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ORIGINAL RESEARCH

Antibiotics Combinations and Chitosan Nanoparticles for Combating Multidrug Resistance *Acinetobacter baumannii*

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Background: Successful treatment of *Acinetobacter (A.) baumannii*-associated infection is complicated by the emergence of multidrug resistance (MDR), particularly in clinical settings. This urges searching for new alternatives to encounter such health problem.

Aim: This study aimed to evaluate certain antibiotic combinations and CNPs either alone or in combination of some selected antibiotics for the purpose of combating MDR *A. baumannii* clinical isolates.

Methods: A total of 51 *A. baumannii* clinical isolates were recovered from discharged clinical specimens of the Clinical Microbiology Central Laboratory of AL Kasr Al Aini hospital, Cairo, Egypt. Conventional standard Lab tests were used for identification followed by *rec*A gene testing for confirmation. Antimicrobial susceptibility tests were conducted out according to CLSI guidelines. Genotypic analysis using Enterobacterial Repetitive Intergenic Consensus-polymerase chain reaction (ERIC-PCR) of the respective isolates showed that they were clustered in nine clones. The prepared CNPs were characterized by dynamic light scattering and HR-transmission electron microscope imaging. Antibiotic combinations and co-effect of CNPs with some selected antibiotics (either each alone or in combination of two) were evaluated using the Checkerboard microdilution and minimum inhibitor concentration decrease factor (MDF) methods, respectively.

Results: The recovered 51 *A. baumannii* clinical isolates were MDR (100%) of these 92% (47/51) were extensively drug resistance (XDR). Combinations of colistin (CT)+meropenem (MEM) and MEM+tigecycline (TGC) showed synergism in 77.7% and 44.4% and additive effects in 22.3% and 55.6% of the tested MDR *A. baumannii* isolates (n=51), respectively. However, CT+TGC combination showed antagonism. CNPs exhibited good inhibitory activity (inhibition zones ranged from 24 to 31 mm) against selected nine MDR *A. baumannii* isolates (one isolate from each clone). The MIC of CNPs at concentrations (ranging from 1 to 5 mg/mL) were from 0.16 to 0.25 mg/mL, indicating good in vitro antimicrobial activities. CNPs (5 mg/mL) when combined with CT, TGC or MEM, CT+MEM and TGC+MEM significantly increased the susceptibilities of the MDR *A. baumannii* isolates to these antibiotics by 88.8%, 66.6%, 100%, 77.7%, and 44.4%, respectively. No significant effects were observed when CNPs (5 mg/mL) were combined with CT+TGC.

Conclusion: The current study demonstrated the significant in-vitro activities of CNPs either alone or in combination with CT, TGC or MEM, CT+MEM and TGC+MEM and the successful combinations of MEM either with CT or with TGC against the MDR *A. baumannii* pathogens. However, further in vivo studies should be conducted to verify such activities and their potential use in human.

Keywords: *A. baumannii*, multidrug resistance, chitosan nanoparticles, antibiotic combinations, meropenem, colistin, tigecycline

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Introduction

A. baumannii is a Gram-negative, non-fermentative, *coccobacillus* and is considered to be one of the major causative agents of nosocomial infections. It is figured in the "critical" category of World Health Organizations (WHO) priority pathogens list for development of new antibiotics.[1] *A. baumannii* is one of the six superbugs" identified by the Infectious Diseases Society of America as "ESKAPE".[2] *A. baumannii* has been implicated in a diverse range of infections including, pneumonia, bacteremia, wound and burn infection, urinary tract infection and meningitis.[3,4] It is conspicuously prevalent in intensive care units where frequent epidemics have been tremendously problematic to control.[3,4] The rapid emergence and worldwide distribution of drug resistant *A. baumannii* as a foremost nosocomial pathogen highlights its successful adaptation clinical settings and health-care ecosystem.[5] Many studies have shown that the biofilm formation is the reason behind the survival of *A. baumannii* in harsh environments and high resistance to various antibiotics. Several mechanisms are considered key factors in the high resistance of biofilms such as: (a) impaired drug diffusion, (b) enzyme-caused neutralizations, (c) heterogeneous function, (d) slow rate of growth, (e) persistent cells, and (f) alterations in microbial phenotypic and genotypic features.[6–9]

Phenotypic identification of *A. baumannii* clinical isolates should be confirmed using genotypic methods via detection of *rec*A gene by polymerase chain reaction (PCR)[10] followed by genotyping confirmation using the Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR). ERIC-PCR analysis can differentiate MDR *A. baumannii* strains at the clonal level to confirm their clonal relationship. This helps controlling these resistant strains and tracing their epidemics.[11]

As known, the main strategy for treating infectious diseases is antimicrobial agents; however, the misuse of antimicrobial agents accelerates the emergence of resistance which in turn leads to serious losses such as financial cost, societal cost, and cost of death.[12,13] *A. baumannii* can evolve antibiotic resistance through several mechanisms, including enzymes inactivating antibiotics, alteration of the target or cellular functions due to mutations, formation of biofilms and reduced entry into the target site of bacteria (Efflux pump).[14] To combat the bacterial resistance, many scientists are trying to develop and explore new antimicrobials, however, it is not an easy process to have efficient and approved one.[15–18]

As known in literature, carbapenems, colistin and tigecycline still retain activities against MDR *A. baumannii*.[19–21] Carbapenems such as meropenem cause bacterial cell death by binding covalently to penicillin-binding proteins (PBPs) involved in bacterial cell wall biosynthesis. Colistin, owing to its unique mechanism of action, that is disruption of bacterial outer membrane lipids as well as tigecycline which inhibits bacterial protein synthesis by acting on the 30S ribosomal subunit and prevents amino acids from incorporating and elongating peptide chains.[19–21]

Another promising strategy for overcoming the microbial resistance is the use of nanoparticles. Chitosan nanoparticles generally consist of biodegradable polymers or lipids which are biocompatible and are non-toxic. But, the possible toxic effect can never be denied due to their large surface area and smaller size relative to the cellular components, and proteins can lead to adverse tissue reaction and cause toxicity such as toxicity towards a murine melanoma cell line, several tumor cell lines and gastric carcinoma cell line.[22–24]

The major processes underlying the antibacterial effects of NPs are disruption of the bacterial cell membrane, penetration of the bacterial cell membrane and induction of intracellular antibacterial effects, including interactions with DNA and proteins.[25–27] Chitosan nanoparticles (CNPs) acquired extraordinary biological action particularly against MDR pathogens due to its minute size, quantum effect, elevated sorption capacity and good antimicrobial activity by binding positively charged chitosan to negatively charged bacterial cell wall surfaces such as lipopolysaccharides. This binding has led to the alteration of the bacterial membrane permeability, causing leakage of intracellular constituents and cell death, binding to DNA in bacteria causing inhibition of DNA replication and cell death and Chitosan acts as a chelating agent that selectively binds to trace metal elements causing toxin production and inhibiting microbial growth.[27–29] Characterization CNPs can be performed appropriately by Dynamic Light Scattering (DLS) and High resolution -Transmission electron microscope imaging (HR-TEM).[30–32] Till now, few studies had been conducted to explore the antimicrobial activities of either antibiotic combinations or combination of CNPs with certain antibiotics against MDR or XDR clinical pathogens.[19–22,28] Therefore, this study aimed to evaluate certain antibiotic combinations and CNPs either alone or in combination of antibiotics for the purpose of

combating MDR *A. baumannii* clinical isolates, the life threatening pathogens with limited therapeutic options.

Methods

Specimen Collection and Identification of the Recovered Clinical Isolates

A total of 51 *A. baumannii* isolates were obtained from the Microbiology Central Lab of AL Kasr Al Aini hospital, Cairo, Egypt between January, and June 2020. These isolates were recovered from 730 different discharged clinical specimens including, pus, urine, sputum, bronchial lavage, and cerebrospinal fluid according to the hospital records. Identification of the isolates were carried out using conventional techniques (colony morphology, culture using a specific media ChromAgar, and biochemical tests). The isolates were cultured on chromAgar (when the color changes from yellow to red, it indicates that the isolate is *A. baumannii*). The identification of isolates was also confirmed using the automated system, Vitek-2 (bioMérieux, Marcy L'Etoile, France) and PCR analysis of the *rec*A gene as previously described.[33] Negative control (PCR reaction with chromosomal DNA of *A. baumannii* ATCC 17978 but without *rec*A primers) and positive control (PCR reaction with chromosomal DNA of *A. baumannii* ATCC 17978 as a PCR template plus *rec*A primers) were used for quality control.

Antimicrobial Susceptibility Testing and MDR Definition

The obtained bacterial isolates were evaluated for susceptibility to the antimicrobial agents recommended by the Clinical and Laboratory Standards Institute, 2018.[34] Susceptibility tests were performed using the Kirby–Bauer disk diffusion method on Mueller–Hinton agar (Hi media, India) using the following antimicrobial disks (Bioanalyse, Turkey): Piperacillin (PIP, 100 µg), piperacillin/tazobactam (TPZ, 10/100 µg), ampicillin-sulbactam (SAM, 10/10 µg), cefepime (FEP, 30 µg), ceftriaxone (CRO, 30 µg), Amikacin (AK, 30), gentamicin (CN, 30 mcg), ciprofloxacin (CIP, 5 mcg), imipenem (IMP, 10 µg), meropenem (MEM, 10 µg), doxycycline (DO, 30 µg), Trimethoprim-sulfamethoxazole (SXT, 1.25/23.7 µg), tigecycline (TGC, 15 µg). Susceptibility to colistin (CT) was examined via minimum inhibitory concentration (MIC) measurement using E-test (Bioanalyse, Turkey) according to manufacturer's recommendations. The reference *A. baumannii* ATCC 17978 and *E. coli* ATCC 25922 strains were used as a quality control. The MIC of TGC, CT, MEM (products of Merck, Darmstadt, Germany) were carried out using microbroth dilution method according to CLSI guidelines, 2018).[34] MDR phenotype was inferred as described by Magiorakos et al.[35]

Molecular Typing of Recovered Isolates

ERIC-PCR was carried out on 51 *A. baumannii* isolates to investigate the clonal relationship, clonal expansion, and their diversity.[36] Genomic DNA was extracted using the Genomic DNA Purification Kit (Thermo Fisher Scientific, UK) according to the manufacturer's recommendations. ERIC-PCR was carried out using the ERIC-1 primer (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC-2 primer (5′-AAGTAAGTG ACTGGGGTGAGCG-3′) primers as previously described.[36] Analysis of ERIC-PCR dendrogram was constructed using the UPGMA clustering method, Bionumerics program version 7.6 (Applied Maths). The Percentage of similarity among 51 isolates of *A. baumannii* was calculated using Jaccard's Coefficient.[37]

Evaluation of Antibiotic Combinations

The MIC of MEM, TGC and CT was determined using the broth microdilution technique according to the CLSI guidelines 2018.[34] In vitro combinations of MEM+TGC, MEM+CT and TGC+CT were performed in 96-well microdilution plates and evaluated using the checkerboard method. Two-fold Serial dilutions of antibiotic aqueous solutions were prepared starting from (1024 µg/mL) to (0.25 µg/mL) using standard laboratory powders of the antibiotics (CT, MEM and TGC). A 0.5 McFarland standards of bacteria used and inoculated into Mueller-Hinton broth medium. The plates were incubated at 37 °C for 24hr. The sum of the fractional inhibitory concentration (ΣFICs) was calculated as described by Hsieh et al.[38] The combination is considered synergistic when ΣFIC is ≤ 0.5, additive when ΣFIC is > 0.5 and ≤ 1, indifferent when ΣFIC is >1 and ≤ 4, and antagonistic when ΣFIC is > 4.[39]

Preparation of Chitosan Nanoparticles (CNPs)

About 5 mg/mL of low molecular weight chitosan (Sigma-Aldrich, Darmstadt, Germany, CAT, 448869) was suspended in 10 mL of 1% v/v acetic acid and the pH was adjusted between 4.6 and 4.8 using 10 N NaOH. A total of 0.1 g of sodium tripolyphosphate was dissolved in 100 mL of distilled water. CNPs were produced suddenly while adding the

tripolyphosphate solution dropwise to the chitosan solution under uninterrupted mixing. The manufactured CNPs were purified at 10,000 g for 20 min by centrifugation. Then, the pellet was collected and the CNPs were washed with distilled water then freeze-dried.[40]

Characterization of CNPs

Dynamic Light Scattering (DLS; Zeta Sizer Characterization)

The prepared CNPs were characterized by DLS where, the particle size distribution and zeta potential were measured through DLS with Zetasizer Nano S (Malvern, UK). The analysis was carried out at a scattering angle of 90° at a temperature of 25°C using nanoparticles dispersed in deionized distilled water (1 g of sample was dissolved in 25 mL of deionized water and then sonication is done in sonics Vibra cell Sonicator, UK for 15 min). Particle size distribution of the nanoparticles is reported by intensity as previously reported.[41]

Transmission Electron Microscope Imaging (HR-TEM)

Chitosan nano- suspension was prepared in Ultrasonicator (SB-120DTN, Taiwan) for 15 min then particles were deposited from a dilute aqueous suspension onto (200 mesh) Cu grid with the support of a 10 nm thickness carbon film. After solvent evaporation, Cu grid was placed in double title grid holder and tested under Transmission Electron Microscope (HR-TEM Tecnai G20, FEI, Netherlands) as previously described.[42]

Antimicrobial Activity of CNPs

This was done using two methods:

Well-Cut Diffusion Technique

Purified colonies of MDR *A. baumannii* isolates from overnight plates were picked and inoculated on Mueller Hinton medium. After solidifying, wells were punched out using 0.7 cm cork borer. Then, 100 µL of chitosan nanoparticles were pipetted into each well. All plates were incubated at 37°C for 12 h. After incubation, the radius of clear inhibition zone around each well was measured in mm as previously determined.[43–45]

Broth Dilution Technique

Broth dilution assays were used to determine the MIC of the CNPs against MDR *A. baumannii* isolates. Two-fold serial dilutions of CNPs were prepared using Mueller Hinton broth (starting concentrations were 1, 2.5 and 5 mg/mL). To prepare the inoculum, all the bacterial cell suspensions were adjusted to 0.5 McFarland0.5 ($1–2 \times 10^8$ cfu/mL), then 100 µL of each MDR *A. baumannii* was used for inoculating the tubes under aseptic condition. The tubes were then mixed and incubated for 24 hr at 37°C. After 24 hr of incubation, the MIC was calculated.[40,46]

Evaluation of CNPs-Antibiotic Combinations

In vitro evaluation of CNPs (5 mg/mL) in combination with MEM, TGC and CT (each alone or MEM+TGC, MEM+CT and TGC+CT) against the selected MDR *A. baumannii* was determined by calculating the MIC decrease factor (MDF) as previously reported.[46] In brief, the MIC of each of MEM, TGC or CT (each alone or MEM+TGC, MEM+CT and TGC+CT) was determined using the agar diffusion technique according to the CLSI guidelines 2018.[34] Then, the same MICs of the respective antibiotics was determined but in the presence of CNPs (5 mg/mL) in each well. The MDF of each isolate was calculated according to the following formula $MDF=MIC_{without\ CNPs}/MIC_{with\ CNPs}$. An MDF value equal or greater than 4 was defined as a significant inhibition according to the protocol of Huguet et al.[47]

Results

Specimen Collection and Identification of the Recovered Clinical Isolates

A total of 51 identified *A. baumannii* isolates were obtained from the Microbiology Central Lab of AL Kasr Al Aini hospital. The PCR analysis of the *rec*A gene (425 bp) of the respective isolates is shown in Figure S1 (Supplementary File).

Antimicrobial Susceptibility Findings

Antibiogram analysis of the 51 *A. baumannii* clinical isolates against the 14 tested antimicrobial agents is delineated in Table S1 (Supplementary File). Results revealed that, all the tested *A. baumannii* clinical isolates were MDR (100%) and 92% (47/51) were XDR. The MDR isolates (n=51) were 92–100% resistance to PIP, TPZ, SAM, FEP, CRO, AK, CN, CIP, IMP, MEM, DO, and SXT. However, lowest resistance was observed to TGC and CT (4% each) (Table S1 Supplementary File).

ERIC-PCR Analysis of Recovered Isolates

ERIC-PCR analysis of the 51 MDR *A. baumannii* isolates is shown in Figure S2 (Supplementary File). Dendrogram analysis using BioNumerics fingerprint data software and unweighted pair group method with arithmetic averages at

97% similarity on 51 isolates of *A. baumannii*; the different clusters at 97% similarity are arbitrarily designated as Clusters 1–9, clusters 1 and 7 are the largest group representing the most prevalent clones of *A. baumannii* and its variants among the tested isolates (Figure 1).

Evaluation of Antibiotic Combinations

Based on dendrogram analysis, nine selected isolates coded, A31, A35, A20, A8, A3, A25, A11, A26, and A42 representing the 9 clusters (1–9) were selected for evaluating the two antibiotic combinations (CT+MEM, TGC+MEM and CT+TGC). The MIC values (µg/mL) of the tested antibiotics either alone or in combinations are shown in Table S2 (Supplementary File). The FICI of each of the tested isolates is delineated in Table 1. The FICI values of two tested antibiotic combinations against the nine MDR *A. baumannii* are demonstrated in Figure S3 (Supplementary File). Total percentage of synergy, additive, and antagonistic effects of two tested antibiotic combinations (CT+MEM, TGC+MEM and TGC+CT) against MDR *A. baumannii* (n=9) is shown in Figure S4 (Supplementary File). Results revealed that the CT +MEM and TGC+MEM combinations gave synergy in 77.7% (7/9) and 44.4% (4/9) of the tested isolates (n=9). On the other hand, CT+TGC gave 100% antagonism on the tested isolates (n=9).

Characterization of CNPS

DLS (Zeta Sizer Characterization)

As shown in Figure 2A and B), the zeta potential was positive at 37.7mV and the average size of CNPs at selected concentration was 441.7 ± 58 nm.

Transmission Electron Microscope Imaging (HR-TEM)

As displayed in Figure 2C, the TEM images have displayed the morphological properties and surface appearance of CNPs. The CNPs have virtually spherical shape, smooth surface, and size range of about 80–500 nm.

Antimicrobial Activity of CNPs

Well-Cut Diffusion Technique

Based on dendrogram analysis, nine MDR *A. baumannii* isolates (coded A31, A35, A20, A8, A3, A25, A11, A26, and A42) representing the 9 clusters (1–9) were used for this test. Results of this test is delineated in Figure S5 (Supplementary File) and in Table 2.

Broth Dilution Technique

The result of the MIC by broth dilution assay of the CNPs prepared at three concentrations 1, 2.5 and 5 mg/mL is outlined in Table 3. The MIC of the tested MDR *A. baumannii* isolates (n=51) was in the range of 0.16 to 0.5 mg/mL as presented in Table 3.

Evaluation of CNPs-Antibiotic Combinations

Evaluation of CNPs (5 mg/mL) in combination with MEM, TGC and CT (each antibiotic alone) or with combination of two antibiotics including, MEM+TGC, MEM +CT and TGC+CT against nine MDR *A. baumannii* isolates (coded A31, A35, A20, A8, A3, A25, A11, A26, and A42) representing the 9 clusters (1–9) are shown in Table 4. Of these, 8 isolates (88.8%) were XDR. Results revealed that, CNPs 5 mg/mL, when combined with CT, TGC or MEM, significantly increased the susceptibilities of the MDR *A. baumannii* isolates to these antibiotics by 88.8%, 66.6% and 100%, respectively. Moreover, CNPs (5 mg/mL), when combined with CT+MEM, TGC+MEM, significantly increased the susceptibilities of the MDR *A. baumannii* isolates to these antibiotics by 77.7%, and 44.4%, respectively. No significant effects were observed when CNPs (5 mg/mL) were used in combination with CT +TGC (Table 4).

Discussion

A. baumannii is an opportunistic pathogen of relevant medical importance responsible for the various recalcitrant nosocomial infections worldwide, predominantly in the critically ill patients.[48] *A. baumannii* is a MDR "red alert" pathogen with limited therapeutic options and therefore, impose life threatening conditions.[49] In this study, we aimed to explore the activity of chitosan nanoparticles (CNPs) and to evaluate certain antibiotic combinations for the purpose of combating resistance mediated by this nightmare pathogen. Accordingly, a total of 51 *A. baumannii* clinical isolates were recovered from 730 different discharged clinical specimens including, pus, urine, sputum, bronchial lavage, and cerebrospinal fluid according to the hospital records of the Clinical Microbiology Central Laboratory of AL Kasr Al Aini hospital, Cairo, Egypt. Conventional standard Lab tests were used for identification followed by testing for the *rec*A gene for confirmation. Detection of *A. baumannii* in microbiological laboratories is usually based on both phenotypic and genotypic methods.[10,50] The accuracy, speed, identification, and interpretation of genotypic methods are

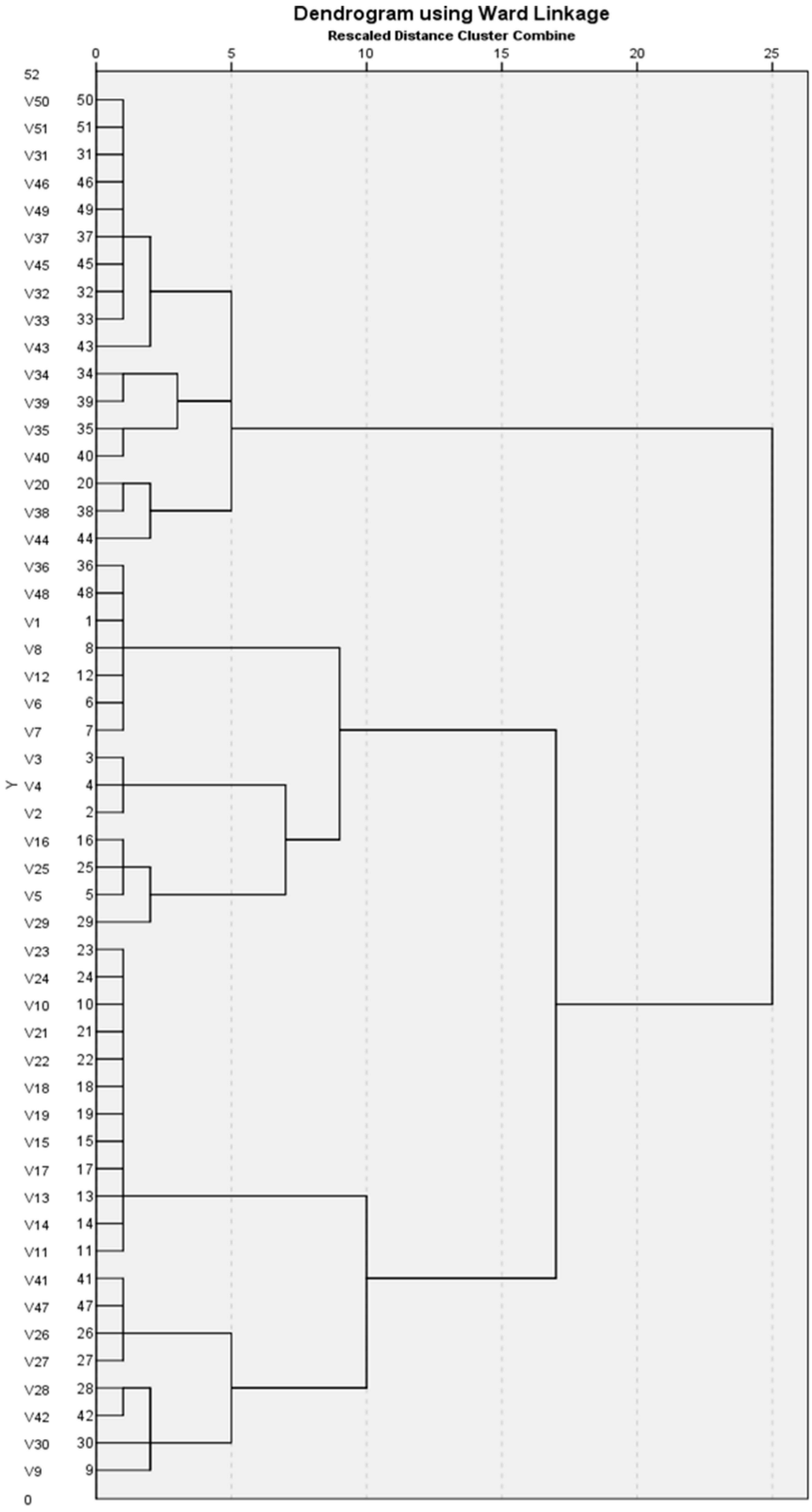

Figure 1 Dendrogram of ERIC-PCR analysis of the 51 isolates of *A. baumannii*; the different clusters at 97% similarity are arbitrarily designated as Clusters 1–9.

Table 1 FICI Values of Two Tested Antibiotic Combinations (CT +MEM, TGC+MEM and TGC+CT) Against Nine Selected MDR *A. baumannii*

Isolate Code	∑FIC					
	CT +MEM	Int.	TGC +MEM	Int.	CT +TGC	Int.
A3	0.257	S	0.515	D	8.0	A
A8	0.253	S	0.503	D	12.0	A
A11	0.257	S	0.265	S	6.0	A
A20	0.257	S	0.503	D	10.0	A
A25	0.253	S	0.503	D	12.0	A
A26	0.503	D	0.315	S	5.3	A
A31	0.507	D	0.503	D	12.0	A
A35	0.257	S	0.257	S	8.0	A
A42	0.128	S	0.257	S	8.0	A

Notes: ∑FIC, Sum of the fractional inhibitory concentration; Int., (interpretation); S, Synergism ≤0.5; D, Additive >0.5 ≥1; I, Indifference >1 and ≤4.0; and antagonistic when ΣFIC is > 4.

Abbreviations: CT, colistin; MEM, meropenem; TGC, tigecycline.

higher than those of phenotypic identification methods.[11] Bacterial genomes containing repeated sequences such as the ERIC sequence which can be used for epidemiological purpose to evaluate similarity between the isolates and their diversity as well as origin.[51,52] In our study, the ERIC-PCR analysis of the recovered 51 MDR *A. baumannii* clinical isolates indicated that they bunched in nine clusters based on the obtained fingerprinting. This indicates that, there was cross-transmission within hospitalized patients. Based on the obtained findings, it can be stated that ERIC-PCR is a reliable method to demonstrate the clonal relatedness among *A. baumannii* recovered from different specimens of different patients.[36,37] The findings of this study are similar to the studies reported by Ying et al and Hammoudi et al who were able to cluster *A. baumannii* strains based on their genetic relatedness and confirmed cross contamination in the clinical settings.[51,53]

Antimicrobial susceptibility tests were carried out on the obtained 51 MDR *A. baumannii* clinical isolates against a panel of antimicrobial agents composed of 14 different antimicrobial agents according to CLSI guidelines, 2018.[34] The rational of antimicrobial selection to be enrolled in this study, was based on the international and empirical guidelines which defined the antimicrobial agents involved in the treatment protocols of *A. baumannii*-associated infections.[1,34] The tested isolates exhibited high percentage of resistance which ranged from 92 to 100% towards cefepime, gentamicin, ceftriaxone, amikacin, imipenem, ciprofloxacin, trimethoprim sulfamethoxazole, ampicillin /sulbactam, doxycycline, piperacillin / tazobactam and meropenem. Results revealed that, all the tested *A. baumannii* clinical isolates were MDR (100%) of these, 92% were XDR. However, they showed lower resistance towards TGC and CT (4% of isolates were resistant). Accordingly, all *A. baumannii* isolates included in our study were MDR and 92% of these were XDR according to international standard definitions for the acquired resistance.[31] The MDR status reported in the current study agrees with the findings of other two recent studies carried in Iraq.[54,55] In this regard, two strategies have been performed to combat the resistance of *A. baumannii* which are the use of antibiotics combinations as well as CNPs either alone or in combination of the antibiotics that showed activities against the respective pathogens according to the results obtained from antimicrobial susceptibility tests. The antibiotic combinations were evaluated by checkerboard microdilution method as previously determined.[38,39] The MICs of the antibiotics in the combinations were significantly reduced as compared to the MICs of each drug alone, and thereby gave synergism. In this study, the CT-MEM combination demonstrated a synergistic effect in 77.7% of the isolates, the findings of this study are like studies reported by other researchers.[56,57] The TGC-MEM combination showed a synergistic and additive effects for 44.4% and 55.6% of the tested isolates, respectively. The findings of this study are like that of a recent study conducted by Li et al who evaluated the addition of TGC in combination with MEM against *A. baumannii* isolates in Heilongjiang Province in China.[18] The CT-TGC combination showed antagonist effect for 100% of the isolates. This finding disagrees with a study carried by Li et al who evaluated the addition of TGC in combination with CT against *A. baumannii* isolates.[18] The variation in the results may be due to differences in the time at which the studies were conducted, as well as differences in the geographic areas or it could be due to physicochemical interaction of the two antibiotics. The second strategy for overcoming MDR resistance that has been evaluated in this study was the use of nanoparticles. CNPs have antimicrobial activity against bacteria, fungi, and viruses. Interestingly, nanoparticles containing low molecular weight chitosan were previously verified to have high activity against Gram negative bacteria than Gram positive bacteria.[25] In general, nanoparticles offer many distinctive advantages such as, reducing acute toxicity, overcoming resistance, and lowering cost, when compared to conventional antibiotics.[58,59] There are different methods used for synthesis and characterization of nanoparticles either alone or in combination with different materials aiming to improve their antimicrobial activity.[60–67] In this study, CNPs were prepared and characterized by DLS (to measure

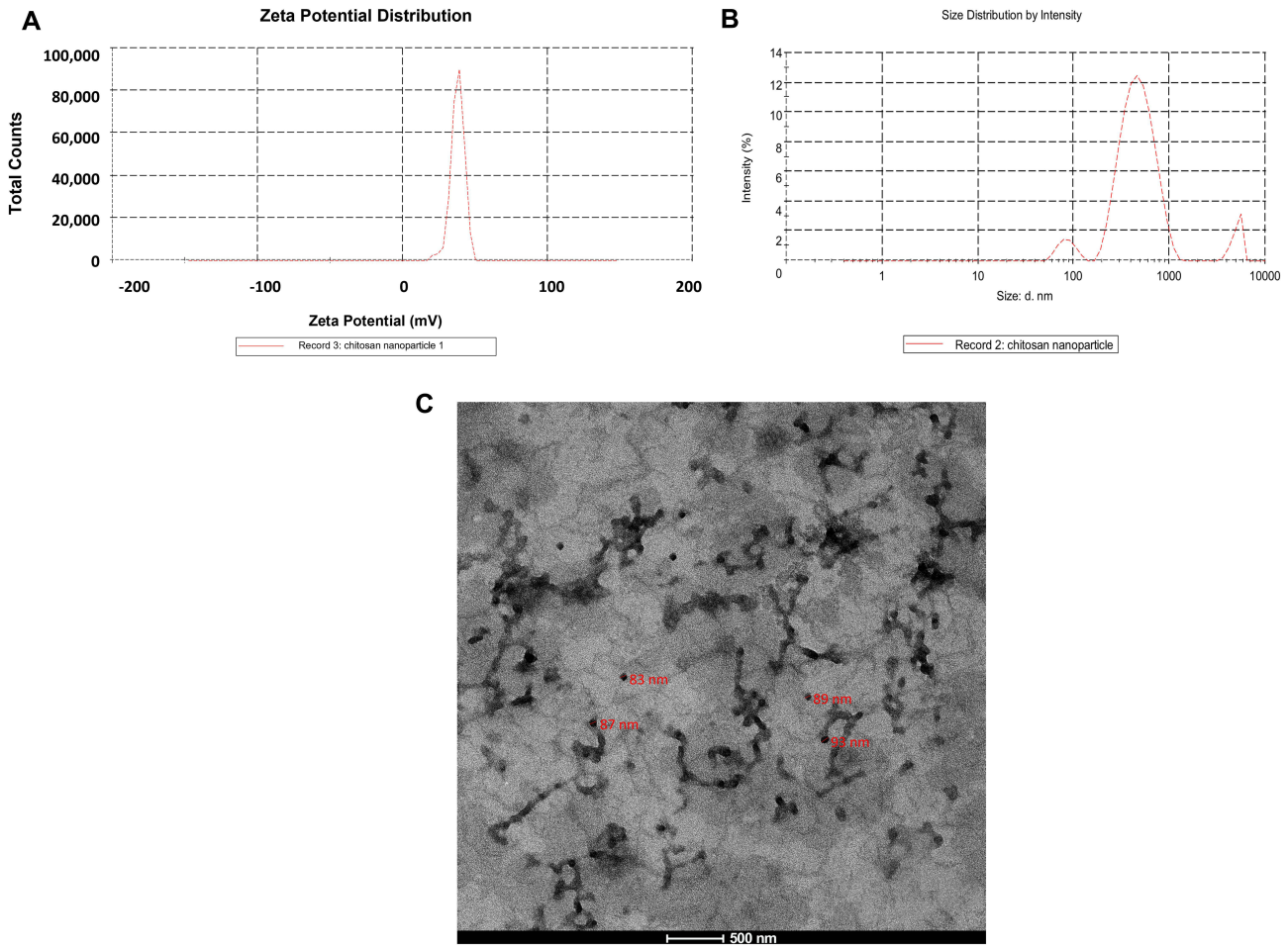

Figure 2 Characterization of chitosan nanoparticles. (**A**) Zeta potential characterization, (Surface net charge) of CNPs (+37.7 mV), (**B**) Zeta Sizer characterization of CNPs (441.7 nm), (**C**) HR-TEM micrograph of CNPs.

hydrodynamic diameter in the nanometer range) and confirmed via imaging the formed CNPs through HR-TEM. The size of CNPs at selected concentration was 441.7 nm and Zeta potential positive 37.7mV which mean that the formed nanoparticles were stable and of smaller particle size with positive charge on the surface of the CNPs indicating high sorption capacity and good antimicrobial properties.[29] The HR-TEM images have shown the morphological properties and surface appearance of nanoparticles. Morphologically, the CNPs prepared were found to be spherical in shape and of smooth surface indicating good properties of the synthesized CNPs as previously reported.[68,69] The CNPs formed in this study exhibited good antimicrobial activities against nine selected MDR *A. baumannii* (representing the nine ERIC-PCR clusters). The MIC of CNPs at concentrations in the range of 1–5 mg/mL, were found to be in the range of 0.16–0.25 mg/mL which were very promising results. The difference in the antimicrobial activities against MDR *A. baumannii* were dependent on the different concentrations of the tested CNPs. Our results are in line with other two previous studies conducted by Cobrado et al[70] and Pourhajibagher et al.[71] Interestingly, Pourhajibagher et al proved that CNPs produced

Table 2 Average of Inhibition Diameter (Mm) ±SD of CNPs Against MDR *A. baumannii* Isolates (n=9)

MDR *A. baumannii* Isolate Code	Average of Inhibition Diameter (mm) ±SD
A3	25± 0.35
A8	30± 0.24
A11	24± 0.26
A20	25± 0.25
A25	25± 0.25
A26	30± 0.25
A31	25± 0.3
A35	30± 0.3
A42	31± 0.25

Table 3 MIC Values CNPs (1, 2.5, 5 mg/ml) Against the MDR *A. baumannii* Isolates (n=51) by Broth Dilution

Concentrations of CNPs (mg/mL)	MIC (mg/mL)	MDR *A. baumannii* Isolate Code
1	0.5	A1, A5, A6, A7, A8, A9, A10, A11, A12, A13, A14, A15, A16, A17, A18, A19, A20, A21, A22, A23, A24, A26, A27, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38, A39, A40, A41, A43, A44, A45, A46, A47, A48, A49, A50, A51
	0.25	A2, A3, A4, A25, A28, A42
2.5	0.31	A1, A2, A3, A5, A6, A7, A9, A10, A12, A13, A14, A15, A16, A17, A19, A20, A21, A22, A24, A25, A26, A27, A28, A30, A31, A32, A33, A34, A35, A36, A37, A38, A39, A41, A42, A44, A45, A47, A48, A49, A50
	0.16	A4, A8, A11, A18, A23, A29, A40, A46, A51
5	0.16	A2, A3, A4, A6, A7, A8, A9, A10, A11, A12, A14, A15, A17, A18, A19, A20, A21, A22, A23, A24, A25, A26, A27, A28, A29, A30, A31, A32, A33, A34, A35, A36, A37, A38, A40, A41, A42, A44, A45, A46, A47, A48, A49, A51
	0.31	A1, A5, A13, A16, A39, A43, A50

Table 4 Effects of CNPs (5 mg/ml) on the MIC of MEM, TGC and CT (Each Alone or in Combinations)

Isolate Code	CT		TGC		MEM		CT+MEM		TGC+MEM		CT+TGC	
	MIC (µg/mL)	MDF	MICs (µg/mL)	MDF	MICs (µg/mL)	MDF	MICs (µg/mL)	MDF	MICs (µg/mL)	MDF	MICs (µg/mL)	MDF
A3	0.5	4	0.5	4	16	4	0.064	4	0.25	2	4	2
A8	0.125	8	0.125	4	16	4	0.064	4	0.25	2	4	3
A11	0.25	4	0.5	4	4	8	0.032	8	0.066	4	3	2
A20	0.25	8	0.125	4	16	4	0.064	4	0.25	2	4	2.5
A25	0.125	8	0.25	2	16	4	0.015	16	0.25	2	4	3
A26	0.125	8	1.0	3	4	16	0.25	2	0.04	8	5.0	1
A31	0.5	2	0.25	2	16	4	0.25	2	0.25	2	4	3
A35	0.25	4	0.125	16	8	8	0.032	8	0.064	4	8	1
A42	0.25	4	0.25	8	4	16	0.032	4	0.032	8	8	1

Abbreviations: MIC, minimum inhibitory concentration; MDF, MIC decrease factor; CT, colistin; MEM, meropenem; TGC, tigecycline.

significant reduction of 93.2% on the viable count of planktonic and of 55.3% on the biofilm formation of *A. baumannii* strains as compared to the control group.[71] Furthermore, the activities of CNPs (5 mg/mL) when combined with CT, TGC or MEM, CT+MEM and TGC+MEM were evaluated via measuring the MDF as previously reported.[48] Based on our findings, the CNPs at a concentration 5 mg/mL gave maximum antibacterial activity against the 51 MDR *A. baumannii* clinical isolates (MIC was 0.16 mg/mL against 44 isolates and 0.31 mg/mL against 7 isolates). This result was in accordance with a previous study conducted in 2020.[40] Accordingly, CNPs at a concentration 5 mg/mL was selected to study their co-effects with above-mentioned antibiotics. Our results showed the respective CNPs-antibiotics combinations significantly increased the susceptibilities of the MDR *A. baumannii* isolates by 88.8, 66.6, 100, 77.7, and 44.4%, respectively. No significant effects were observed when CNPs (5 mg/mL) were combined with CT+TGC. The obtained results were in accordance with many recent studies conducted in 2021 on the antimicrobial activities of different nanoparticles either alone or in combination with other antimicrobials have proved activity against various pathogens. These included, Casein-silver NPs combined with TGC against *A. baumannii*,[72] antibiofilm and anti-virulence potential of silver NPs against MDR *A. baumannii*,[73] Cu:Ag bimetallic NPs for antibiotic-resistant bacteria,[74] Lignin-Capped silver NPs,[75] Smaller Copper Oxide Nanoparticles against MDR Bacteria,[76] colistin-integrated chitosan nanoparticles.[77] Our results revealed that CNPs when combined with MEM significantly increased the susceptibilities of the MDR *A. baumannii* isolates by 100% as

compared to MEM alone. Our result is supported by the findings of another study that revealed, meropenem-loaded CNPs exhibited both in vitro and in vivo activities against a wide range of Gram positive and Gram negative MDR pathogens with a great potential for overcoming antimicrobial resistance.[78] The encapsulation of CNPs plus antibiotics as well as their in vivo evaluations will be an important our prospective work.

Conclusion

The current study demonstrated the significant in vitro activities of CNPs either alone or in combination with CT, TGC, MEM, CT+MEM and TGC+MEM antibiotics. Combinations of CT+MEM and MEM+TGC showed synergism in 77.7% and 44.4% and additive effects in 22.3 and 55.6% of the tested MDR *A. baumannii* isolates (n=51), respectively. CNPs (5 mg/mL) exhibited good inhibitory activities (MIC was from 0.16 to 0.31 mg/mL) against nine MDR *A. baumannii* isolates that were selected according to the results of the ERIC-PCR. CNPs (5 mg/mL) when combined with CT, TGC or MEM, CT+MEM and TGC+MEM significantly increased the susceptibilities of the MDR *A. baumannii* isolates by 88.8%, 66.6%, 100%, 77.7%, and 44.4%, respectively. However, no significant effects were observed when CNPs (5 mg/mL) were combined with CT+TGC. The obtained finding will guide the physicians for the management of MDR *A. baumannii*-associated infections. However, further in vivo studies should be conducted to verify such activities and their potential use in human.

Data Sharing Statement

All the data supporting the findings are included in the manuscript.

Ethical Clearance

The study protocol was reviewed and approved by the institutional ethics committee, Faculty of Pharmacy, Ain Shams University (ENREC-ASU-2019-272). This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki.

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Disclosure

The authors declare that they have no competing interests in this work.

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