Ann Ig 2020; 32(1): 72-80 doi:10.7416/ai.2020.2332

Antibacterial activity and mechanism of action of chitosan nanofibers against toxigenic *Clostridioides (Clostridium) difficile* Isolates

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Key words: Chitosan nanofibers, Clostridioides difficile, antibacterial effect, electron microscopy, atomic force microscopy

Parole chiave: Nanofibre di chitosano, Clostridioides difficile, effetto antibatterico, microscopio elettronico, microscopia a forza atomica

Abstract

Background. Clostridioides difficile *a Gram-positive, obliged anaerobic, rod-shaped spore-former bacterium, causes a wide spectrum of diseases, ranging from mild, self-limiting diarrhoea to serious diarrhea. Chitosan, a natural polysaccharide, is largely known for its activity against a wide range of microorganisms. Chitosan, in the form of nanofibrils (nanofibrilated chitosan), consists of separated fibers which can be suspended easily in aqueous media.*

Study design. *This paper, for the first time, aims to evaluate the antimicrobial activity of chitosan nanofibers against* C. difficile *isolates.*

Methods. *Chitosan nanofibers were characterized through scanning electron microscopy and atomic force microscopy. Minimum inhibitory concentration and minimum bactericidal concentration of chitosan nanofibers against toxigenic* C. difficile *isolates (with resistance gene: ermB, tetM and tetW) was determined by the standard broth microdilution method.*

Results. *The Miniumum Inhibitory Concentration of chitosan nanofibers for two toxigenic isolates with resistance genes ermB, tetM and tetW, two toxigenic isolates ermB+ tetM+ and the standard strain ATCC 700057 was similar and equal to 0.25 mg/mL. The minimum bactericidal concentration for all isolates was 0.5 mg/mL.*

Conclusions. *The results demonstrated that chitosan nanofibers exhibit potent antimicrobial activities against multiple toxigenic* C. difficile *isolates, and the antibacterial effect of chitosan nanofibers against* C. difficile *isolates with ermB, tetM and tetW resistance genes indicates that interfering with the synthesis of proteins is not the mechanism of action of chitosan nanofibers.*

Introduction

Clostridioides difficile (synonymous: *Clostridium difficile*) is an anaerobic Gram-positive spore-forming bacillus responsible for a wide spectrum of diseases, from mild post-antibiotic diarrhea to pseudomembranous colitis (PMC) that

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can be severe and lead to complications such as toxic megacolon, septic shock, and even death (1, 2). *C. difficile* became medically important when it was found to be the substantial leading agent of morbidity and mortality among people receiving antibiotics in hospitals and in the community (3). There are many risk factors for *C. difficile* infection (CDI); age above 65 years, previous hospitalization, recent antibiotic therapy (particularly with broad-spectrum antibiotics with activity against anaerobes), immunosuppression and proton pump inhibitors (4, 5). The most important *C. difficile* virulence factor, the two large clostridial toxins A and B (TcdA and TcdB), are then produced. Toxin A (*tcdA*) is a known enterotoxigenic causative substrate for diarrhea and toxin B (*tcdB*) is a well known as cytotoxin enzymes which damage the human colonic mucosa (6, 7). The presence of *C. difficile* producing both toxin A and B correlates best with CDI occurrence and is responsible for the intestinal symptoms (8). Although all isolates are usually susceptible to metronidazole and vancomycin, recent reports of *C. difficile* isolates with significantly reduced susceptibility and even resistance to these antibiotics suggest a potentially serious problem with the continued use of these agents to treat CDI (9). Therefore, the development of alternative treatment strategies in addition to novel diagnostic approaches for CDI have become increasingly important. Chitosan, is a natural cationic polysaccharide composed of randomly repeating units of β(1, 4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit) (10, 11). Chitosan is hypoallergenic and exhibits antibacterial properties due to its chemical structure, mainly the positively charged amino groups along its backbone, so it can be used in various scientific and medical fields (12). Nanofiber technology represents a significant object for recent material research studies. A nanofiber has a characteristic morphology, an extremely high surface-to-volume ratio, and unique optical and mechanical properties (13). However, the extent of, and the mechanism by which chitosan or nanofibers of chitosan inhibit *C. difficile* or other anaerobe organisms and its destructive effects on bacterial physiology have not been investigated. Besides first line antibiotic treatments, which target *C. difficile* but also species of the normal microbiota, new therapeutic approaches are needed to treat CDI and prevent recurrences (14, 15). These new strategies must protect the normal intestinal microbiota and its barrier effect and limit rise in antibiotic resistance. Moreover, it has been proved that cumulative exposure to any kind of antibiotics increases the risk of developing CDI. The aim of the present work was to morphological analyze chitosan nanofibers and investigate the effects of chitosan nanofibers against antibiotic resistance and toxigenic *Clostridioides difficile* isolates.

Methods

Characterization of chitosan nanofibrils

Nanofibrilated chitosan in the form of aqueous gel (2.5% w/v in water, pH 7.2 and 85 % degree of deacetylation) was purchased from Nano Novin Polymer Co., (Sari, Iran). In order to characterize the size and morphology of chitosan nanofibers, scanning electron microscopy (SEM) and atomic force microscopy (AFM) were utilized. For AFM imaging, the aqueous gel of chitosan nanofibers was diluted by distilled water and then air dried on the glass slide. AFM was carried out in tapping mode under ambient air conditions (NanoWizard®2, JPK Instruments, Germany). SEM imaging was done through an XL-30 scanning electron microscope (Philips, Germany) with magnification up to 5000X). Prior to microscopy, the sample surface was coated with a gold-palladium layer (~8 nm

thickness) using a sputter coater (Q150R-ES, Quorum Technologies, UK). Average fiber diameter in each sample was estimated by examining 50 fibers using Image J software.

Sample collection and bacterial isolation

A total of twelve non-duplicated toxigenic *C. difficile* isolates from the diarrheal stools of patients who were admitted to Namazi hospital (Shiraz, Iran) in 2018 were used in this study. All fecal specimens were inoculated on selective cycloserine cefoxitin-fructose agar plates (CCFA) after heat shock treatment and incubated in an anaerobic jar at 37ß C for 48 h. Brain heart infusion (BHI), L-cysteine and yeast extract were purchased from Merck Co (Germany). *Clostridium difficile* agar medium was also bought from Acumedia NEOGEN co. (USA). Cycloserine and cefoxitin, were also bought from Mast (UK). Ordered primers were synthesized by Pishgam Company (Tehran, Iran).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) of four antibiotics, including metronidazole, clindamycin, tetracycline, and chloramphenicol were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) (16). Brucella agar supplemented with hemin (5 μg/mL), vitamin K1 (10 μg/mL), and 5% horse blood was used for the tests. *C. difficile* ATCC 700057 was used as a control strain for the susceptibility tests. Antibiotic resistance and susceptibility were determined using the breakpoints defined by the CLSI.

DNA extraction and initial testing

Genomic DNA was isolated from two or three colonies of *C. difficile* and DNA was extracted using a DNA Extraction Kit (GeneAll, Korea) according to the manufacture's recommendation. The extracted DNA was stored at -20°C. All the isolates were examined for *tpi* gene for confirmation of *C. difficile*. Primers for molecular confirmation are given in **Table** 1. After initial denaturation (at 95°C for 5 min), amplification conditions were: denaturation at 95°C for 30 seconds, annealing at 53°C for 1 min, and extension at 72°C for one minute. This was repeated for 30 cycles in a Block assembly 96G thermocycler (Applied Biosystems Veriti™ Thermal Cycler, USA), with a final extension of 72°C for 5 min. Agarose gel electrophoresis was used to detect amplified DNA products. A volume of 5 μl amplified DNA PCR products were subjected to electrophoresis at 50 V in horizontal gels containing 1% agarose with Tris-borate buffer (45 mM Tris-borate, 1 mM EDTA). The gel was stained with safe stain load dye (CinnaGen Co., Iran) exposed to ultraviolet light to visualize the amplified products.

Amplification of toxin genes and antimicrobial resistance genes

PCR was performed to detect toxin genes of *C. difficile*. The primers sets used were NK104 and NK105 for the toxin B gene (*tcdB*), *tcdA-F* and *tcdA-R* for the toxin A gene *(tcdA*) (6, 7). The genes encoding resistance to chloramphenicol (*catD*), MLS_B (*ermB*) (17), tetracycline (*tetM, tetW*) (18), and metronidazole (*nim*) (19), were detected by PCR method using specific primers (Table 1). *C. difficile* ATCC 700057 was used as a control strain for the susceptibility tests.

Nanofibrillated chitosan Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

Minimum inhibitory concentration (MIC) assay was performed according to the guidelines of the Clinical Laboratory and Standards Institute for Anaerobes. Five clinical isolates (2 isolates tcdA+ tcdB+ ermB+ tetM+ tetW+, 2 isolates tcdA+ tcdB+ ermB+ tetM+ and the strain ATCC 700057) of *C. difficile* were overnight-grown at 37°C in an anaerobic jar in BHIS,

Table 1 - The list of primers used in this study

Gene	Primer name	Primer sequence	Product size (bp)	References
tpi	tpi-F	5´- AAAGAAGCTACTAAGGGTACAAA -3´	230	(Lemee et al., 2004)
	tpi-R	5´- CATAATATTGGGTCTATTCCTAC -3´		
tcdA	tcdA-F	5´- AGATTCCTATATTTACATGACAATAT -3´	369	(Lemee et al., 2004)
	tcdA-R	5´- GTATCAGGCATAAAGTAATATACTTT -3´		
tcdB	NK104	5´- GTGTAGCAATGAAAGTCCAAGTTTACGC -3´	203	(Kato et al., 1991)
	NK105	5´- CACTTAGCTCTTTGATTGCTGCACCT -3´		
nim	NIM-3	5´- ATGTTCAGAGAAATGCGGCGTAAGCG -3´	458	(Trinh et al., 1996)
	NIM-5	5´- GCTTCCTTGCCTGTCATGTGCTC -3´		
ermB	E5	5´- CTCAAAACTTTTTAACGAGTG -3´	711	(Spigaglia et al., 2004)
	E6	5´- CCTCCCGTTAAATAATAGATA -3´		
tetM	TETMd	5´- TGGAATTGATTTATCAACGG -3´	1,000	(Rupnik et al., 2009)
	TETMr	5´- TTCCAACCATACAATCCTTG -3´		
tetW	WRC1	5´- CATCTCTGTGATTTTCAGCTTTTCTCTCCC -3´	457	(Rupnik et al., 2009)
	WRC2	5´- AGTCTGTTCGGGATAAGCTCTCCGCCG -3´		
catD	CL1	5´- ATACAGCATGACCGTTAAAG -3´	500	(Spigaglia et al., 2004)
	CL2	ATGTGAAATCCGTCACATAC -3´		

brain heart infusion supplemented with L-cysteine (0.1% (wt/vol), and yeast extract (5 mg/mL). 50 µl of chitosan nanofibers (concentration of chitosan nanofibers was 4 mg/mL) was added in 50 µL of BHIS, so the first concentration for investigating the antimicrobial activity of chitosan nanofibers was 2 mg/mL, and then this solution was serially diluted (2-fold) 7 times in 96-well microplates. In the last stage, 5×10^6 CFU of the strains of *C. difficile* (mentioned above) in final suspension were inoculated on all concentrations. Concentrations of chitosan nanofibers ranged from 0.016 to 2 mg/mL. Moreover, inoculated bacteria in BHIS without chitosan nanofibers and BHIS alone were used as positive and negative controls, respectively. Plates were then incubated under anaerobic conditions for 24 h at 37°C. To determine the MBC, bacterial suspensions from each well were streaked onto BHIS agar and incubated 24 to 48 hours.

Results

Size and morphology of chitosan fibers

The AFM and SEM images of chitosan nanofibers are shown in Figure 1. The AFM image clearly showed individual fibrils of chitosan (Figure 1A). These fibers showed a diameter between 50-110 nm with a mean of 87 nm. The SEM image again indicated the fibrous morphology of chitosan (Figure 1B). However, the individual fibrils were not clearly distinguishable in the SEM image. The average size of separated individual fibers (red arrows in Figure 1B) was measured and reported as mean fiber diameter. In addition to individual fibers aggregated and bundled fibrils are observable in the SEM image (white dotted arrows in Figure 1B). The aggregation of nanofibers and the formation of bundles was an unavoidable phenomenon during the sample preparation step. The aggregated fibrils were not taken into account for measurement of average fiber diameter. From the SEM image, the fibers mean diameter was calculated to be

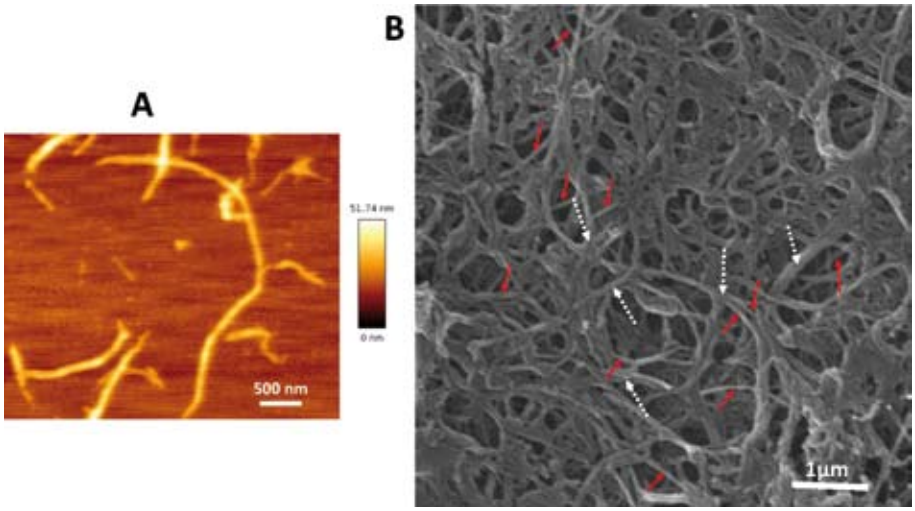

Figure 1 - Atomic force microscopy (A) and scanning electron microscopy (B) images of chitosan nanofibers. Red arrows indicate separated individual fibers and white dotted arrows indicate aggregated and bundled fibrils.

75 nm, which was almost in agreement with AFM results.

Antibacterial activity of chitosan nanofibers against C. difficile isolates

According to MIC results, resistance to tetracycline (MIC ≥16 µg/mL) and clindamycin (MIC ≥8 µg/mL) was observed in 8 and 7 isolates, respectively. None of the strains showed resistance to other antibiotics tested. All of the clinical isolates were *tcdA*-positive and *tcdB*-positive, so 12 isolates were toxinogenic. The erythromycin ribosomal methylases genes of class B (*ermB*), which commonly mediate resistance to macrolide–lincosamide–streptogramin B (MLSB) family was detected in 7 isolates. Eight isolates were *tetM* positive, 2 isolates *tetW* positive (*tetM* and *tetW* usually confer resistance to tetracycline), and all *C. difficile* isolates were negative for the presence of *nim* and *catD* genes that commonly mediate resistance to metronidazole and chloramphenicol, respectively (Table 2). The MIC of chitosan nanofibers for two toxigenic isolates with resistance genes *ermB, tetM* and *tetW*, two toxigenic isolates *ermB+ tetM+* and standard strain ATCC 700057 was similar and equal to 0.25 mg/mL. MBC for all isolates was 0.5 mg/mL.

Table 2 - Toxin genes and resistance genes of *C. difficile* isolates

No. of isolates	Toxin genes	Resistance genes
1	*tcdA, tcdB*	*ermB, tetM*
2	*tcdA, tcdB*	*ermB*
3	*tcdA, tcdB*	*ermB, tetM, tetW*
4	*tcdA, tcdB*	*ermB, tetM*
5	*tcdA, tcdB*	-
6	*tcdA, tcdB*	*tetM*
7	*tcdA, tcdB*	*ermB, tetM*
8	*tcdA, tcdB*	*tetM*
9	*tcdA, tcdB*	*ermB*
10	*tcdA, tcdB*	*ermB, tetM, tetW*
11	*tcdA, tcdB*	-
12	*tcdA, tcdB*	*tetM*

Discussion

The search for new antimicrobial agents is a major concern today, because of the increasing development of drug resistance

to human pathogens and the presence of undesirable effects of certain antibacterial agents (20). It should be noted that, in recent years, the ability of *C. difficile* to tolerate several commonly prescribed antibiotics, its production of potent cytotoxins (toxin A, toxin B), and its high rate of recurrence have resulted in CDIs becoming a healthcare concern worldwide (21, 22). Similar to what happens to various bacterial pathogens, alternative strategies for treatment or prevention of *C difficile* infections are required (23). A multidisciplinary tactic to drug discovery should be identified, involving the generation of new molecular diversity from natural product sources, and providing the best solution to the current productivity problems in the scientific society involved in drug discovery and development (24, 25). As described in previous studies, Chitosan exhibits various potential biological activities, such as antitumor, immunostimulatory and antibacterial properties; and the antimicrobial effectiveness of chitosan and its derivatives was most pronounced towards aerobic Gram negative and Gram positive bacteria (26, 27). The current study reports, for the first time, some antibacterial effects of chitosan nanofibers on antibiotic resistance of toxigenic *Clostridioides difficile* isolates, considered as a representative of the most common anaerobic bacteria causing infections in hospitalized patients. Metronidazole is the first choice for mild to moderate, and it is a nitroaromatic pro-drug that needs the reduction of the 5-nitro group of the imidazole ring for the cytotoxic turn for bacterial cells (28). Nitroimidazole (*nim*) genes usually conferred resistance to Metronidazole (29). For macrolide–lincosamide–streptograminB (MLSB) antibiotics, ribosomal methylation is the most common mechanism of resistance to the antibiotics of the MLSB family in *C. difficile* (28, 30). Class B of erythromycin ribosomal methylases genes (*ermB*) commonly mediate resistance to these antibiotics, even if rarely other *erm* genes in *C. difficile* isolates have been detected (17, 31). Recent papers indicate that *C. difficile* resistance to tetracycline is commonly due to the protection of the ribosomes from the action of antibiotic (28, 32). *tetM* is the predominant class in *C. difficile* isolates, but other *tet* genes have been identified too. Especially in *C. difficile* isolates, obtained from humans and animals, the co-presence of both *tetM* and *tetW* have been described (18). Resistance to chloramphenicol is not so widespread in *C. difficile* isolates (30), and resistance to chloramphenicol is usually conferred by a *catD* gene, encoding for chloramphenicol acetyltransferase (17, 33). We found that 8 *C. difficile* isolates displayed tetracycline resistance (MIC ≥16). They carried at least one *tetM* or *tetW* gene and two strains had both genes (Table 2), and 7 isolates were resistant (MIC ≥8) to clindamycin.

Previous studies have indicated that chitosan displayed the highest inhibitory effects against Gram-positive bacteria (34) and the antibacterial tests carried out by turbidity and well inhibition zone showed that the chitosan is consistently more active against the Gram-positive bacteria than Gram-negative bacteria (35, 36). Newly, much attention has been given to the electrospinning process as a unique technique because it can produce polymer nanofibers with diameters ranging from several micrometers to tens nanometers, according to the polymer and the treatment conditions. In electrospinning, a high voltage is used to create electrically charged jets of a polymer solution (37). These jets dry for the form nanofibers, which are collected on a non-woven fabric target (38). These nanofibers have considerable interest in various types of applications, because they have many useful properties, such as a high specific surface and a high porosity (13, 38). So the high specific surface area of chitosan nanofibers results in good contact with *C. difficile* isolates, significantly inhibiting their

growth and killing them. In the present study, MIC of chitosan nanofibers on multiple clinical isolates (that include: two toxigenic isolates with resistance genes *ermB, tetM* and *tetW*, two toxigenic isolates *ermB+ tetM+* and the standard strain ATCC 700057) was similar and equal to 0.25 mg/mL, and we revealed that the MBC for all isolates was 0.5 mg/mL. There is no specific report on the antimicrobial effects of nanoparticles on *C. difficile* isolates, so this is the first study to investigate the effects of nanoparticles on *C. difficile* isolates. Yang et al (39) extended the antibacterial activity of lauric acid on multiple clinical isolates of *C. difficile*, that included both toxigenic and non-toxigenic strains, and they revealed that the MIC and MBC ranged from 0.312 to 0.625 mg/mL. Therefore, chitosan nanofibers have antimicrobial activity on *C. difficile* isolates at lower concentrations compared to lauric acid. As with all nanoparticles, chitosan nano-fibers are less effective than antibiotics. However, due to various potential biological activities of chitosan nanofibers, such as immunostimulatory, derived from natural sources and antibacterial activity against *C. difficile* isolates, it could be used as a good alternative strategy for treatment or prevention of CDI.

Several mechanisms are accounted for the antibacterial effects of nanoparticles: e.g., they react with the sulfhydryl groups and cause respiration blockage and cell death, rupture via attachment to the negatively charged bacterial cell wall, proton motive force destruction or bind with DNA molecules and lead to helical disruption (40, 41). As seen in our findings, the antibacterial effect of chitosan nanofibers on *C. difficile* isolates with *tetM, tetW* and *ermB* resistance genes indicates that interfering with the synthesis of proteins is not the mechanism of action of chitosan nanofibers, because isolates are resistant to MLSB family and tetracycline, due to the presence of resistance genes *ermB, tetW* and *tetM,* respectively.

Conclusions

The current study**,** for the first time, evaluated the antimicrobial activity of chitosan nanofibers against *C. difficile* isolates as a representative of the most common anaerobic bacteria causing infection in hospitalized patients. From this study, it can be concluded that chitosan nanofibers exhibit potent antimicrobial activities against multiple toxigenic *C. difficile* isolates. Due to various potential biological activities of chitosan nanofibers, such as immunostimulatory, derived from natural sources and antibacterial activity against *C. difficile* isolates, it can be used as a good alternative strategy for treatment or prevention of CDI. Also, the antibacterial effect of chitosan nanofibers against *C. difficile* isolates with *tetM, tetW* and *ermB* resistance genes indicates that interfering with the synthesis of proteins is not the mechanism of the effect of chitosan nanofibers.

Funding: This work was supported by Shiraz University of Medical Sciences [grant numbers 97-01-01-17280]. This manuscript was extracted from Ph.D. thesis of M. Shahini Shams Abadi under the supervision of Dr. N. Hadi.

Ethical aspects: This study was approved by the Ethics Committee of Shiraz University of Medical Sciences (Register code: IR.SUMS.REC.1397.661).

Acknowledgment: The authors wish to thank Dr. Nasrin Shokrpour at the Research Consultation Center (RCC) of Shiraz University of Medical Sciences for his invaluable assistance in editing this manuscript.

Conflict of interest: The authors declare that they have no conflict of interest.

Riassunto

Attività antibtterica e meccanismo d'azione delle nanofibre di chitosano nei confronti dei ceppi tossigeni di Clostridioides (Clostridium) difficile

Premessa. Il *Clostridioides difficile*, un batterio Gram positivo, sporigeno, anaerobio obbligato, a forma di bastoncello, è responsabile di una vasta gamma di patologie, che spaziano da una diarrea leggera ed autoli-

mitante ad una forma di diarrea assai grave. Il chitosano, un polisaccaride naturale, è ben noto per la sua attività di contrasto verso un'ampia gamma di microorganismi e, sotto forma di nanofibre (chitosano nanofibrato) consiste di fibre separate tra loro che facilmente vanno in sospensione in ambiente idrico.

Disegno dello studio. Questo lavoro, per la prima volta, si è sforzato di valutare l'attività antimicrobica delle nanofibre di chitosano verso ceppi di *C. difficile*.

Metodi. Le nanofibre di chitosano sono state caratterizzate mediante microscopio elettronico a scansione e microscopio a forza atomica. Le minime concentrazioni inibenti e le minime concentrazioni battericide delle nanofibre di chitosano nei confronti dei ceppi isolati di *C. difficile* tossigeni (con geni di resistenza *ermB*, *tetM* e *tetW*) sono state determinate con il metodo standard di microdiluizione in brodo.

Risultati. La minima concentrazione inibente delle nanofibre di chitosano verso due ceppi tossigeni di *C. difficile* in possesso dei geni di resistenza *ermB*, *tetM* e *tetW*; due ceppi tossigeni *ermB+* e *tetM+;* ed il ceppo standard ATCC 700057, è risultata simile tra loro e pari a 0.25 mg/mL. La minima concentrazione battericida per tutti questi ceppi è risultata pari a 0.5 mg/mL.

Conclusioni. I risultati ottenuti dimostrano che le nanofibre di chitosano sono caratterizzate da una potente azione antibatterica verso diversi isolamenti di *C. difficile* tossigeni, e che l'effetto antibatterico delle nanofibre di chitosano verso i ceppi di *C. difficile* in possesso dei geni di resistenza *ermB*, *tetM* e *tetW* indica che l'interferenza con la sintesi proteica non è il meccanismo d'azione delle nanofibre di chitosano.

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