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Evaluation of antibacterial efficiency of chitosan nanoparticles on *Salmonella bongori*

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ABSTRACT

Salmonella bongori, which belongs to the genus of *Salmonella*, is a gram-negative, rod-shaped bacterium, typically considered a microbe of cold-blooded animals and causes salmonellosis in human being characterized by cramping and diarrhea. These bacteria become resistant to antibiotics and make some problems in the treatment. Researches needed to find a more appropriate and accurate way to get help from chitosan nanoparticle technology. Therefore, the aim of this study was to evaluate the antibacterial effect of chitosan nanoparticles against *Salmonella bongori*. After culture and preparation of *Salmonella bongori*, ionic gelation method was used to produce chitosan nanoparticles. Hole plate and tube dilution methods were used in qualitative determination of antimicrobial activities against *Salmonella bongori*. Finally, zeta's analysis assay, dynamic optical scanning, and electron microscopy performed to evaluate nanoparticles. Low molecular weight chitosan nanoparticles were produced by analyzing the results of optical dynamics scattering (111.7 nm), zeta analysis (20.8 mV) and microscopy (<200 nm). The diameter of the non-growth halo at different concentrations revealed chitosan and antibiotic nanoparticles had a high and effective effect against bacteria. According to the results, there was a significant relationship between the chitosan nanoparticles resistance and antibiotics against bacteria. On the other hand, the nanoparticles antibacterial properties were higher than antibiotics, which can be deduced from chitosan nanoparticles for controlling diseases and destroying resistant bacterial species.

Key words: *Salmonella bongori*; chitosan nanoparticles; antibiotic resistance

Introduction

Salmonella serovars are predominately pathogenic *Enterobacteriaceae* that are considered to have differed from a common ancestor with Escherichia coli, 100 million years ago (Doolittle et al., 1996). *Salmonella* is the most frequent reason for infectious illness in the world. There are more than 2500 serovars of *Salmonella* and all are potential pathogens (Ravishankar et al., 2010). The genus *Salmonella* currently two classes; *S. bongori* and *S. enterica* (Kauffmann, 1966; Le Minor et al., n.d.; Ryan et al., 2017). *S. bongori* have been described to infect humans (Giammanco et al., 2002), the species is predominantly correlated with cold-blooded animals whereas serovars causing disease in humans and other warm-blooded animals mostly belong to *S. enterica* subspecies *enterica*. Compared to *E. coli*, many genes, which are unique to *Salmonella* serovars, are found on large discrete genomic islands that constitute prophage elements and specialized SPIs (*Salmonella* Pathogenicity Islands) (Schmidt & Hensel, 2004). These *Salmonella*-specific roles involve many genes needed for the full expression of virulence and some of these were obtained by *S. enterica* following the split from *S. bongori*. Several features of *S. bongori* recommend that this species may lie someplace between E. coli and *S. enterica*. For instance, *S. enterica* encodes two complete type III secretion systems encoded by SPI-1 and SPI-2 (Cirillo et al., 1998; Hensel et al., 1998), whereas *S. bongori* lacks SPI-2, which is required for optimal replication within macrophages (Gog et al., 2012). *S. bongori*, formerly separated from an African frog in 1972 and includes 21 representatives of the 23 known serovars. The data of scientific researches reveal that S. bongori possesses only a basic set of ancestral *Salmonella* virulence functions and lacks various metabolic pathways that determine *S. enterica*. Although, *S. bongori* has not remained

functionally static, however, it has acquired a stock of 12 T3SS candidate effector proteins, 10 of which are not found in other salmonellae but are significantly similar to known effectors found in enteropathogenic *Escherichia coli* (EPEC) strains (Fookes et al., 2011).

Nanoparticles are small particle size with positive surface charges, improving their durability in the presence of biological cations and their antibacterial activities because of the interaction with negatively charged biological membranes and site-specific targeting in vivo (Qi et al., 2004). Nanoparticulate systems that enhance the selectivity of antibiotics in phagocytic cells have been studied elsewhere (Briones et al., 2008). It was found that chitosan nanoparticles could restrain the growth of different bacteria examined. Chitosan nanoparticles were newly used as a vector for aminoglycosides (Lu et al., 2009). Chitosan is a polycationic polymer with more than 5000 glucosamine units. This biopolymer is collected from the chitin (N-acetyl-D-glucosamine polymer) recovery of shrimp and crab shells using alkaline deacetylation (López-Cervantes et al., 2007). Chitosan is a safe, biodegradable and biocompatible composite with antimicrobial features. The published studies on the antimicrobial activity involve particularly potent effects against bacteria (*Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*) and fungi (*Piricularia oryzae*, *Botrytis cinerea*, *Fusarium oxysporum*) (Bento et al., 2009; Dutta et al., 2009). However, the antimicrobial activity of chitosan depends on numerous factors, such as the pH of the medium, the temperature and the presence of different food components, and the type of chitosan (deacetylation degree, molecular weight) (Devlieghere et al., 2004). Three mechanisms have been suggested as an elaboration of the antimicrobial characteristics of chitosan. I) change in the cell permeability because of interaction between the polycationic chitosan and the electronegative charges on the cell surface (Martínez-Camacho et al., 2010). II) Chitosan plays as a chelating agent that selectively joins crucial metals and nutrients inhibiting the microbial growth (Wu et al., 2018). III) Chitosan, of low molecular weight, interacts with DNA and interferes with the mRNA synthesis (Rabea et al., 2003).

To the best of our knowledge, the utility of chitosan nanoparticles against *S. bongori* has not yet been reported and therefore is highly warranted. The aim of this study was to examine the antibacterial effect of chitosan nanoparticles against *S. bongori*.

Materials and Methods

Culture and preparation of S. bongori

The antibacterial activity of the chitosan nanoparticles was assessed against *S. bongori* (ATCC® 43975™) bacteria which was obtained from the Iranian Research Organization for Science and Technology (IROST). This microorganism was grown in Tryptic Soy Broth (TSB) at 37 °C in an atmosphere including 5% CO_2. Next, growth was verified using Gram staining, catalase test, optochin, and bacitracin tests. The bacteria were stored at -80°C until used in the study.

Preparation of chitosan nanoparticles

Chitosan was obtained from the Pasteur Institute of Iran (Tehran, Iran). In the present work, the chitosan nanoparticles were synthesized from the chitosan using sodium tripolyphosphate as a crosslinking agent by ionotropic gelation technique. Originally, in order to create the comparable chitosan solution, about 1.5 g of chitosan suspended in 200 mL of 2% acetic acid solution was kept under magnetic stirring process for about 20 min. Then to the above-prepared chitosan solution, 0.8 g of sodium tripolyphosphate dissolved in 107 mL of conductivity water was added dropwise and mixed well for about 30 min to reach stability. A milky colored emulsion like the appearance of chitosan nanoparticles was formed upon the ionic cross-linking between the sodium tripolyphosphate and chitosan solution. After reaching stability, the suspension was formed in above-mentioned conditions. The nanoparticles were separated by centrifugation at 20 000 g and 14°C for 30 min, freeze-dried and stored at 5 ± 3°C.

Electron microscopy analysis

After the preparation of the synthesized chitosan nanoparticles, the characterization of the nanoparticle was analyzed by scanning electron microscope (SEM). The particle size and morphology of synthesized nanomaterials were determined using a field emission scanning electron microscope (FE-SEM, 15 kV, model 54160, Hitachi, Japan).

Hole diffusion method

This assay was conducted using a suspension with 0.5 McFarland standard turbidity. Holes of 6 mm diameter were then made on the Mueller Hinton agar (Merck) plate (8 mm thick) inoculated by flooding and filled with 50 µL of chitosan nanoparticles and antibiotics mixture at concentrations of 0.25, 0.5, 1, 2, and 4 mg/mL. The plates containing the *S. bongori* and mentioned mixture were incubated at 37°C for 24 and. The antimicrobial activity was assessed by measuring the inhibition zone (IZ) near each hole.

Assays for antibacterial activity

The minimum inhibitory concentration (MIC) of chitosan nanoparticles was determined by a turbidimetric method. In this method, a number of test tubes each carrying 5.0 mL of Muller–Hinton broth (MHB, Difco, England) were autoclaved for 15 min at 121°C. Chitosan nanoparticles powder was carefully quantified and added to distilled water. The pH of the samples in suspension in distilled water was about 6.5. To the first tube, 5.0 mL of chitosan nanoparticles (1 mg/mL) suspension was added. After mixing, 5.0 mL of the mixture

was transferred to the second tube, and similar transformations were repeated. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The tubes were inoculated under aseptic conditions with 50 µL of the freshly prepared bacteria suspension. The positive control was given with doxycycline, and the blank control tubes were only contained Muller– Hinton broth. After mixing, the tubes were incubated at 37°C for 24h. The tubes were then analyzed for the obvious signs of growth or turbidity. The lowest concentration of chitosan nanoparticles that inhibited the growth of bacteria was considered as the minimum inhibitory concentration or MIC.

The minimum bactericidal concentration (MBC), or the lowest concentration of chitosan nanoparticles that destroys 99.9% of bacteria, was determined by examining the live organisms in those tubes from the MIC test that revealed no growth 20. A loopful from each of those tubes were inoculated on EMB (Eosin–Methylene Blue) agar and checked for signs of growth. The growth of bacteria confirms the presence of these bacteria in the original tube. On the contrary, if no growth was recognized, the original tube contained no living bacteria, and the chitosan nanoparticles were considered as being bactericidal at that concentration.

Statistical Analysis

All analyses were replicated three times and data were displayed as the mean ± standard deviation. Statistical analysis was conducted with the analysis of variance (ANOVA) procedure using Graf Pad Prism 7 software (GraphPad Software Inc., CA, USA). The differences among the experimental data were determined using multiple comparisons of individual means by pairwise t-tests. A value of $P < 0.05$ was considered statistically significant.

Results

The size of chitosan nanoparticles (low molecular weight) was obtained using an electron microscope less than 200 nm. The average size of the nanoparticles was determined 111.1 nm by using dynamic light scattering (DLS) (Figure 1). Furthermore, the dispensary index of chitosan nanoparticles was determined by Zeta analysis (20.8 mV) which is shown in Figure 2.

Table 1 shows the antimicrobial activity of chitosan nanoparticles (0.25, 0.5, 1, 2, and 4 mg/mL) in hole plate method.

The MIC exhibited by chitosan nanoparticles against *S. bongori* are given in Table 2. Obtained results revealed that chitosan nanoparticles are generally more active on *S. bongori* had higher MIC values (99% death).

Figure 1. *The size of chitosan nanoparticles with low molecular weight.*

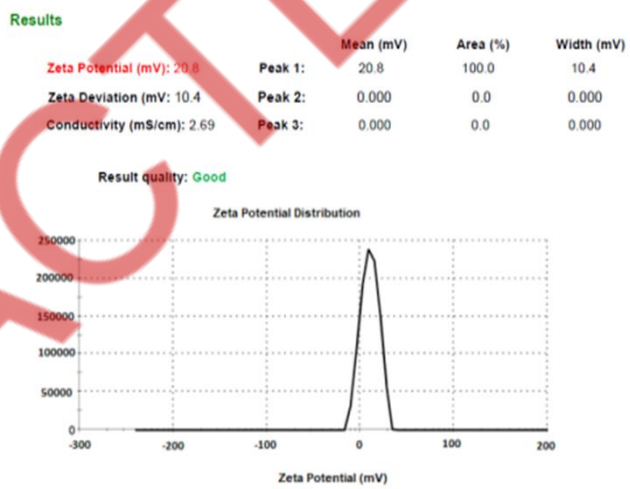

Figure 2. *The zeta potential of chitosan nanoparticles with low molecular weight.*

Discussion

Salmonellosis is gastroenteritis caused by the infection with different serotypes of *Salmonella* bacteria. In recent years, different types of *Salmonella* have become more and more resistant to commonly used antibiotics. Despite significant advances in health and control of the food chain, *Salmonella* transmission continues to affect communities around the world. In 2007, the annual incidence of salmonellosis was 14.9 per 100,000 population. Salmonellosis and typhoid fever are increasingly associated with travel to developing countries (currently 72% of about 400 cases a year) (Chen et al., 2013). Infection sources include India (30%), Pakistan (13%), Mexico (12%), Bangladesh (8%), Philippines (8%), and Haiti (5%). The incidence of *Salmonella* infection was high in South and Central Asia, Southeast Asia, and possibly in South Africa (100 per 100,000 population per year).

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Table 1. *The diameter of the inhibition zone in different concentrations of chitosan nanoparticles and antibiotics*.

Concentration	Average Hole Diameter ± SEM (mm)				
	Chitosan nanoparticles	Gentamicin Antibiotic	Streptomycin Antibiotic	Nanoparticles with Gentamicin Antibiotic	Nanoparticles with Streptomycin Antibiotic
0.25	7 ± 0.2	6 ± 0.2	5 ± 0.1	9 ± 0.2	8 ± 0.2
0.5	9 ± 0.2	8 ± 0.3	7 ± 0.2	12 ± 0.2	11 ± 0.2
1	12 ± 0.3	11 ± 0.2	10 ± 0.2	16 ± 0.2	14 ± 0.1
2	17 ± 0.1	13 ± 0.3	12 ± 0.3	19 ± 0.1	18 ± 0.1
4	19 ± 0.3	16 ± 0.3	14 ± 0.3	23 ± 0.1	20 ± 0.1

Table 2. *The Minimum Inhibitory Concentration (MIC) and the Minimum Bactericidal Concentration (MBC).*

Material	MIC (mg/mL)	MBC (mg/mL)
Chitosan nanoparticles	0.5	1.5
Gentamicin antibiotic	1.5	2.5
Streptomycin antibiotic	1	3

The rest of Asia, Africa, Latin America and Oceania (except Australia and New Zealand) typically had a mean fever rate (10-100 per 100,000 population), while the incidence in other parts of the world was low (10 cases Per 100,000 population) (Bhan et al., 2005; Crump et al., 2004; Linam & Gerber, 2007).

Chitosan is a natural polymer with specific characteristics, including antimicrobial activity, non-toxicity, and biodegradability, which has attracted great attention for years (Rodríguez-Núñez et al., 2012). Chitosan nanoparticles have smaller sizes than chitosan and this property could make it unique (Chávez de Paz et al., 2011; Du et al., 2009). Pioneer studies have described the antimicrobial potential of chitin, and chitosan during 1980-1990s (Goy et al., 2009). Most studies have justified that chitosan can be used in active food packaging as an antimicrobial agent (Ye et al., 2008). Some studies have shown that chitosan has anti-adhesive properties and antibacterial actions against streptococci such as *S. mutans* (Bae et al., 2006; Sano et al., 2003).

In 2004, a group of researchers led by Lifeng investigated the antibacterial effects of chitosan nanoparticles on *E. coli*, *Salmonella* colitis, *Staphylococcus aureus* and *Salmonella typhimurium*. They produced chitosan nanoparticles using the ionic gelation method and examined its effect on bacteria. Their nanoparticles were tested by zeta analysis and electron microscopy, which yielded results like the present study. Further, the minimum inhibitory concentration and the minimum concentration of chitosan nanoparticles were measured, with an average inhibitory concentration of 0.25 mg/mL and a minimum inhibitory concentration of 1 mg/mL for bacteria (Qi et al., 2004). The results of the present study were similar for *S. bongori*, and it is concluded that chitosan nanoparticles significantly control the growth of microorganisms.

In a study in 2008, Barzegar et al., investigated the antimicrobial activity of chitosan in mayonnaise, they showed that adding 0.2% of chitosan to mayonnaise increases its shelf life because chitosan has high antimicrobial properties, it can be used as a preservative (Barzegar et al., 2008). In Ma et al. study, the antimicrobial activity of chitosan nanoparticles against *E. coli* and *Staphylococcus aureus* was measured by Hole-plate method. The results of this study showed the diameter of the inhibition zone 0.8 and 6.8 mm for *E. coli* and *Staphylococcus aureus* respectively. They revealed that the antibacterial activity of chitosan nanoparticles improved with increasing concentrations (Ma et al., 2010).

The antimicrobial effects of chitosan nanoparticles against Streptococcus mutant bacteria were performed in 2011 by Luis Paz et al. Nanoparticles were prepared based on different molecular weights and antimicrobial effects were investigated. The results of this study showed that chitosan nanoparticles with high molecular weight have a small antimicrobial effect (20-25% cell degradation), while chitosan nanoparticles treated with low molecular weight had a high antimicrobial effect (more than 95% cell degradation) (Chávez de Paz et al., 2011). In the present study, low-molecular-weight chitosan nanoparticles were prepared that had high antimicrobial properties. In 2011, Chung et al. investigated the antibacterial effects of water-soluble chitosan against *Staphylococcus*, *Listeria monocytogenes*, *Bacillus cereus*, *E. coli*, *Shigella* and *Salmonella typhimurium*. They used water-soluble chitosans, as in the present study, against these bacteria in pH 5 and 7. They showed that the best PH for the antimicrobial activity of chitosan was between 5 and 6 (Chung et al., 2011).

In a study in Egypt by Ebrahim et al. an investigation was carried out on the effects of gentamicin, tetracycline, ciprofloxacin and chitosan nanoparticles against Staphylococcus aureus and *E. coli*. They produced chitosan nanoparticles with ion gelation method at pH 5.5. The results of this study showed the diameter of the inhibition zone 6.5 and 5.5 mm for nanoparticles and antibiotics respectively. After mixing chitosan nanoparticles with antibiotics, the diameter of the inhibition zone varied between 21 and 22 mm. The simultaneous effect of nanoparticles with antibiotics will give rise to a very strong and valuable antibacterial property (Sobhani et al., 2017). In another study, Irie et al. optimized

the effect of chitosan nanoparticles with amoxicillin against *Helicobacter pylori*. The results showed that chitosan nanoparticles with amoxicillin can target the drug effectively to reach *Helicobacter pylori*. These results indicate that chitosan nanoparticles containing multi-layer amoxicillin have a high potential for effective treatment of *Helicobacter pylori* infection. According to the results of this study, chitosan nanoparticles and antibiotics can be used to target other bacteria (Arif et al., 2018).

Conclusion

In conclusion, there was a significant correlation between the resistance of antibiotics and chitosan nanoparticles. The antibacterial effect of chitosan nanoparticles was higher in bactericidal and bacteriostatic antibiotics. So it can be concluded that chitosan nanoparticles might be used to cope with diseases to eliminate bacterial resistant species.

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