How to Cite:
Khan, F., Shah, D. A., Ahmad, S. S., Fahimullah, F., Mazhar, M., Khan, S., Mughal, H. S., Khan, F., & Bukhari, N. T. (2023). Chitosan improves the efficacy of gentamicin against MDR and biofilm-forming URO pathogenic E. coli. *International Journal of Health Sciences*, *7*(S1), 2686–2697. https://doi.org/10.53730/ijhs.v7nS1.14566

Chitosan improves the efficacy of gentamicin against MDR and biofilm-forming URO pathogenic *E. coli*

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***Abstract*---**Uropathogenic *E. coli* is the most common cause of urinary tract infections (UTIs), accounting for 80-90% of community-acquired UTIs and 30-50% of hospital-acquired UTIs. Uropathogenic *E. coli* that form biofilms are linked to chronic and persistent inflammation, resulting in severe and recurring UTIs. Biofilms promote antibiotic resistance and the horizontal transfer of virulence genes, promoting the formation of multidrug-resistant organisms. This study aimed to combine low-molecular-weight chitosan with aminoglycoside

gentamicin to improve its efficacy against biofilm formation and MDR *E. coli*. Different strains of bacteria were isolated from urine samples of different patients at the Ayub Teaching Hospital, Abbottabad, out of which 16 were identified as *E. coli* by API (Analytical Profile Index) 20E. The antibiotic sensitivity profile was determined using the disk diffusion method, and the results showed that a total of 10 isolates were found to be multidrug-resistant MDR (N=62.5%). Biofilm formation was tested using the TCP method, a total of 7 isolates were found to be strong biofilm producers (N=44%). Gentamicin exhibited the highest inhibitory activity against 10 isolates of *E.coli*, with MIC-p ranges of 4 and 2 µg/ml, respectively, while 6 isolates showed resistance to gentamicin with MIC-p ranges >512 µg/ml. The combination of chitosan with gentamicin demonstrated greater potency, with MIC-p ranges of 2 and 1 against gentamicin-sensitive isolates and MIC ranges of 4 and 2 against gentamicin-resistant and MDR *E. coli*. The results of MIC-b showed that gentamicin inhibited biofilm formation in 5 isolates at a much higher concentration (2048 µg/ml), but the combination of gentamicin with chitosan enhanced its activity against biofilms, reducing MIC-b to 128, 64, and 32 µg/ml, respectively. Similarly, the minimum biofilm eradication concentration (MBEC) of gentamicin was found to be >2048 µg/ml, but the combination with chitosan reduced it to 128 and 64 µg/ml. This study concludes that the combination of chitosan with different front-line antibiotics may enhance efficacy against multidrug-resistant bacteria and biofilm-forming pathogens, which cause prolonged and severe infections.

***Keywords*---**Biofilm, Chitosan, MIC-p, MIC-b, MBEC, UPTis, TCP, API.

1. Introduction

Urinary tract infections (UTIs) are growing more difficult to treat, owing to rising recurrence rates and resistance to first-line medications. (Ponnusamy *et al.*, 2012). Every year, 150-250 million cases of UTIs are recorded worldwide, (Anusha and Sundar, 2020) 90% of all UTIs, including nosocomial and community-acquired infections, are caused by Uropathogenic *E. coli* (UPEC). (Foxman, 2002) UPEC colonizes the urinary bladder as an opportunistic intracellular pathogen, causing a variety of clinical symptoms ranging from cystitis to severe pyelonephritis. (Mulvey *et al.*, 2000). Their ability to form a biofilm is linked to hospital-acquired infections, particularly catheter-associated urinary tract infections (CAUTI). Since biofilms possess a higher concentration of polysaccharides, they render immune components and antibiotics ineffective. In contrast to the planktonic state, bacterial cells in consortia possess greater abilities to tolerate antibiotics and remain challenging in clinical settings (VA *et al.*, 2013). Gentamicin was introduced in 1963 as the first clinically useful broad-spectrum antimicrobial agent, and it remains one of the most powerful antibiotics for the treatment of serious infections (Edson and Terrell, 1999). Chitosan exhibits anti-biofilm activities and the ability of chitosan to damage biofilms formed by microbes such as *Listeria monocytogenes, Bacillus cereus,*

Staphylococcus aureus, Salmonella enterica, and Cryptococcus neoformans has been documented (Martinez *et al.*, 2010; Orgaz *et al.*, 2011). It is generally accepted that the combination of chitosan with other antimicrobial components such as antibiotics could enhance its antimicrobial activity and thereby reduce the development of antibiotic-resistant strains (Huang *et al.*, 2011). Several studies have demonstrated antimicrobial resistance among UPEC with increasing trends to the most commonly used antibiotics such as ciprofloxacin, trimethoprim-sulphamethoxazole among others (Neupane *et al.*, 2016).Understanding the link between biofilm formation, the presence of virulence genes and antimicrobial resistance distribution in UPEC strains is key to designing effective strategies and measures for the prevention and management of UTIs especially severe, recurrent and complicated UTIs (Donelli and Vuotto, 2014).

2. Materials and Methods

2.1. Bacterial strain confirmation and chemicals

Clinical suspected samples of E. *coli* were collected from Ayub Teaching Hospital, Abbottabad. All the samples were obtained from different urine specimens, *and the experiment was performed in the microbiology lab at Ayub Teaching Hospital, Abbottabad*. The collected samples were then confirmed microbiologically by culturing them on Nutrient Blood and MacConkey agar. Additionally, biochemical confirmation was carried out using the API 20E for proper identification. The collected *E. coli* samples were prepared in nutrient broth containing 40% glycerol and stored at -80°C for further experiments. *E. coli* ATCC25922 was obtained from the National Institute of Health Sciences, Islamabad (NIH). A 96-well tissue culture plates (Nested Biotechnology), Mueller-Hinton broth (MHB) and Tryptic Soy Broth (TSB) Acetic acid and other chemicals and reagents used in this study were sourced from Worldwide Scientific, Rawalpindi.

2.2. Antimicrobial susceptibility testing

Antibiotic susceptibility testing was performed using using the Kirby-Bauer method on Mueller–Hinton agar plates (BIOPHARMA) according to the guidelines and breakpoints of the Clinical and Laboratory Standard Institute (FR, 2012) while for colistin disc elusion method was used. The antimicrobial discs used, which were all obtained from Oxoid (UK). The following antibiotics were used at different concentrations to confirm multidrug-resistant (MDR) *E. coli*: gentamicin, cefepime, levofloxacin, meropenem, aztreonam, and colistin. *E. coli* ATCC 25922 was use as a negative control.Strains that showed resistance to three or more classes of antibiotics were considered MDR *(Magiorakos et al., 2012).*

2.3. Quantification and detection of biofilm formation

Tissue culture plate method was performed as described by (Christensen *et al.*, 1982) for quantitative measurement of biofilm production. Using a micro titer assay. A single colony from each subculture plate on blood agar was inoculated in a glass tube containing two ml TSBglu. The tubes were incubated overnight at 36°C ± 1 under aerobic conditions. Two hundred micro litres from each of the

inoculated TSBglu tubes were aseptically transferred in the wells of a flat bottomed micro well plastic plate. The inoculated micro well plastic plate was incubated overnight at 36°C ± 1 without sealing of the plate for proper oxygenation**.** Next day, the contents were discarded by inverting the plate and striking it on filter paper. The micro well plastic plate was washed once by adding 200 µl PBS (pH 7.2) into each well and then discarded. Then 200ul of freshly prepared sodium acetate (2%) was added to each well (for biofilm fixation) for 10 minutes and then discarded. This was followed by adding 200 µl crystal violet (0.1%) to each well for biofilm staining. The Plates was kept at room temperature for 30 minutes, and then the stain was discarded. The washing step was repeated once more. Finally, the plate was left to dry at room temperature for one hour, after which, the absorbance was read on a spectrophotometer at 620 nm OD.

2.4. Preparation of stock solution of chitosan and gentamicin

Low molecular weight chitosan (Mw = 60–120 kDa) and gentamicin were obtained from Sigma Aldrich. The chitosan stock solution was prepared following the procedure described in previous literature (Costa et al., 2017). Specifically, 7 mg/L of low molecular weight chitosan was added to 10 mL of 1% glacial acetic acid (w/v) and stirred overnight to ensure thorough mixing (Costa et al., 2017). The pH of the solution was adjusted to 6.9 using 10N NaOH, and the chitosan solution was then stored at 4°C for future use. For the preparation of the gentamicin stock solution, the stock solution for MIC-P (minimal inhibitory concentration for planktonic cells) was prepared at a concentration of 512 µg/mL, as per the method described by (Jakobsen et al., 2007) Additionally, for MIC-B (minimal inhibitory concentration for biofilm-associated cells), a gentamicin stock solution was prepared at a concentration of 2048 µg/mL, following the protocol outlined by (Rafaque et al., 2020). The experiments were conducted under sterile conditions to ensure the accuracy and reliability of the results.

2.5. Determination of MIC-p

Briefly, gentamicin was diluted in a 96-well micro titer plate from 512 to 2µg/ml and chitosan solution at a fixed concentration 7mg/l concentrations was added. An aliquot of 100 µl of the bacterial suspension adjusted to a 0.5 McFarland standard was inoculated and incubated at 37 °C for 24 h. The MIC is defined as the lowest concentration of chitosan and combined with gentamicin that can inhibit visible bacterial growth (Felipe *et al.*, 2019).

2.6. Determination of MIC-b and MBEC

For the measurement of MIC-b, 75 µL of standardized bacterial suspension was inoculated in a 96-well micro titer plate. The plate was incubated at 37°C for 24 hours. After incubation, the plates were washed with phosphate buffer saline (PBS). Two-fold serial dilutions of gentamicin, ranging from 0.5–2048 µg/mL were prepared and 100 µL of appropriate concentration of each antibiotic was added to each well of a micro-titer plate and chitosan was added to each well at a fixed concentration the plate was incubated for 18–24 hours at 37°C. MIC-b for each tested sample was visually estimated as the lowest concentration of antibiotic capable of inhibiting the biofilm formation of planktonic bacteria (Rafaque *et al.*,

2020). Subsequently, for the measurement of MBECs, the treatment procedure was essentially the same as described for MBIC except, after the incubation step, wells without visible growth were scraped thoroughly and particular attention was given to the edges of wells. Scraped material was transferred to 1 mL PBS. Each sample was briefly vortexes to disrupt biofilm and a 100 µL sample was subsequently plated on a fresh tryptic soy agar (TSA) plate. Antibiotic concentration on which no bacterial growth was observed on the TSA plate was considered MBEC (Wang *et al.*, 2016).

3. Result and Discussion

3.1. Culture collection and confirmation through APIE20

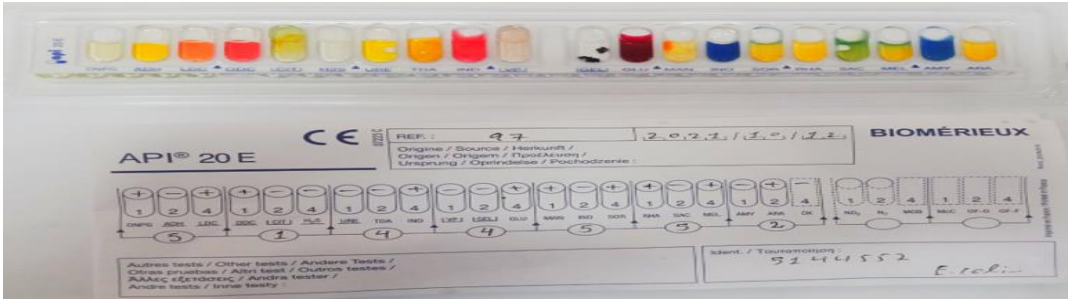

Figure-1 API kit E20 shows biochemical confirmation of *E.coli*

3.2. Antimicrobial susceptibility testing

Antimicrobial resistance (AMR) poses a grave threat to public health and is attributed as the third major cause of mortality worldwide. Rise in infections caused by multidrug-resistant MDR pathogens prompted WHO to declare a list of 12 priority AMR pathogens in 2017, of which, carbapenem-resistant *Enterobacteriaceae* (*Escherichia coli, Klebsiella spp, Serratia spp, Proteus spp*) fall under critical priority group (Sundaramoorthy *et al.*, 2019).as shown in Table 1, a total of 16 isolates of *E. coli* were tested for their antimicrobial susceptibility using the disk diffusion method. Out of these, 10 (62.5%) isolates were found to be multidrug-resistant (MDR). These MDR isolates displayed resistance to gentamicin, cefepime, meropenem, colistin, aztreonam, and levofloxacin. Our work is most similar to the previous study reported (Ramírez-Castillo *et al.*, 2018) 63.3% isolate of uropathogenic *E. coli* were found to be MDR. The remaining 6 (37.5%) isolates were identified as non-MDR. Among these, 6 (37.5) *E. coli* isolates were resistant to gentamicin. The highest resistance was observed against cefepime and levofloxacin (10,62%) and cepefime (9,56%). This is most similar to the previous study (Hassanshahi *et al.*, 2020) The highest rate of resistance to levofloxacin (66.7%) has been reported among patients having urinary tract infections with *E. coli* isolates. Another previous study reported that the resistance rate in E. coli isolates towards cepefime was found to be 22%, which is not consistent with our work. This difference can be attributed to the variation of resistance patterns to antimicrobials based on their usage(Siddiqui *et al.*, 2013) This difference can be attributed to the variation of resistance patterns to antimicrobials based on their usage (Siddiqui *et al.*, 2013), another previous

study reported(Ansari *et al.*, 2015) that meropenem resistance in isolates of E. coli was found to be 60–75%.The least resistance was observed, as only 2 (12.5%) were resistant to colistin.

Table-1 show antibiotic sensitivity profile of *E. coli* isolates

Sample id	Isolation Source	MDR	Resistance against
90	Urine		
91	Urine	Positive	Gentamicin, Cefepime, Meropenem, Levofloxacin
92	Urine		
93	Urine		Colistin
94	Urine		
95	Urine		
96	Urine	Positive	Cefepime, Meropnem, Levofloxacin
97	Urine	Positive	Gentamicin, Cefepime, Meropenem, Levofloxacin
98	Urine	Positive	Gentamicin, Cefepime, Meropenem, Levofloxacin
99	Urine	Positive	Gentamicin, Cefepime, Aztreonam, Levofloxacin
100	Urine	Positive	Cefepime, Meropnem, Levofloxacin
101	Urine	Positive	Gentamicin, Cefepime, Meropenem, Levofloxacin,
102	Urine	Positive	Cefepime, Meropenem, Levofloxacin, Colistin
103	Urine		Gentamicin
104	Urine	Positive	Cefepime, meropenem, aztreonam, levofloxacin
105	Urine	Positive	Gentamicin, Cefepime, Meropenem, Aztreonam, Levofloxacin

3.3. Quantification and detection of biofilm by TCP method

Among the 16 isolates, a total of 7 isolates of E. coli were found to be strong biofilm producers using the tissue culture plate method, representing 44% of the total. Additionally, four isolates of E. coli were observed to produce weak biofilms on the tissue culture plate, accounting for 31% of the total isolates. One isolate exhibited no biofilm-producing capabilities, while 3 isolates were classified as moderate biofilm producers, making up 19% of the total. the TCP method was found to be the most sensitive, accurate and reproducible screening method for the detection of biofilm formation and has the advantage of being a quantitative model to study the adherence on biomedical devices (Mathur *et al.*, 2006).

Table 2 show different degree of biofilm formation according to cut-off OD

Mean OD value	Biofilm formation
<0.007	Non
0.007-<0.0140	Weak
0.0140-0.028	Moderate
>0.028	Strong

Many persistent infections are caused by biofilm-producing bacteria, which are difficult to eliminate. *E. coli* biofilm formation enhances colonization and increases UTI. These infections may be challenging to treat due to multiple drug resistance.

Biofilm development in *E. coli* enhances colonization and increases the rate of UTIs, which can be difficult to treat due to multidrug resistance (MDR). Biofilm prevalence among Uropathogenic *E. coli* (UPEC) varies from 60% to 70%. (Sharma *et al.*, 2009). Similar work was performed by (Karigoudar *et al.*, 2019) 89% isolate of *E.coli* isolated from catheterized patients were strong biofilm producers 49% from non-catheterized patients were biofilm producers on TCP methods 26% of the isolate were non-biofilm producers. Another study (Karigoudar *et al.*, 2019) reported biofilm producing percentage of TCP was found to be 69% of *E.coli.*

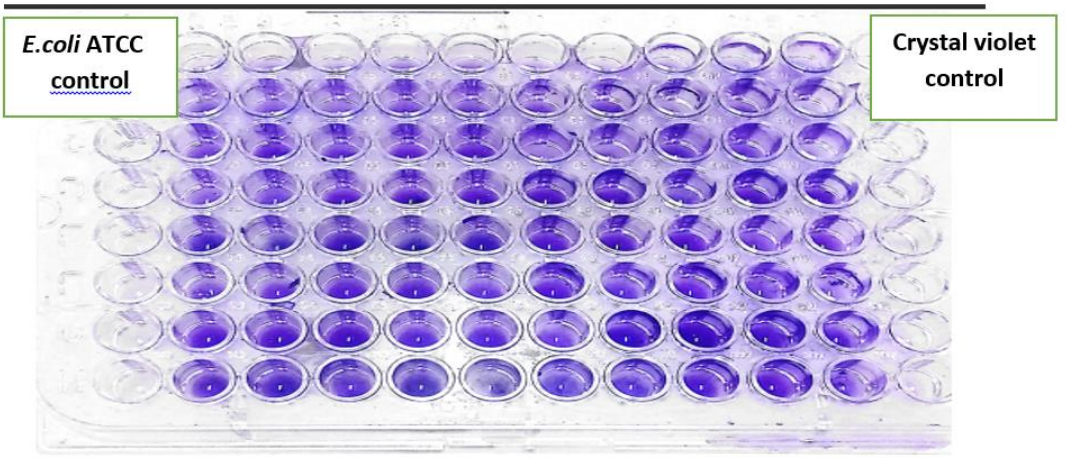

Figure 2. biofilm detection by TCP method well No 1 and 12 have negative of crystal violet and TSB and *E.coli* ATCC only

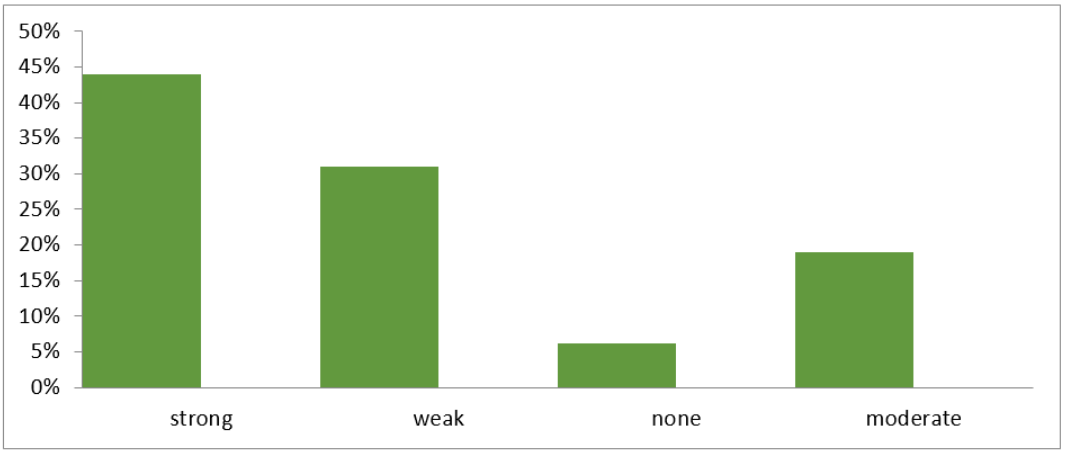

Figure 3. Graph shows biofilm producing % of *E.coli* isolates by TCP methods

3.4. Determination of MIC –p

To determine whether the combination of chitosan with gentamicin improved its efficacy against multidrug-resistant (MDR) and gentamicin-resistant isolates of *E. coli.* We tested a total of 17 *E. coli* isolates, which included one isolate of *E. coli*

ATCC25922, a reporter strain, to determine the minimum inhibitory concentration (MIC) of chitosan when combined with gentamicin.it was observed that the addition of chitosan at a concentration of 7 mg/ml enhanced the efficacy of gentamicin against the gentamicin-resistant strain, with MIC values of 2, 1, and 4 µg/ml, compared to>512 for gentamicin alone. Additionally, the combination of chitosan improved the activity of gentamicin against MDR isolates of *E. coli*, with MIC values of 2 and 4 µg/ml, respectively. The MIC range for *E. coli* ATCC was determined to be 4 µg/ml for gentamicin, and the addition of chitosan reduced the MIC to as low as 2 µg/ml. Similar work was performed by (Tin et al., 2009) used different types of chitosan in combination with various antibiotics. The MIC of low molecular chitosan in combination with sulfamethaxezole against Pseudomonas aeruginosa was found to be 8 µg/ml. Their work is the closest similarity to our study. Another previous study was conducted by (Breser et al., 2018), where the MIC of chitosan combined with cloxacillin against Staphylococcus was 0.125 and 0.25, respectively indeed, LMW chitosan's antibacterial activity has been related to its interaction with electronegative chemicals in the bacterial cell's core. (Costa *et al.*, 2014). Chitosan activity is influenced by a variety of factors, where molecular weight and the type of microorganisms evaluated play important roles (Verlee *et al.*, 2017). The antibacterial action of chitosan is presumably due to interactions with the bacterial surface, either the cell wall or the outer membrane, and it involves electrostatic attraction by peptidoglycans in Gram-positive bacteria (Sahariah and Másson, 2017). Chitosan is a polymer with a positive charge and the presence and density of this cationic charge are believed to be responsible for the efficient binding of chitosan to the anionic components present in the bacterial membrane (Raafat *et al.*, 2008).

Table 2: Shows MIC-p of gentamicin and combination chitosan

Sample ID	MIC-p Chitosan+Gentamicin µg/ml	MIC-p Gentamicin µg/ml	MDR
90	2	>512	
91	2	4	Positive
92	2	4	
93	1	2	
94	1	4	
95	4	8	
96	2	4	Positive
97	2	>512	Positive
98	4	>512	Positive
99	4	4	Positive
100	4	4	Positive
110	4	>512	Positive
102	2	4	Positive
103	4	>512	
104	2	4	Positive
105	4	>512	Positive
E.coli ATCC 25922	2	4	

3.5. Determination of MIC-b and MBEC of chitosan and combine with gentamicin

Chitosan exhibits anti-biofilm activities and the ability of chitosan to damage biofilms formed by microbes has been documented (Orgaz *et al.*, 2011). Chitosan has been shown to penetrate biofilms due to the ability of cationic chitosan to disrupt negatively charged cell membranes as microbes settle on the surface (Rabea *et al.*, 2003; Carlson *et al.*, 2008). Several factors accounted for the extraordinary resistance of biofilm bacteria to antibiotics. One factor that is generally conceded to play a role in antibiotic resistance is the inability of the antibiotic to penetrate biofilms, thereby reducing the antibiotic available to interact with biofilm bacteria. Given chitosan has been shown to penetrate and damage biofilm (Orgaz *et al.*, 2011). total of 7 strong biofilm-producing isolates were tested for their Minimum biofilm Inhibitory Concentration MIC-b. Chitosan at a concentration of 7 mg/m enhanced the antibiofilm activity of gentamicin up to 32 µg/ml. Three strong biofilm producer isolates of E. coli were inhibited at a concentration of 32 µg/ml, while one strong biofilm producer isolate of E. coli was inhibited at a concentration of 128 µg/ml. gentamicin at a higher concentration of 2048 µg/ml inhibited the growth of four strong biofilm-producing isolates, with three strong biofilm producers exhibiting complete resistance to gentamicin. The MIC-b for these isolates was found to be >2048 µg/ml. In a previous study conducted by (Breser et al., 2018) the MIC-b of chitosan combined with cloxacillin against Staphylococcus biofilms was found to be 32, 48, and 24 µg/ml, respectively. The results of the MBEC testing revealed that the MBEC of gentamicin for all seven isolates was >2048 µg/ml. However, when combined with chitosan, the MBEC of gentamicin was reduced to 128, 256, and 64 µg/ml, which is consistent with the previous work reported by (Rafaque et al., 2020) for E. coli isolates, where the MBEC was also found to be >2048 µg/ml.

Table 3: Shows MIC-b and MBEC of gentamicin and combine with chitosan

Sample ID	MIC-b-Combination µg/ml	MIC-b-Gentamicin µg/ml	MBEC (Combine) µg/ml	MBEC Gentamicin µg/ml
91	64	>2048	128	>2048
97	128	2048	128	>2048
98	32	2048	64	>2048
100	32	2048	128	>2048
102	32	2048	256	>2048
104	64	>2048	128	>2048
105	64	>2048	256	>2048

4. Conclusion

Taken together, our data demonstrate that the combination of specialized chitosan with aminoglycoside antibiotics such as Gentamycin can effectively inhibit E. coli biofilm formation and the growth of MDR and gentamicin-resistant E. coli. Further studies will be needed to design chitosan gentamicin-based Nano-antibiotics and assess their effects in vitro.

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