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Research Paper

Synthesis, Characterization and Effect of the Antibacterial Activity of Chitosan Nanoparticles on Vancomycin-Resistant *Enterococcus* and Other Gram Negative or Gram Positive Bacteria

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Abstract: *Chitosan, a non-toxic and biocompatible natural polymer has been used as an absorption enhancer for poorly absorbable drugs. Biodegradable polymers such as chitosan have been used extensively in biomedical fields in the form of sutures, wound covering and as artificial skin and antibiotic delivery for infection disease. The aims of research is Synthesis, characterization and effect of the antibacterial activity of chitosan nanoparticles on Vancomycin-Resistant Enterococcus and other gram negative or gram positive Bacteria. Chitosan was synthesized. Then chitosan nanoparticle is characterized. Size and zeta potential of nanoparticle was determined by DLS and SEM microscope method. The antibacterial activity of chitosan nanoparticle derivatives prepared on Vancomycin-Resistant Enterococcus, Staphylococcus aureus and Escherichia coli was examined by well diffusion and micro titer method according CLSI. Size and zeta potential of nanoparticle were 210 nm and + 11 mv by DLS. Chitosan nanoparticle has antibacterial activity on bacteria. According to well diffusion test, the highest inhibition zone was recorded against Staphylococcus aureus, lower diameter of inhibition zone was showed against Enterococcus faecalis and Escherichia coli, respectively. MIC and MBC results were showed the highest inhibitory of chitosan nanoparticles against Staphylococcus aureus. This nanoparticle can be used antibacterial agent. It can affect synergism with antibiotics. Chitosan nanoparticle will apply to pharmacology for drug delivery and caring.*

Keywords: Chitosan, Nanoparticle, Antibacterial Activity, Drug Resistant.

Introduction

Gram-positive organisms, including *S. aureus* and *Enterococcus*, were among the top 10 most frequently isolated organisms based on the recent update from the National Healthcare Safety Network (NHSN) surveillance of antimicrobial-resistant pathogens associated with healthcare-associated infections reports. *Enterococcus* sp., the fermentative Gram-positive cocci, are part of human and animal intestine normal flora (1).However, with gaining resistance to different clinical antibiotics such as vancomycin (with prevalence of 12%), they have received attention (2).They are regarded as well as the second common nosocomial infections cause of endocarditis and a variety of serious infections caused by Gram-positive bacteria, such as enterococci, methicillin-resistant *Staphylococcus aureus*(i.e. MRSA) and *Clostridium difficile*, since 1970. These organisms were found to be among the most commonly isolated organisms in hospitals. Vancomycin has been used heavily in the last two decades, resulting in the emergence of enterococci resistant to glycopeptide antibiotics (3,4).The most important vancomycin resistant genes of *Enterococcus* sp., include *vanA, vanB. vanA* and *vanB* genes are located on transposones*Tn1546* and *Tn1547*either on plasmids or chromosomes. Identification and Characterization of effective compounds on antibiotic resistant genes of isolates appear necessary (5, 6, 7). Chitosan is a polysaccharide that is found in many crustaceans shells. Chitosan has been selected as a carrier for drug delivery especially macromolecules due to its physical and chemical properties. From a technical point of view, positive charge and solubility of chitosan in water is very important (8). These factors can cause interactions between chitosan and negatively charged surfaces in aqueous environment or with charged membrane of microorganisms, moreover, the interactions are gaining the attention in then nanotechnology field. Chitosan is a non-toxic, biocompatible and biodegradable compound that is approved with food and drug administration (FDA). This compound has received considerable attention according to its minimal systemic toxicity and has been used to deliver peptide or drug in applied sciences (9). In the past decade, the advent and spread of antibiotic-resistant strains has become a major concern, in addition, this increase as an important problem in medicine has threatened public health (10). Today the uncontrolled spread of antibiotic resistance in bacterial strains is a major challenge in Community (4, 7). Based on these problems, production and presentation of antimicrobial compounds have been provided. As chitosan is a compatible compound with human body, studies on its antimicrobial properties provide a useful field in order to replace the results of this investigation to control and treat bacterial infections due to applications listed. The aim of this study was to synthesize, characterize and effect of the Pharmaceutical activity of chitosan on vancomycin resistant *Enterococcus* and other on gram negative or gram positive bacteria.

Experimental

1. Synthesis of Chitosan Nanoparticles

Chitosan was purchased from Sigma-Aldrich. Chitosan nanoparticles solution were obtained upon the addition of Tripolyphosphate (TPP) aqueous solution (1mg/ml) to chitosan solution (2mg/ml) stirred at room temperature (rate of 300 rpm) (11).

2. Characterize of Nanoparticles

2.1 Electrical Charge and Size of Nanoparticles

Particle size, electrical distribution, uniformity and the zeta potential of chitosan nanoparticles were determined using Dynamic light scattering (DLS) or Zeta sizer instrument (Malvern). Zeta potential,

that is, surface charge, can greatly influence particle stability in suspension through electro static repulsion between particles and with a wave length of 633 nm.

2.2 Transmission Electron Microscope (TEM)

Size, shape, distribution and uniformity of chitosan nanoparticles were determined by Electron Microscope.

3. Bacterial Strains and Cultivation

Staphylococcus aureus ATCC 25923, *Escherichia coli* ATCC 25922, *Enterococcus faecalis* ATCC 51229 and *Pseudomonas aeruginosa* PAO1 were obtained from Reference Laboratory in Tarbiat Modares University, in Tehran- Iran. Clinical strains of *Enterococcus faecalis* and *facum* harboring *VanA* and *VanB* genes were used for antimicrobial tests.

4. Extraction of DNA from Clinical Isolates of *Enterococcus*

Genomic DNA of clinical Enterococcus and standard strains was obtained using the genomic DNA Extraction Kit (China Gen, Iran) following the manufacturer's instructions. The PCR Reaction for *VanA* and *VanB* genes was performed. Standard strain of *Enterococcus faecalis* (ATCC 51229) was used for reaction.

5. Determination of Antibacterial Activity of Chitosan nanoparticles against Experimental Strain

5.1. Susceptibility to Chitosan Nanoparticles by Using Well Diffusion Test

Antibacterial activity of chitosan nanoparticles was determined by using two methods: well diffusion test by measuring inhibitory zones and broth dilution by measuring minimum inhibitory concentration (12).

In well diffusion test, wells are punched on the surface of plates that was Inoculated with bacteria and 50 µl of chitosan nanoparticles were injected into the wells. The plates were incubated for 18 hours at 37° C. Diameter of inhibitory zones was determined in mm. An injected well with 50 ml of normal saline was used as negative control. Gentamicin and Oxacillin (MAST Co. UK) were used as positive control drugs. In this study all experiments were done in triplicate.

5.2. Minimum Inhibitory Concentration (MIC)

To determine the minimum inhibitory concentration of chitosan nanoparticles against bacterial strains micro broth dilution method was used (12). 100 µl of Muller Hinton broth (Merck, Germany) was added to each ELISA microplate. After preparation of chitosan nanoparticles, 100 µl of a 1:2 dilution were added to the first well and after mixing, 100 µl of the mixture were transferred to the second tube and the similar transformations were repeated. Then, 10 µl of each bacterial suspension (10^6cfu/ml) was added to the separate tubes. Control tube was placed without chitosan nanoparticles and bacteria. The microplates were incubated for 18 hours at 37°C. MIC (minimum inhibitory concentration) was defined as the lowest concentration at the end of18 hours of incubation for bacteria growth.

5.3. Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal (MBC), 10 µl of bacterial suspension in each well was cultured on Muller Hinton agar (Merck, Germany) and to investigate the growth of bacterial plates were incubated for 18 hours. Low concentration of the aqueous and ethanol, in which 99.9% of the bacteria did grow was considered as MBC (12). All experiments were done in triplicate.

6. Results

6.1. Nanoparticles Size

Size, dispersion and uniformity of chitosan nanoparticles were determined by DLS (Malvern). Size of chitosan nanoparticles with an average of 210 nm was showed with three replications (Figure1). DLS data was showed that more than 95% of samples were in the range of 210 nm and this peak was greater than about 5% of the rest in the range of 20 to 70 nm. The average peak was around 45nm.

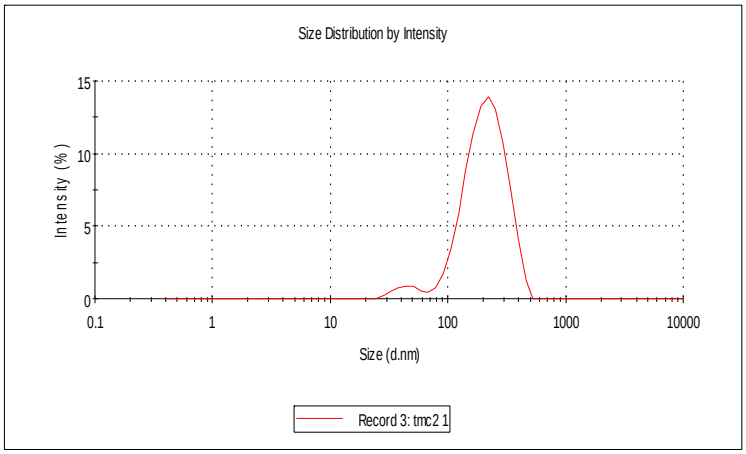

Figure 1: The size of chitosan nanoparticles by DLS (nm)

6.2. Electrical Charge of Nanoparticles:

Electrical charge, distribution and uniformity of chitosan nanoparticles were determined by Zeta sizer. Figure 2 was showed electrical charge of chitosan nanoparticles.

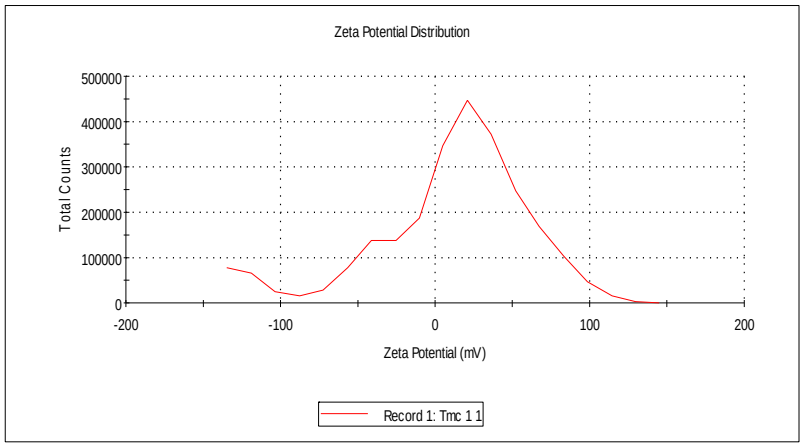

Figure 2: Electrical charge of chitosan nanoparticles by Zeta sizer (mV)

6.3. Transmission Electron Microscopy

Size, shape, uniformity and distribution of chitosan nanoparticles were determined by transmission electron microscopy and zeta sizer. TEM image of nanoparticles was showed figure 3. The size of particles ranges from 100 to150 nm was obtained.

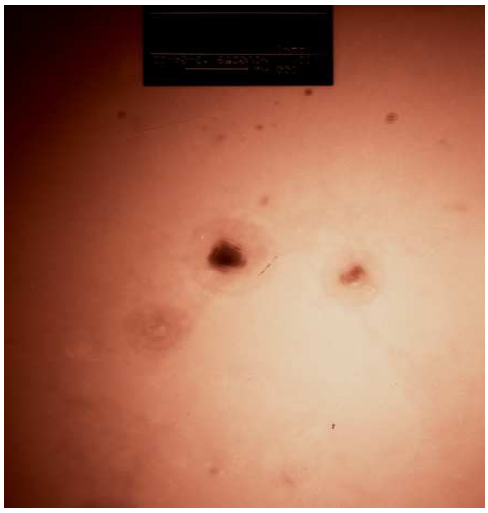

Figure 3: Transmission electron microscopy (TEM) image of chitosan nanoparticles

6.4. Identification of *Van A* and *Van B* Genes in Clinical Isolates

Van A and Van B genes were shown in clinical isolates (Fig.4). Fragment length for Van A and Van B product genes in presence of 100bp marker was around 737bp and 420bp, respectively.

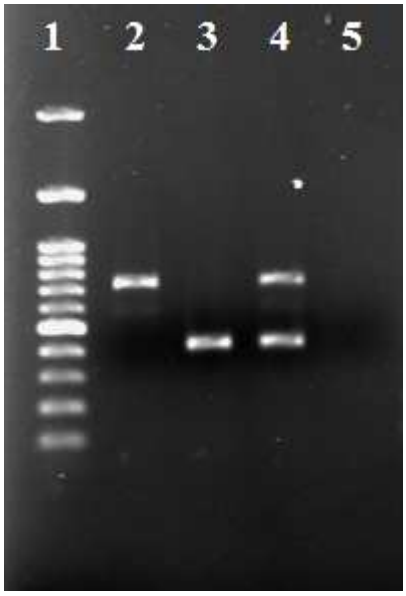

Figure 4: Specific PCR amplification products of the *van A* and *Van B* genes of *Enterococcus sp.* clinical isolate Lane 1: marker 100bp; Lane2: *van* A-positive control, clinical isolate; Lane 3: *van* B-positive control, clinical isolate; Lane 4: *van* A and *van* B-positive control, clinical isolate; Lane 5: negative control

6.5. The Results of Antibacterial Activity of Chitosan nanoparticles against the Experimental Strains

After the period of time the culture of microorganisms were examined and results were recorded. Some examples of nanoparticles that were injected into the reaction medium did not show any response.

Some of them showed small and the others indicated large diameter of inhibition zones. Antibacterial test results were given in Table1. According to well diffusion test, the highest inhibition zone was recorded against *Staphylococcus aureus* ATCC 25923 with 21 diameter (figure4) while, lower diameter of inhibition zone was showed against *Enterococcus faecalis* ATCC 51229 and *Escherichia coli* ATCC 25922, respectively. The lowest inhibition zone was recorded against *Pseudomonas aeruginosa.*

Table 1: Agar diffusion test results: MIC (μg/ml) and MBC (μg/ml) of Chitosan against different control strains of Bacteria

Inhibition Zone Diameter (mm) Inhibitory and bactericidal effects
Agar Broth

Bacteria	chitosan nanoparticles	Positive control		MIC (mg/ml)	MBC (mg/ml)
		*Gentamicn	*Oxacillin		
Enterococcus Faecalis	18±0.4	0	0	0.03	-
Escherichia coli	19±0.5	21±0.3	14±0.8	0.07	0.15
Staphylococcsaureus	21±0.3	12±0.8	22±0.9	0.03	0.07
Pseudomonas aeruginosa	12±0.4	15	0	-	-
Enterococcus Clinical isolate No.1:*Van*A-positive	14±0.2	0	0	-	-
Enterococcus Clinical isolate No.2: *Van*A-positive	15±0.6	0	0	-	-
Enterococcus Clinical isolate No.3: *Van*A-positive	14±0.4	0	0	-	-
Enterococcus Clinical isolate No.1: *Van*B-positive	14±0.3	0	0	-	-
Enterococcus Clinical isolate No.2: *Van*B-positive	14±0.3	0	0	-	-
Enterococcus	13±0.4	0	0	-	-

Clinical isolate No.3: *Van*B-positive *Enterococcus* Clinical isolate No.1: *Van*Aand *Van*B-positive	13±0.4	0	0	-	-

*stock dilution (0.77mg/ml), Gentamicin (10µg), Oxacillin (1 µg).

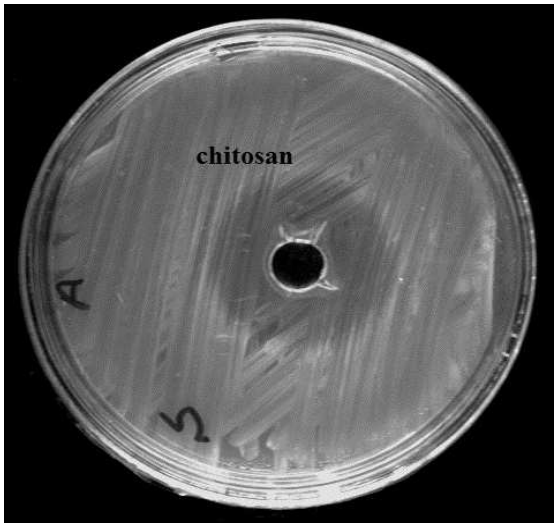

Figure 5: Well diffusion test against control *Staphylococcus aureus*

MIC and MBC results were showed the highest inhibitory of chitosan nanoparticles against *Staphylococcus aureus*. Different concentrations of MIC were undertaken on different strains. Over 98% of the bacteria were killed at the concentration of 0.38 mg/ml. The highest inhibitory effect was showed against *Staphylococcus aureus* at dilution of 0.5 (0.03 mg/ml concentration) (Fig 6). The lowest inhibitory effect was showed against *p. aeruginosa.* The inhibitory effect on vancomycin resistant enterococcus spp. bacteria was showed moderate.

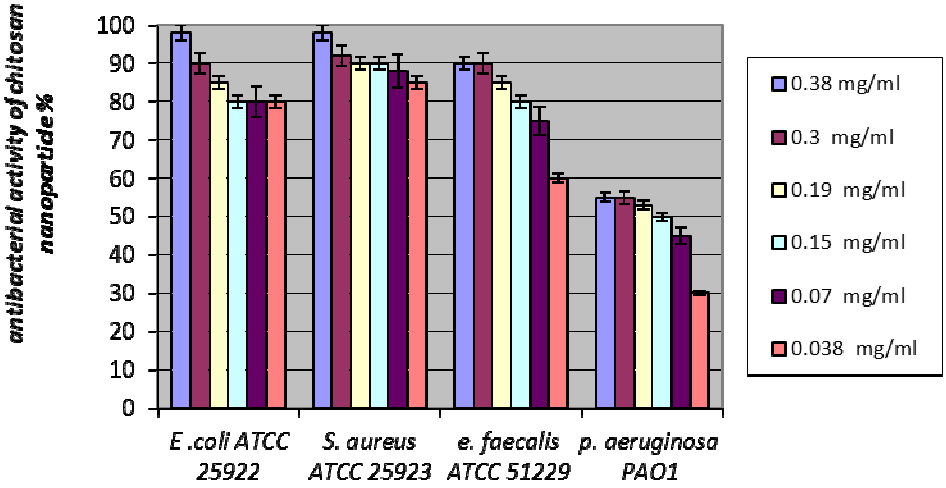

Figure 6: Antibacterial effect of different concentrations of chitosan nanoparticle against standard bacteria of *E. coli, S. aureus* and *E. faecalis* for 18h at 37 °C. The initial cell number was 10^6CFU/mL. Each value was expressed as mean ± SD (n = 4).

7. Discussion

Antibiotic resistance is present in bacteria and this resistance can be transmitted from one species to another (20). Vancomycin resistant enterococci (VRE) is characterized as one of the main causes of nosocomial infections in severe and Immunodeficiency patients (21). Furthermore, resistant enterococci in the human society acts as a source of nosocomial infections (13). Chitosan with excellent biological and physicochemical characteristics has received attention as a drug delivery vehicle (19). In recent years, with the advent of multidrug-resistant organisms, Searching for safe and effective antimicrobial agents in order to prevent and treat a variety of bacterial infections has been more explicit (14). Researchers are now looking for useful compounds which can increase effectiveness of antimicrobial agents. In this research, chitosan nanoparticles were synthesized. Size of them was verified by DLS and transmission electron microscopy then the results of these methods were confirmed with each other. The results were indicated that the diameter of particle was around 200±10nm. Various effects of chitosan nanoparticles have been reported against different organisms. Caner et al. were showed that chitosan polymer was effective against *Bacillus subtilis* and *Pseudomonas aeruginosa*, which the inhibitory zone was 17and 14 mm, respectively. Saifi *et al*. showed that from a total of 638 enterococcal isolates that were collected from urine samples in Iran, multi-drug resistant (MDR) to most prevalent antimicrobials was present in 29% and 72% of the *Enterococcus faecalis* and *Enterococcus faecium* isolates, respectively (15). Zhanel *et al*. Studied about antimicrobial-resistant pathogens in intensive care units in Canada during 2005 to 2006. They were showed that vancomycin-resistant enterococci (VRE) made up 6.7% (11/255) of all enterococcal isolates and 88.2% of VRE had the vanA genotype (16). The prevalence of vancomycin resistant enterococci (VRE) was 12 % consisting of *Enterococcus faecalis* (6 %) and Enterococcus faecium (22%) which was investigated by Emaneini *et al*. All of them were vanA phenotype (17). In this study, the antibacterial activity of chitosan nanoparticles was investigated against strains of standard strain and clinical isolate of *Enterococcus faecalis*, standard strain *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. The results were showed an excellent antibacterial activity of these nanoparticles against experimental strains. Standard strain of *Staphylococcus aureus* was showed the highest sensitivity toward nanoparticles. Chitosan with a wide range of antimicrobial activity was showed a different inhibitory efficiency against Gram positive and Gram negative bacteria, and fungi. Antibacterial activity was a complex stage between Gram positive and Gram negative bacteria due to different characteristics of their cell surfaces. It has been reported that the antibacterial effect of chitosan on gram positive bacteria is higher than gram negative ones (1). This is probably due to the outer membrane of gram negative bacteria (2) and in present study, this hypothesis was confirmed by *Staphylococcus aureus* (1, 2) Then this effect was found in *Enterococcus faecalis* and *Escherichia coli.* MIC and MBC tests were also showed that then a no particles had an inhibitory effect against *Staphylococcus aureus* even at 1.10 (50µg/ml) dilution. Also a significant inhibitory effect was observed against *Escherichia coli* and *Enterococcus faecalis* at the other dilutions. Two hypotheses for the antimicrobial action have been postulated. There are as follows:

(1) In *S. aureus*, perhaps the chitosan nanoparticle with positive charge can interact with the cell surface or essential nutrients so as to inhibit the growth of bacteria or can interfere with anionic channels.

(2) In *E. coli*, low molecular weight chitosan can enter the cell through pervasion or interfere with anionic channels.

8. Conclusion

As, bacterial resistance has been increased and resistant factor has been transmitted easily from resistant bacteria into sensitive one, so use of polymer such as chitosan to deliver drugs to an appropriate location in biological systems has a lot of interests. Chitosan was degraded over time in biological systems, but the destruction range of chitosan is adjustable by controlling the degree of

diacetylation. This feature helps drug release into the body in a controlled way and increases its influence. The unique properties of Chitosan nanoparticles encouraged its use as a carrier to deliver therapeutic drug such as antimicrobial drugs.

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