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Ultrastructural Analysis of Chitosan Antibacterial Activity against Clinical Isolates of *Staphylococcus aureus* and *Escherichia coli*

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Abstract

The continuous search for an antimicrobial agent led to the identification of potential antimicrobial biomaterials based on polymers naturals, such as chitosan (CS). However, the mechanism of action of antibacterial activity of CS for gram-positive and gram-negative bacteria was not completely elucidated. The aim of this work is to report the antibacterial activity of CS through ultrastructural analyses of the clinical isolates *Staphylococcus aureus* and *Escherichia coli* by Transmission Electron Microscopy. The CS has a bactericidal action against *S. aureus* and *E. coli* which alters its cellular ultrastructure, such as with collapsed cell walls, condensed chromatin and the increase of intracellulares structures like vacuoles and cell debris. In this way, the CS represents a potential model for the future design of antibacterial in order to control bacterial resistance of patients in hospital settings.

Keywords

Antibacterial Activity, Chitosan, Ultrastructure, *Staphylococcus aureu*, *Escherichia coli*

1. Introduction

Infectious diseases are one of the leading causes of death in the world and high rates of morbidity and mortality. Antimicrobial resistance and lack of new alternative antibiotics are able to exacerbate this situation [1]. The continuous search for antimicrobial agent led to the identification of potential antimicrobial biomaterials based on polymers naturals, as chitosan polymer poly-cationic [2] [3]. Chitosan (CS), a

polysaccharide is formed by residues of 2-amino-2-deoxy-D-glucan obtained by deacetylation of chitin found in fungi, insects and crustaceans [4]. The CS has been studied due to its wide range of biological activities; among them the antibacterial activity has stood out [5] [6] [7].

Chitosan has increasingly become an important biomaterial used for antibacterial purposes. Several mechanisms have been proposed for the antibacterial action of CS among which the most accepted model is due to the electrostatic interactions between the positive charge of the amino group in the CS molecule (pH < 6.3, chitosan pKa value) and negative charges on the surface of bacterial cells, promoting osmotic imbalances and inhibition of the growth of microorganisms [8] [9]. However, the mechanisms of action of antibacterial activity of chitosan for gram-positive bacteria such as *Staphylococcus aureus* and gram-negative such as *Escherichia coli* were not completely elucidated. Lee and Wu [8] [10], have demonstrated the antibacterial effects of medium molecular weight CS against *E. coli* and *S. aureus*, but failed to elucidate its mechanism of action. Some research has suggested that the antibacterial property of chitosan would decrease with increasing its molecular weight [11] [12].

S. aureus is a common skin gram-positive microorganism, second major contaminating bacteria in food products and the most notorious microorganism that causes surgical infections [13]. *E. coli* is one of the bacteria responsible for the largest number of intestinal and urogenital infections. Urinary tract infection (UTI) affects millions of children and adults around the world. Some strains of *E. coli* can cause diarrhea, while others cause urinary tract infections, respiratory diseases, and pneumonia and other diseases. *E. coli* can also cause more serious infections, such as hemorrhagic colitis (HC) and hemolytic uremia [14] [15].

The indiscriminate use of antibiotics has generated microorganisms resistant to antibacterial drugs, requiring the investigation and development of new alternatives of treatment with more potent drugs [16] [17] [18]. In view of the increase in the cases of *S. aureus* multiresistance and increasing number of *E. coli* infections, the objective of this work is to report the antibacterial activity of chitosan and evaluating the action against gram-positive and gram-negative clinical isolates at the ultrastructural level by Transmission Electron Microscopy.

2. Materials and Methods

2.1. Materials

Commercial Chitosan with deacetilation degree of 90.43%, Medium Molecular Weight (MMW), was purchased from SP Farma (São Paulo-Brazil). Acetic acid, sodium hydroxide, peptone, agar and all other chemicals of analytical grade were obtained from Sigma Chemical Company Ltd. All other chemicals were of analytical grade and were used without further purification.

2.2. Microorganisms

The tested microorganisms were *Staphylococcus aureus* IC133 and *Escherichia*

coli IC08 (clinical multi-resistant bacteria isolates of patients from intensive care unit—ICU) obtained from Departamento de Antibióticos—UFPE, Brazil. The bacteria were maintained on Nutrient Agar (NA) slants at 4˚C and were grown in nutrient broth incubated over night at 37˚C.

2.3. Antibacterial Activity

The *E. coli* and *S. aureus* clinical isolates were grown in nutrient broth incubated the CS solutions at concentrations from 0.1% and 0.5% and were prepared in acetic acid (0.25%). The CS solutions and acetic acid (0.25%) used as control, were filtered through 0.22 μM membrane (Millipore). The cultures obtained were grown in an autoclaved nutrient broth, overnight, at 200 rpm/37˚C to obtain cell suspension containing optical density (OD) 1.0. The resulting cultures were diluted for a final OD 1.0 in 5 ml of nutrient broth which contained chitosan in a final concentration of 0.1% and 0.5%, incubated overnight at 200 rpm/37˚C. The samples were removed after 6 and 24 hours later (adapted Liu et al. 2004) [19]. Biomass determination was assayed using Beckman UV640 spectrophotometer at 600 nm and the actual values were calculated based on a calibration curve. The inhibitory effects in cell growth were calculated related to the growth in medium without CS.

2.4. Transmission Electron Microscopy (TEM)

The *E. coli* and *S. aureus* was prepared for TEM as follows: One milliliter of cultures, at the final optical density of 2.0 A600/100mL of nutrient broth was added into CS solution, to give the final concentration of the CS 0.1% and 0.5% (w/v). After incubation on a rotary shaker (120 rpm) at 37˚C for 24 h, the suspension was centrifuged. The cells were washed twice with Phosphate Buffered Saline (PBS) 5 mmol pH 7.2 and then fixed with 2.0% glutaraldehyde (v/v) in PBS buffer. The samples were postfixed with 1% (w/v) OsO_4 in 5 mmol L-1 PBS for 1 h at room temperature and washed three times with the same buffer, dehydrated in graded ethanol, then embedded in Epon the low-viscosity embedding medium. Thin sections of the specimens were cut with a diamond knife on an Ultracut Ultramicrotome and the sections were double-stained with saturated uranyl acetate and lead citrate. The grids were examined with a JOEL Transmission Electron Microscope (JOEL-Hitachi, Tokyo, Japan) at an operating voltage of 75 kV.

2.5. Statistical Analysis

All measurements were performed in triplicate and data was presented with the average ± standard error. A two-tailed unpaired t-test was employed to assess the statistical significance of the results for all measurements.

3. Results

3.1. Antibacterial Activity

The antibacterial activity of CS was performed by biomass measurement in spec-

trophotometer in order to observe the inhibitory action of CS at concentrations of 0.1 and 0.5% in clinical isolates of *S. aureus* and *E. coli* on the influence of time exposure. The antimicrobial action of CS against *S. aureus* and *E. coli* showed that CS was more effective against S. aureus in the two concentrations tested at all exposure times, as shown in Table 1.

It was shown that the antibacterial process of chitosan against *E. coli* was gradually increased over time in the gram-negative bacterium, whereas in the gram-positive bacteria there was a decline in inhibitory action as the exposure time increased. In *S. aureus* strains tested with 0.5% CS there was a 70% growth inhibition in 6 h and a smaller inhibition after an exposure of 24 h, with statistical difference ($p < 0.005$). A larger inhibitory action was observed in the 0.1% CS exposure, but with no statistical difference ($p < 0.005$), however when comparing both concentrations, CS of 0.1% had a greater inhibitory action at 6 h than CS 0.5% in the same time interval.

3.2. Ultrastructure Analyses by Transmission Electron Microscopy

In order to understand the mechanism of the antibacterial activity of CS against *S. aureus* and *E. coli*, the Microscopic TEM analysis was carried out to evaluate ultrastructural changes in the bacteria. The antimicrobial effect of unmodified chitosan against *E. coli* at concentrations of 0.1% - 0.5% are shown in Figure 1 and Figure 2.

Figure 1(a) represents the control, growth of the bacterium without CS, where cells with normal structural aspects, with few cytoplasmic inclusions and without perceptible ultrastructural changes. In the images of Figure 1(b) and Figure 1(c) for samples treated with 0.1% CS, isolated bacteria with collapsed cell wall, condensed chromatin, the increase of intracellular structures like vacuoles and cell debris are observed. Morphological changes in the bacteria tested in CS 0.5% were observed; this concentration favored an osmotic imbalance, evidenced by the increase in the larger number of vacuoles inside the bacterium, besides the destructuring of the cell membrane allowing extravasations of the cytoplasmic material (Figure 2).

Figure 3(a) shows *S. aureus* cultured in control medium without the presence of CS, where we observed conserved morphological characteristics, as intact cells

Table 1. The inhibitory effects of CS at concentrations of 0.1% and 0.5% with exposure to time of 6 h and 24 h on clinical isolates from ICU patients *Staphylococcus aureus* IC133 and *Escherichia coli* IC08.

Microrganismos	CS 0.1%		CS 0.5%	
	6 h	24 h	6 h	24 h
E. coli IC08	11.7% ± 3.4%	50.0% ± 4.2%	10.0% ± 0.8%	39.4% ± 2.2%
S. aureus IC133	81.6% ± 7.7%	74.2% ± 6.1%	70.0% ± 5.3%	54.5% ± 8.4%

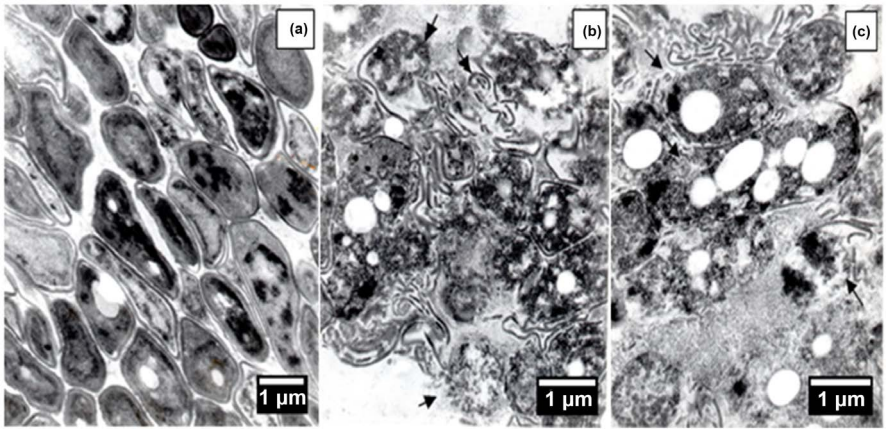

Figure 1. Ultrastructural appearances of *E. coli* IC08 at 24 h exposure with 0.1% CS. (a) Control growth in nutrient broth without chitosan, at 12,000× magnification. (b), and (c) Indicated arrows of disrupted cell wall and the increase of vacuoles (evidenced in the arrows), TEM at 20.000× magnification.

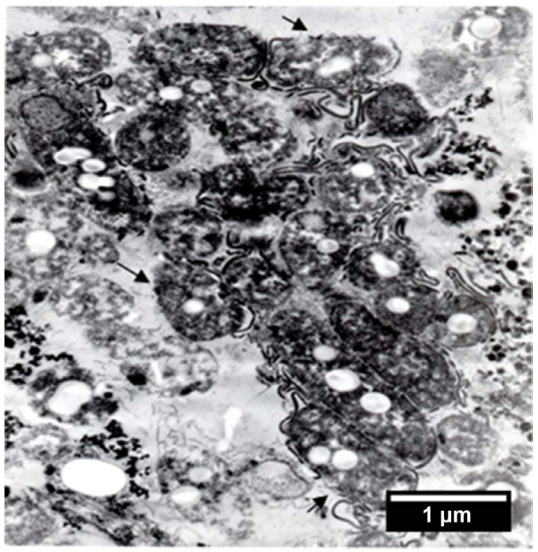

Figure 2. Ultrastructural appearance of *E. coli* IC08 at 24 h exposure with 0.5% CS. Indicated cell rupture (in evidences on the arrows), TEM at 7.000× magnification.

with some cells in process of division. In contrast, **Figure 3(b)** and **Figure 3(c)** show bacteria treated with 0.1% CS, where we observed an antibacterial activity of CS with cell wall lysis, with material dispersed in the medium. **Figures 3(d)-(f)** when bacteria was grown in 0.5% CS, we can observe signs of cellular damage, such as the presence of external clusters on the cell surface, condensed genetic material and thickening of the cell wall as can be visualized in **Figures 3(d)-(f)**.

4. Discussion

Inhibitory effects of the antibacterial action of chitosan on *E. coli* were found by Li (2016) [20], when they observed that the CS of MMW inhibited 10% of the

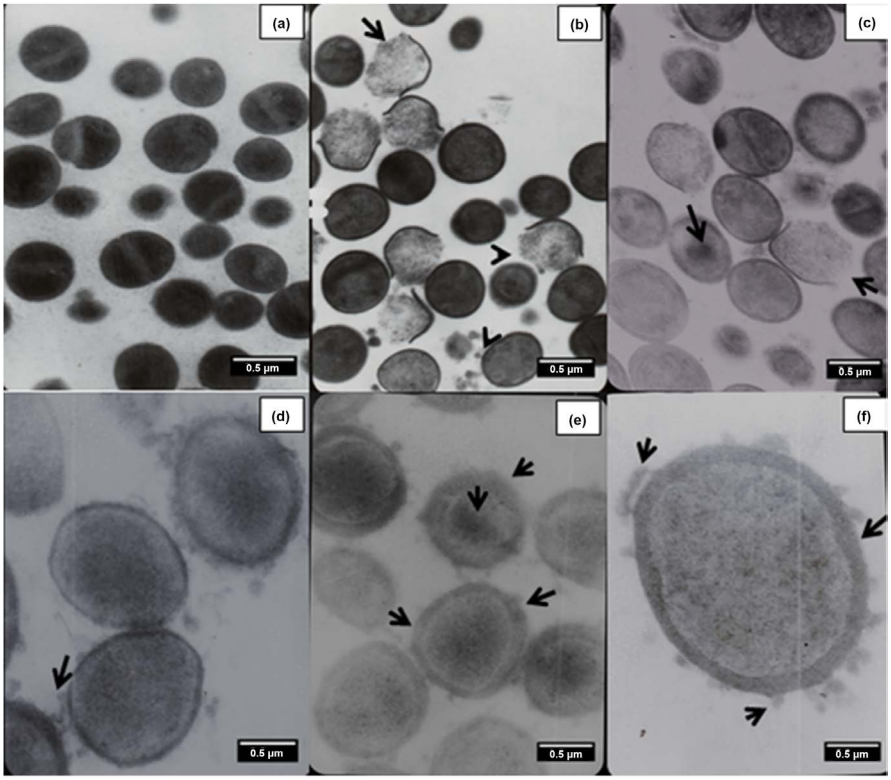

Figure 3. Ultrastructural appearance of *S. Aureus* IC133 at 24 h. (a) Control growth without CS treatment—TEM 20,000×; (b) and (c) Bacteria treated with 0.1% CS, cell wall rupture (black arrows)—TEM 20,000×; (D) Bacteria treated with CS 0.5%, structural changes in the cell wall (black arrows)—TEM 30,000×; (E) Bacteria treated with 0.5% CS, in evidence condensation of the genetic material and thickening of the cell wall (black arrows)—TEM 30,000×; (F) Bacteria treated with CS 0.5%, evidencing thickening of the cellular wall and clusters on the surface—TEM5000×.

cell colonies, after 1 h of exposure, and after 12 h of exposure it would have an inhibitory action of 81% in the number of colonies. Although some authors have reported that CS has a greater inhibitory effect on gram-negative bacteria [21] [22], different authors also describe that CS has inhibitory effects on gram-positive bacteria [19] [23], as observed in our experiments. In our previous studies with same concentrations of 0.1% and 0.5% CS carried out in bovine *S. aureus* strains no antibacterial activity was observed [21]. Different from the results found in our study in *S. aureus* isolates from clinical isolates of patients from the intensive care unit—ICU, where a bacteriostatic action was observed on the growth of the bacteria analyzed.

Similar findings were reported by Liu (2004) [19] and Eaton (2008) [24], when they analyzed the antibacterial activity of MMW CS against *E. coli*. They observed that the bacteria were irregular in shape and had no ruptures in cell wall, unlike our results in which there was rupture of the cell membrane in most bacteria. This is probably due to the time of exposure of the bacteria with CS at different concentrations influenced antibacterial activity as rupture of the bacteria membrane and cell wall, since in our experiment were had 24 h of exposure,

while in the studies conducted by Liu (2004) [19] that period was only of 20 minutes.

In studies carried out by Li (2015) [23], the antibacterial action of native CS in the bacteria *E. coli* and *S. aureus* were observed and it affirmed that there was no alteration in the cellular morphology, but the native CS showed bacterial activity by condensing its genetic material in the medium in *E. coli* ATCC, similar to our results clinical isolate.

It is known that the cell wall of gram-negative bacteria such as *Escherichia coli* contains a thin layer of peptidoglycan (PG), surrounded by a membrane rich in lipopolysaccharides that confers on hydrophobics resistance to some compounds and increase the negative charge of the out membrane cellular [25]. As the surface charge of chitosan is positive, possibly due to elastrotic connections, it must have corroborated for greater damage to the membrane and cellular wall of *E. coli*.

Studies by Masson (2008) [25] have reported that chitosan disrupts the outer membrane function of gram-negative bacteria. Chitosan can therefore sensitize gram-negative bacteria as a bacteriolytic agent. However, the exact mechanism of antimicrobial action is still unknown. The mechanism of action of CS in both *E. coli* and *S. aureus* is possibly due to irreversible membrane and cell wall damage in the bacteria tested. This cellular destabilization may be due to the presence of external clusters which is possibly CS adhered to the cell surface, causing permanent damage, and condensation of the genetic material.

In the study carried out by Eaton (2008) [24] and collaborators, they tested CS MMW in *S. aureus* and did not observe any effect on cell morphology, different from the results found in our study. The author suggested no effect on *S. aureus* due to the thicker layer of peptidoglycan of the cell wall of gram-positive bacteria. Our results also contradict those observed by Raafat (2017) [17], who tested CS LMW in *S. aureus* resistant strains, observing no morphological changes in the ultrastructure of the bacterium and no other actions of CS antibacterial activity in these cells. However in an earlier study by Ramasamy (2017) [26], using gold nanoparticles against *S. aureus*, it was also observed a thickening of the cell wall, premature cell divisions and condensation of the genetic material when viewed in the Electron Microscope.

The mechanism of interaction of CS with the surface of the bacterium may be different for gram-positive and gram-negative bacteria of CS with the surface of the bacterium. The antibacterial activity with CS was observed in other experiments, suggesting inhibitory effects of CS which may have occurred through two possible physical-chemical mechanisms. First, the electrostatic attraction between the cell wall of bacteria and high molecular weight CS, which at high concentrations, the polymer can form agglomerates in the cell wall inhibiting the entry of nutrients into the cell, causing a collapse in the cell wall and extravasation of components [2] [27] [28], as found in our the results. Second, CS may have penetrated bacteria and linked ionically linked itself to cellular DNA. As CS has opposite charge to the genetic material, this suggests that its antibacterial ac-

tivity seems to be mainly caused by the inhibition of DNA transcription, condensing the genetic material, the cell collapsing cell wall, leading to the death of the bacterium [29] [30].

Thus, the bacteriostatic activity and ultrastructural changes here described against *S. aureus* and *E. coli* are probably influenced by their cell wall composition. Nevertheless, the modification of membrane structure, injuries and the increase in thickness of the cell walls visualized in *S. aureus* and *E. coli* treated with CS may indicate an inhibiting action by increase in osmotic pressure, which is created by an increase in the concentration of solutes in the cytoplasm promoting a disruption of the membrane [31].

The use of SEM imaging helped us understand the antibacterial action of chitosan in clinical isolates of *Staphylococcus aureu s*and *Escherichia coli*. It can be inferred that the CS caused the destruction of the cell wall of gram-negative and gram-positive, which led to extravasation of cellular material. The death of the microbial organism may be a result of disruption of the permeability of cell.

5. Conclusion

The antibacterial activity of CS MMW was confirmed against gram-positive and gram-negative bacteria by bactericidal action against *S. aureus* and *E. coli*, destabilizing their intracellular structures and cell wall. It was concluded that CS MMW represents a potential biopolymer as an antibacterial agent in order to combat and assist in the control of bacterial resistance to the hospital environment, especially in the fight against nosocomial infections. Its viable use as a low-cost, non-toxic antimicrobial agent makes it attractive both economically and ecologically. Thus, CS can be used as a possible alternative to conventional antibacterials, reducing side effects on patients.

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Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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