were investigated using crystal violet staining plates, which produced 0.454 values for *P. aeruginosa* and 0.642 for *K. pneumonia* on OD540 after 24 hours of incubation, indicating the production of a robust biofilm (Maruthupandy *et al*., 2020).

The Effect of CS-NPs on Biofilm Development

Our results showed that the addition of sub-mic con (5 mg\ml) of chitosan nanoparticles reduced the *Proteus mirabilis* biofilm formation in a microplate well with inhibition rate 58% (Table 1). Biofilm is composed mainly of extracellular polymeric substances (EPS).

Because of their tiny particle size, nanoparticles may easily enter the EPS matrix (Limoli *et al*., 2014). The current investigation found that the CS-NPs decreased swarming motility and thereby prevented biofilm formation. However, some studies have found that *P.mirabilis* that have the ability to form swarming motility must be biofilm former (Jones *et al.*, 2005).

Chitosan prevented biofilm formation in *E.coli, S. aureus,* K. pneumonia, and *P. aeruginosa*, with a percentage rate of inhibition of up to 98, 97, 94, and 85% respectively, according to (Divya *et al*., 2017). (Muslim *et al*., 2018) demonstrated a significant reduction in biofilm mass in the presence of chitosan, and their findings were validated by light and scanning electron microscopy. While (Fattah *et al*., 2021) discovered the impact of Chitosan nanoparticles on biofilm development by Ps.aeruginosa, and the rate of inhibition was around 84.95%.

Table 1: The effect of CS-NPs on *P. mirabilis* swarming motility and biofilm development.

Swarming motility (Diameter of growth in cm)		Biofilm production (Absorbance in 630 nm)		
Control	Chitosan Nanoparticles	Control	Chitosan nanoparticles	Percentage rate of biofilm Inhibition
8.5	0.5	0.525	0.222	58 %

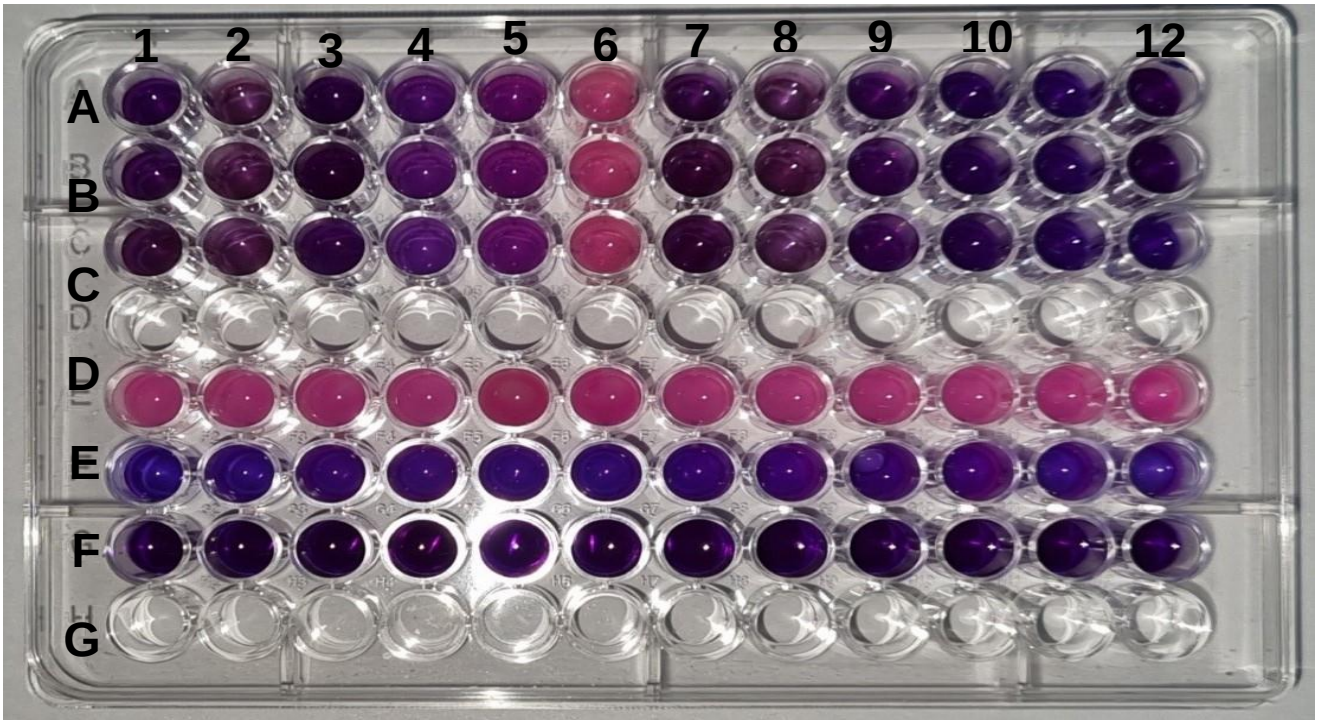
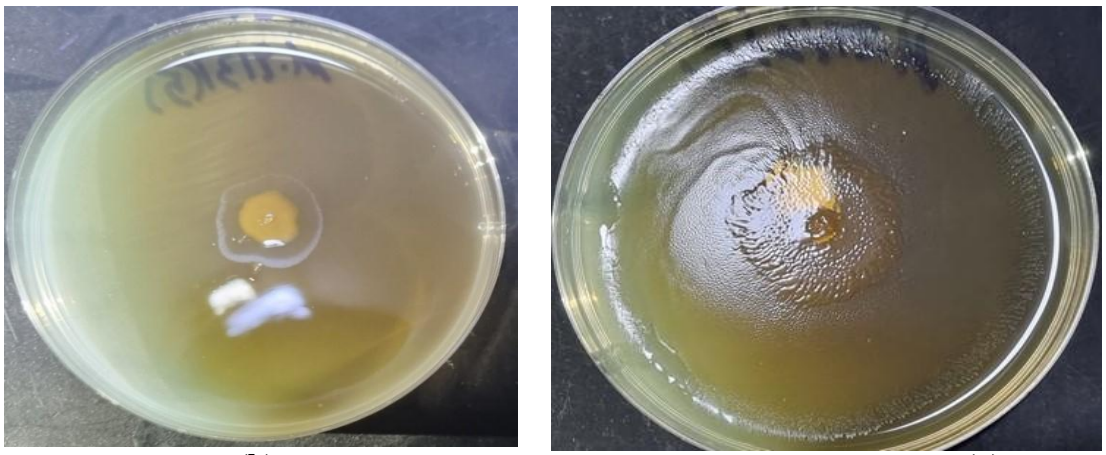


Fig. 1: MIC determination of Chitosan nanoparticles against *Proteus mirabilis*. The rows A, B, and C included tested isolates containing six different doses of Chitosan nanoparticles. This isolate was tested three times. The G row ensures that no contamination occurred during plate preparation since there is no change in the color of resazurin from its native (blue) form to its reduced form (purple). The E row (columns 1,2,3,4,5,6,7,8,9,10,11,12) contained bacterial suspension only as a positive control. The A, B, C rows (columns 7, 8, 9, 10, 11, 12) contained different concentrations of chitosan nanoparticles. The F row (columns 1,2,3,4,5,6,7,8,9,10,11,12) contain broth only as a Negative control.

The highest concentration of CS-NPs in the plate was 40mg/mL, and the lowest concentration was 1.25 mg/ml. The 1, 2, 3 columns showed no color changes; this refers, column 3 was considered as the MIC value. The 4, 5, 6 columns showed color changed this indicates that the isolates were alive. Fig (3).



(b) **(a)**

Fig. 2 :(a) Swarming motility of *Proteus mirabilis*. (b) The effect of sub-MIC Chitosan nanoparticle cons.

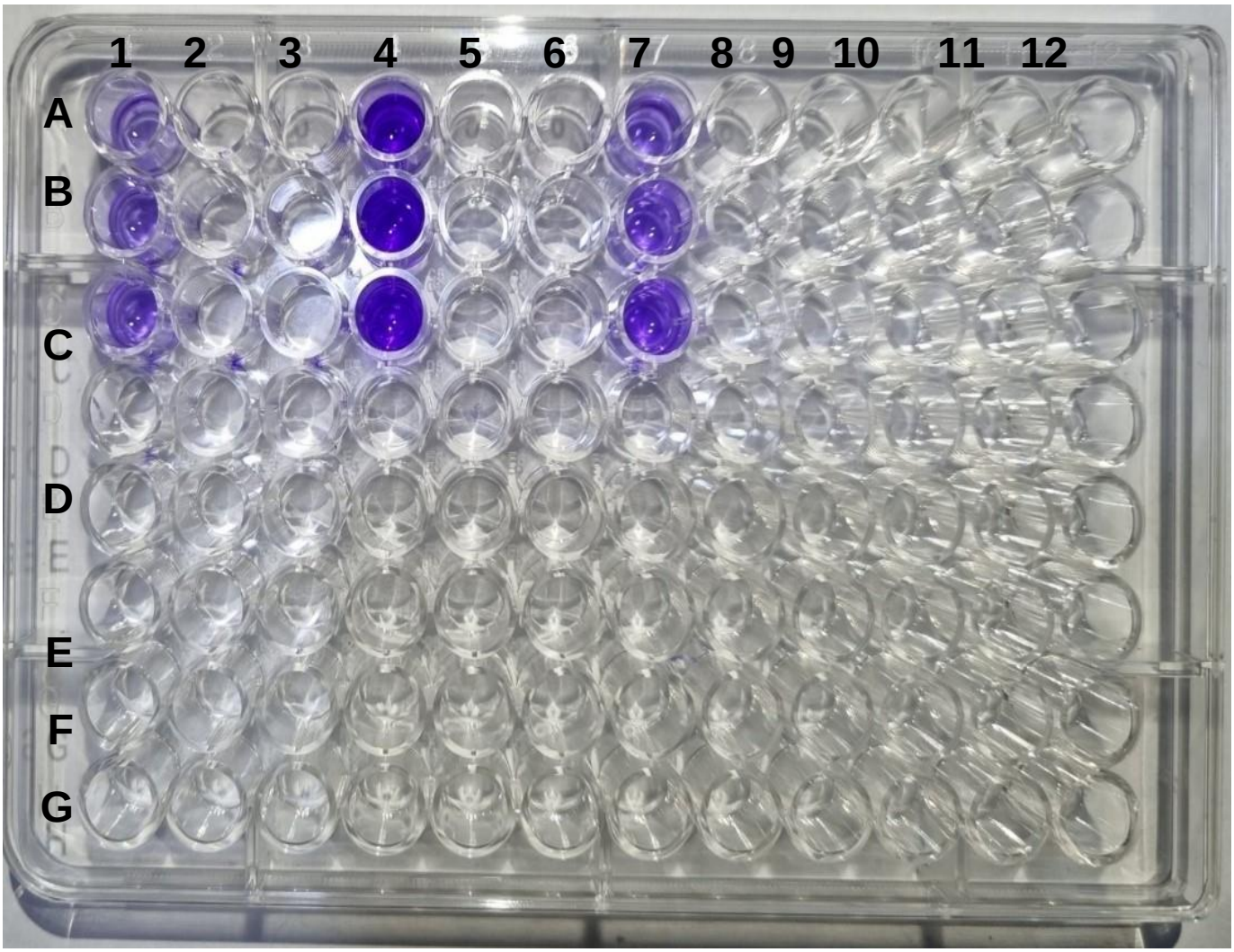


Fig. 3: Microtiter plate test for *P. mirabilis* biofilm development. Isolates were retested 3 times for all cons. Rows A, B, C: column 1 contains simply the broth as a negative control, columns 4 contain the isolate without CS-NPs, and columns 7 contain the same isolate plus sub-MIC CS- NPs.

CONCLUSION

Chitosan nanoparticles were shown to have a considerable influence on *P.mirabilis* pathogenicity which isolated from UTI ,by inhibiting the most critical virulence factors, swarming motility and biofilm formation by used the sub-MIC (CS-NPS) concentration . The FAD rated CS-NPs as generally recognized as safe (GRAS) in 2001. This study is recommended using chitosan in the treatment of UTI caused by *P. mirabilis*, and in the reduced or eliminated catheter- related urinary tract infections (CAUTIS) due to the inability of *P.mirabilis* to migrate on the surface of the catheter.

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تأثير جسيمات الكايتوسان النانوية على النمو، ظاهرة العج، وتكوين الاغشية الحيوية في جرثومة *Proteus mirabilis* المعزولة من التهابات المجاري البولية

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الملخص

تعد جرثومة *Proteus mirabilis* المسبب الثالث لإصابات المسالك البولية الالتهابي ،اجريت هذه الدراسة لتقييم التأثير التثبيطي لجسيمات الكايتوسان النانوية (CS-NPs) على نمو جرثومة *Proteus mirabilis* التي عزلت من مرضى مصابين بالتهاب المسالك البولية وتم تشخيصها بالطرق الروتينية وتأكيد التشخيص باستخدام نظام API فضلا عن التحري عن تأثيرها على حركة العج swarming motility وتكوين الاغشية الحيوية Biofilm formation بوصفها من عوامل الضراوة المهمة لهذه الجرثومة. تم تحديد التركيز المثبط الادنى لجسيمات الكايتوسان النانوية MIC (CS-NPs) لاختبار تأثيرها على نمو السلالة المرضية من جرثومة Proteus *mirabilis* اضافة لذلك تم استخدام التركيز تحت المثبط الأدنى من جسيمات الكايتوسان النانوية Sub-MIC (CS-NPs) لتقييم تأثيرها المثبط على حركة ظاهرة العج وتكوين الاغشية الحيوية. اظهرت نتائج هذه الدراسة ان جسيمات الكايتوسان الناوية CS-NPs تسببت في تثبيط ملحوظ في عوامل الضراوة المهمة بالمقارنة مع معاملة السيطرة بدون اضافة جسيمات الكايتوسان وذلك بحدوث انخفاض متوسط قطر حركة العج من 8.5 الى 0.5 سم. بينما بلغت نسبة تثبيط الاغشية الحيوية 58% والتي تعتبر متوسطة الانتاج للأغشية الحيوية.

الكلمات الدالة: جسيمات الكايتوسان النانوية، ظاهرة العج، تكوين الاغشية الحيوية، *Proteus mirabilis*.