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Research Article

Comparative study of antibacterial activity of narrow-spectrum antibiotic and chemically treated chitosan prepared from giant freshwater prawn waste

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Abstract

Chitosan is a linear polysaccharide composed of a randomly distributed β-(1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It has a number of commercial and possible biomedical uses. Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. This study was conducted to compare the antibacterial activity of chitosan with the combination of chitosan and vancomycin against gram negative *Escherichia coli* and gram positive *Staphylococcus aureus*. OD value measurement technique was undertaken to measure antibacterial activity. After incubation, turbidimetric measurements of bacterial growth of these 2 sets of test tubes were taken after every 4 hours, for 48 hours and then compared. For *S. aureus* and chitosan the average absorbance was found as 0.448, 0.457, 0.492, 0.532, 0.582, 0.645, 0.683, 0.724, 0.751, 0.793, 0.840 and 0.896, while for *S. aureus* with both chitosan and vancomycin, absorbance was 0.267, 0.286, 0.321, 0.346, 0.386, 0.431, 0.467, 0.475, 0.509, 0.541, 0.565 and 0.629. Again for *E. coli* and chitosan the average absorbance was found as 0.407, 0.429, 0.464, 0.506, 0.564, 0.6, 0.645, 0.703, 0.756, 0.815, 0.850 and 0.901, while *E. coli* with both chitosan and vancomycin, absorbance was 0.213, 0.233, 0.322, 0.347, 0.409, 0.446, 0.475, 0.511, 0.545, 0.582, 0.628 and 0.647. Both of these revealed that chitosan and vancomycin together possess higher antibacterial activity against gram positive and gram-negative bacteria than chitosan alone. Comparison of their antibacterial activity against *E. coli* and *S. aureus* revealed that chitosan and vancomycin possess approximately the same antibacterial activity against gram positive and gram negative bacteria.

Keywords: *Escherichia coli*, *Staphylococcus aureus*, vancomycin, *Macrobrachium rosenbergii*, Bangladesh.

Introduction

Chitosan is a linear polysaccharide that consists of (1, 4)-linked 2-amino-deoxy-N- D-glycan. It is a deacetylated form of chitin, the second most abundant polysaccharide found in nature after cellulose [1]. It also exists naturally in a few species of fungi; insects and yeast [2]. Extensive studies have been conducted on the activity of vancomycin on gram positive and gram negative bacteria. To facilitate the genetic investigation, entire genome sequences of the archetypal VRSA (Mu50) and vancomycin-susceptible MRSA strains N315, EMRSA 16 and COL were compared. This research helped to understand the mechanism(s) of vancomycin resistance in *S. aureus* Mu50 and other VRSA strains. Through this study, it was observed whether chitosan increases the antimicrobial activity of narrow spectrum antibiotic vancomycin. The antimicrobial activity of chitosan with that of combined chitosan and vancomycin was compared. The result obtained from the research work will contribute to the evolution of a more active natural antibacterial agent which may be applied to preserve food items, may be used as an antimicrobial agent in pharmaceuticals and may also find uses in agriculture as a more active seed coating, fertilizer, controlled agrochemical release agent. In the drug industry it may be used to formulate weight loss supplements which are already produced in many countries. Chitosan production from shrimp waste also helps to save the environment from serious pollution. This biopolymer can be used as a safe preservative in food. Chitosan may be used as a water purification agent in municipal areas where people always face serious water problems. There is a very realistic chance to get the full potential of this natural biopolymer. After considering the applicability of chitosan and its possible development by vancomycin as well as a narrow spectrum antibiotic, this research work has been undertaken. Thorough review of previous research work in Bangladesh and abroad regarding this topic were investigated. Based on this investigation the objectives of the present research were established as the study of the anti bacterial activity of chemically deacylated chitosan prepared from shrimp and the comparison of anti bacterial activity of chitosan with the combination of chitosan and a narrow spectrum antibiotic vancomycin. Koide (1998) [3] reported that chitin and chitosan *in vitro* show antibacterial and anti-yeast activities. One of the chitosan derivatives, i.e., N-carboxybutyl chitosan, was tested against 298 cultures of different pathogenic microorganisms that showed bacteriostatic and bactericidal activities and there were marked morphological alterations in treated microorganisms when examined by electron microscopy [3]. Conversely, growth inhibition and inactivation of mould and yeasts seems to depend on chitosan concentration, pH and temperature (Rout, 2001) [4].

The antimicrobial activity of chitosan varies depending on their physical properties (degree of deacetylation (DD) and molecular weight), solvent, microorganism species and source. The antimicrobial activity is reported to vary depending on the methods involved in preparation of different DD and molecular weight of chitosan [5, 6]. According to Cuero (1999) [7], the antimicrobial action of chitosan is influenced by intrinsic and extrinsic factors such as the type of chitosan (e.g., plain or derivative), degree of chitosan polymerization, host nutrient constituency, substrate chemical and/or nutrient composition and environmental conditions such as substrate water activity [7]. Extensive research by Tsai and Su (1999) on the antimicrobial activity of chitosan prepared from shrimp against *E. coli* found that higher temperature and acidic pH of food increased the bactericidal effect of chitosan. They also explained the mechanism of chitosan antibacterial action involving a cross-linkage between polycations of chitosan and the anions on the bacterial surface that changes membrane permeability. Chitosan has been approved as a food additive in Korea and Japan since 1995 and 1983, respectively [8].

Materials and Methods

Escherichia coli and *Staphylococcus aureus* strains were tested for the antimicrobial activity of chitosan and vancomycin. Pure culture of *S. aureus* was previously collected from ICDDRB, Dhaka and cultured in MacConkey and nutrient agar media respectively and maintained in the lab. 3% of concentrated (99%) acetic acid was prepared in a conical flask with distilled water to dissolve the powder. 7.5gm of chitosan was placed into two sterile test tubes and 50 ml of 3% acetic acid was poured into each of these gradually. To increase solubility the solution was stirred; heat was applied in water bath at 40^0C. The solution was left at room temperature for 3 days. Stirring and heat was applied in this time with a spirit lamp. Nutrient broth (N.B) was used as culture media in test tubes.

9 ml of nutrient broth media was taken in each of 14 test tubes. Each test tube was always capped with a screw to avoid contamination. Three test tubes were used as standard in spectrophotometer measurement for blank calculation containing media. The upper soluble portion of chitosan sample was added to NB medium in test tube, the pellet portion of the test tube was not taken. 0.5 ml supernatant chitosan solution was taken by micropipette and dispended in each test tube. The pH of chitosan with medium was also adjusted between 6.2-6.5 range to ensure optimum growth of microorganisms using NaOH solution. Five vancomycin discs were added in each test tube.

Two test tubes containing 9 ml N.B media and 0.5ml chitosan solution was mixed. One loop full staphylococci. The loop head was immersed into the broth and mixed well. The same process was followed for the other 2 test tubes with gram negative bacteria. Another 2 test tubes containing NB media, chitosan and vancomycin were taken and inoculated with gram positive bacteria and another 2 test tubes with gram negative bacteria in the same way. Absorbance of nNutrient broth with *S. aureus* and *E. coli* was measured separately at 640 nm. Chitosan was included separately with the both strains for measurement of activity. Vancomycin was incorporated in broth chitosan and the two organisms in which equal concentration of inoculum was used. In each case a blank was prepared. After successful inoculation the test tubes were incubated in an incubator at 37^oC. After each 4 hour intervals, measurements were taken by spectrophotometer up to 48 hrs. Absorbance or OD value can also refer to æindex of refraction [9]. This technique is based on the principle that small particles, such as bacteria, scatter a beam of light passed through the cell suspension. The amount of light scattering is proportional to the concentration of particles in the suspension; because a pure culture of single cells is relatively uniform in size (mass). The number of cells can be measured from the amount of light that reaches a photoelectric cell after passing through the cell suspension. As the bacterial cell population increases, the amount of transmitted light decreases, increasing the absorbance reading on the spectrophotometer. These sequences were repeated for other samples and the respective blank was changed and the absorbance was recorded. OD values were taken after every 4 hours for 48 hours.

Results and Discussion

The recorded absorbance for *S. aureus* and *E. coli* are given in the following tables.

Table1. OD values for *S. aureus* (4h-48h).

Description	Average OD values after incubation of 48 hours											
Hours of incubation	4	8	12	16	20	24	28	32	36	40	44	48
Chitosan + *S. aureus*	0.448	0.457	0.492	0.532	0.582	0.645	0.683	0.724	0.751	0.793	0.840	0.896
Chitosan + *S. aureus* + Antibiotic	0.267	0.286	0.321	0.346	0.386	0.431	0.467	0.475	0.509	0.541	0.565	0.629

The OD value measured for *Staphylococcus aureus* with chitosan demonstrated far higher absorbance than chitosan and vancomycin together. So it is clearly evident that chitosan and vancomycin together possess high level antibacterial properties against *S. aureus*, while chitosan alone possesses lower level of activity. Graphical presentation of the measured OD values for *S. aureus* is shown in Figure 1.

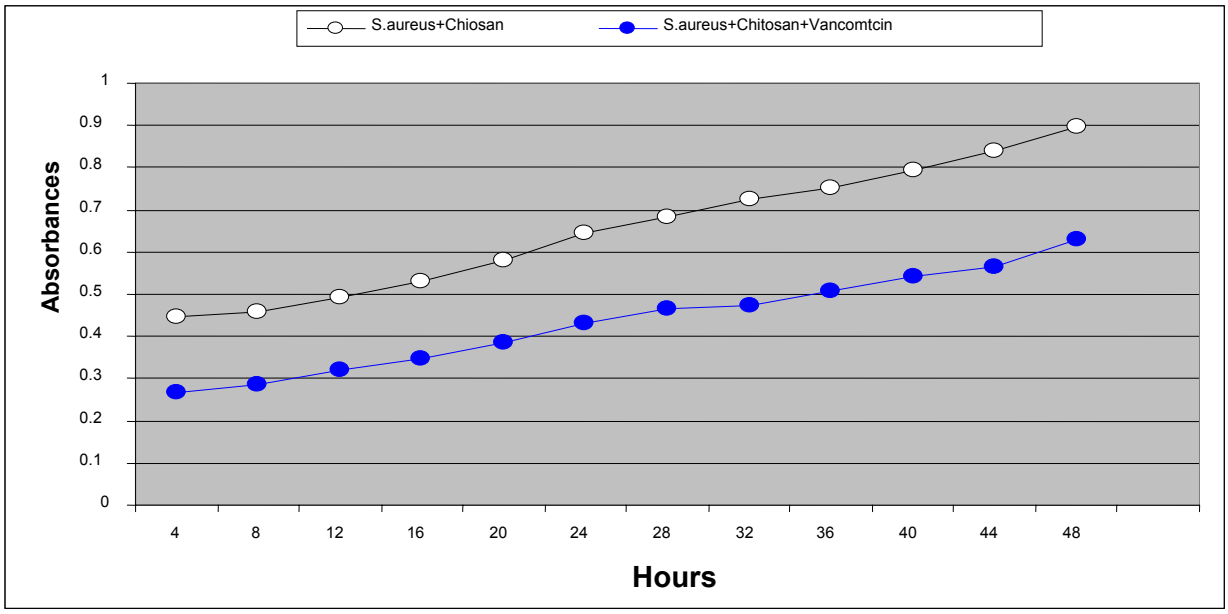

Figure 1. Growth curve for *S .aureus* + chitosan and *S. aureus* + chitosan + vancomycin.

From the above graph it is clearly evident that the growth curve of *S. aureus* with chitosan and vancomycin lies far behind the growth curve of *S. aureus* with chitosan (white). This reveals that growth of *S. aureus* is retarded more by the addition of vancomycin with chitosan.

Table2. OD values for E. coli (4h-48h).

Description	Average OD values after incubation of 48 hours											
Hours of incubation	4	8	12	16	20	24	28	32	36	40	44	48
Chitosan + *E. coli*	0.407	0.429	0.464	0.506	0.564	0.600	0.654	0.703	0.756	0.815	0.850	0.901
Chitosan + *E. coli* + Antibiotic	0.213	0.233	0.322	0.374	0.409	0.446	0.475	0.511	0.545	0.582	0.628	0.647

The OD value measured for *E. coli* with chitosan demonstrated far higher absorbance than chitosan and vancomycin together. So it is clearly evident that chitosan and vancomycin together possess high level antibacterial properties against *E. coli*, while chitosan alone possesses a lower level of activity. Graphical presentation of the measured OD values for *E. coli* are shown in Figure 2.

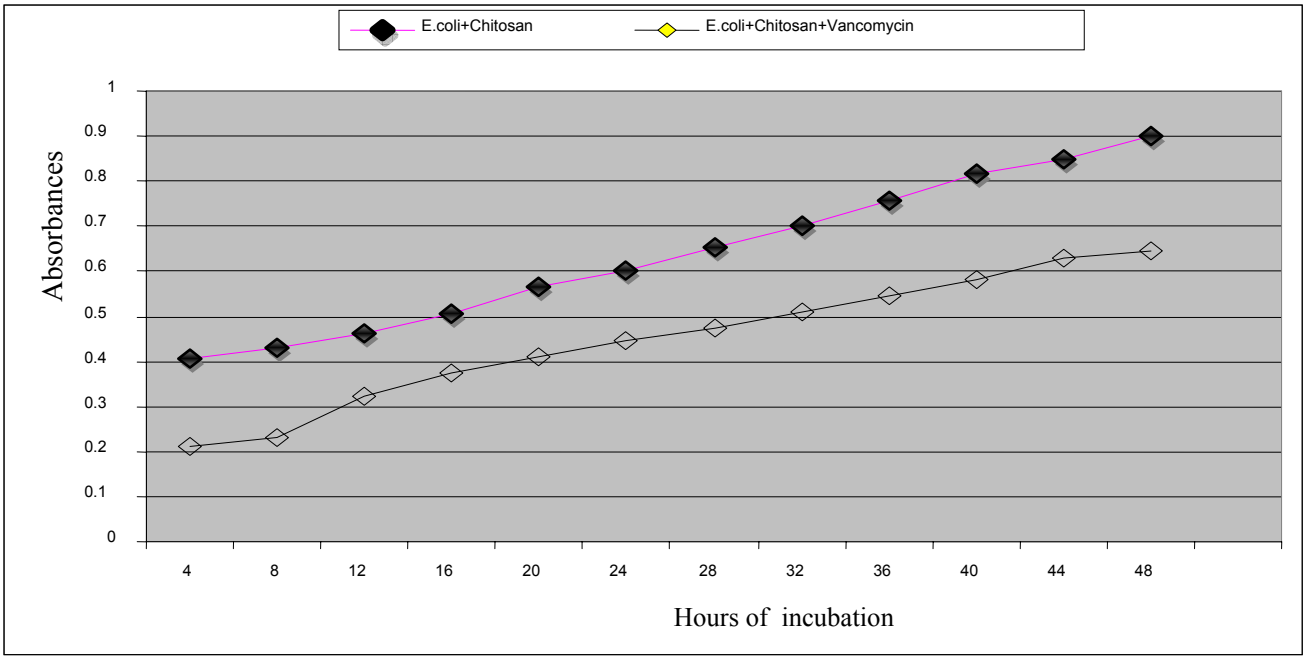

Figure 2. Growth curve for *E. coli* + chitosan and *E. coli* + chitosan + vancomycin.

From the above graph it is clearly evident that the growth curve of *E. coli* with chitosan and vancomycin lies far behind the growth curve of *E. coli* with chitosan. This means growth of *E. coli* is retarded more by the addition of vancomycin with chitosan. A comparative study of antibacterial activity of chitosan and vancomycin against *E. coli* and *S. aureus* is shown in Figure 3.

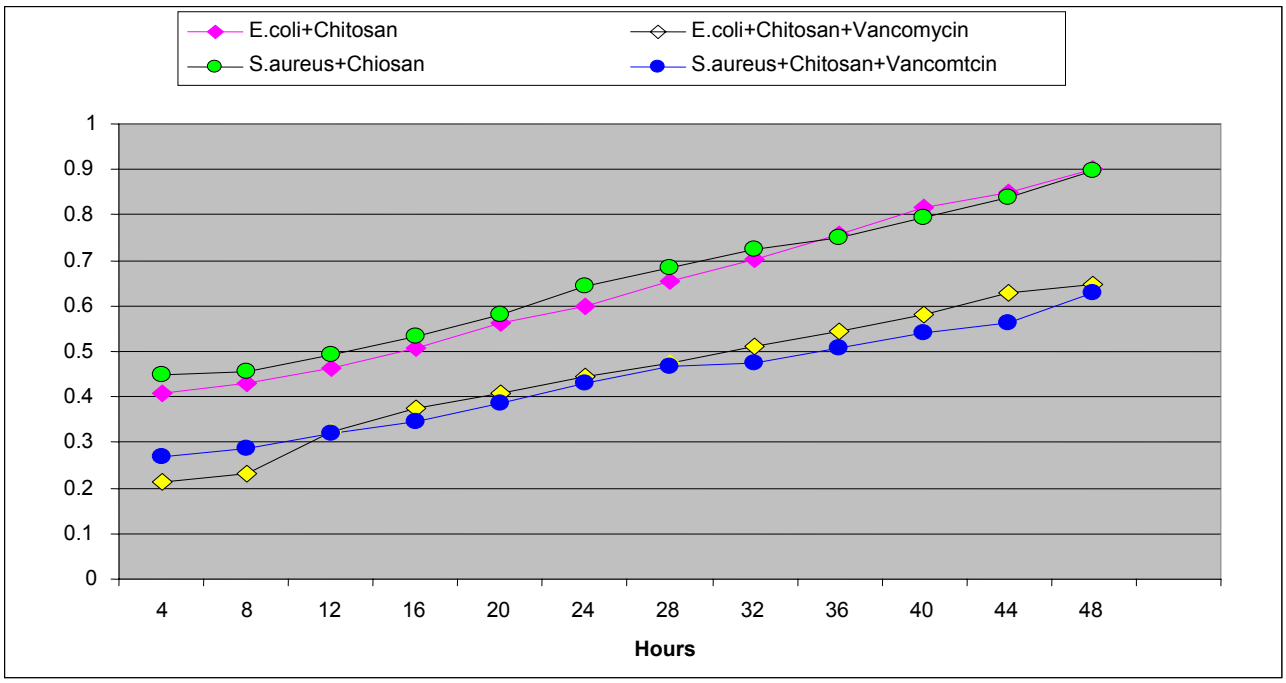

Figure 3. Comparison of the growth curves for *E. coli* and *S. aureus*.

Figure 3 reveals that in the presence of vancomycin at first the growth of gram negative *E. coli* was lower than that of gram positive *S. aureus*. After 12 hours of growth both the bacteria were the same. *E. coli* showed slightly higher growth than *S. aureus*. At some points the OD value for *E. coli* and *S. aureus* were approximately the same. The efficacy of many antibiotics for treatment of infections has become quite limited due to the development of resistance and the threat from antimicrobial-resistant organisms is accumulating and accelerating [10]. Also, the development of resistance to monotherapy is a common problem and dual antimicrobial coverage is often a necessity in *Pseudomonas* infections [11]. Attempts have been made to deal with this problem by using combination therapy [12].

Conclusion

The activity of chitosan is higher against gram positive bacteria than gram negative bacteria. In this study chitosan and vancomycin together showed slightly higher antibacterial effect against gram negative *E. coli*, the difference between antibacterial activity against both the gram positive *S. aureus* and gram negative *E. coli* was negligible. From this research it has been found that chitosan and vancomycin possess approximately equal antibacterial activity against gram positive and gram negative bacteria but when they used together as an antimicrobial agent they work more effectively than chitosan or vancomycin alone. In Table 1 it has been shown that the average OD value of chitosan and *S. aureus* is higher than chitosan and *S. aureus* and vancomycin together. From this it can be concluded the existence of a synergistic effect between chitosan and vancomycin antibiotic. Since chitosan itself acts as an antibiotic, it can certainly increase the activity of vancomycin, which would take the antibiotic technology foreword one step. Chitosan and vancomycin together can also create advanced ways to produce more effective preservatives.

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