

## Evaluation of the Inhibitory Effect of Chitosan Nanoparticles on Biofilm Forming *Escherichia coli* Isolated from Omphalitis Cases

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### ABSTRACT

To experimentally evaluate chitosan nanoparticles (ChNPs) as alternative to antibiotics on controlling biofilm forming *E. coli* isolated from omphalitis cases. A total of 100 yolk sac samples were collected from diseased and dead chicks (1-10 days old) suffered from omphalitis. Yolk sacs were collected from different farms at Assiut city, Egypt. Out of 100 collected samples, a total of 36 *E. coli* isolates were isolated with a prevalence rate of 36%, these isolates belonged to 11 different serotypes, which showed biofilm production that confirmed experimentally by two methods (crystal violet assay and congo red agar), all biofilm producers of *E. coli* serotypes showed resistance to one or more antibiotics by disc diffusion method. Chitosan nanoparticles exhibited superior antimicrobial activity against all the 11 *E. coli* strains in comparison with used antibiotics, the maximum inhibition zone diameter recorded was 32 mm at concentration of 0.1% (ChNPs) and the lowest was 11 mm by Agar well diffusion assay. *In Ovo* colonization with 0.1 % chitosan nanoparticles at the 18<sup>th</sup> day of incubation through amniotic route had positive impacts on bacterial count of recovered *E. coli* isolates ( $2.9 \times 10^5$  CFU) without any adverse effect on hatchability.

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### Introduction

Yolk sac infection is the main cause of chick mortality during the first week of the post-hatching period, and accounting for large economic losses to the poultry industry (Ulmer Franco, 2011). The potential spread of antibiotic resistance pattern via the food chain is of high concern for public health. Even more, alarming is that *E. coli* isolates remain pathogenic potential with ability to form biofilm and these bacteria may persist during food processing and consequently lead to greater risks of food contamination (Pavlickova *et al.*, 2017). Common food borne pathogens also possesses film forming property for use as edible coatings and strong antimicrobial activity (Jeon *et al.*, 2002; Jan *et al.*, 2012).

Biofilm is a great threat as it gives bacteria ability to acquire transmissible genetic elements, protect bacteria from being damaged by therapeutic agents such as disinfectants and antibiotics, therefore, bacteria can develop antibiotic resistance that is difficult to eliminate (Davey and O'Toole, 2000;

Nachammai *et al.*, 2016). In addition, bacterial cells in biofilms are usually more resistant to sanitizing agents than planktonic cells of the same species.

Chitosan is a non-toxic, biodegradable polymer of high molecular weight similar to cellulose of a plant fiber. Chitosan is a natural material with excellent physicochemical properties. It is environmentally friendly and has bioactivities such as immune-enhancing effects and antibacterial activities against various food spoilage microorganisms.

Nanotechnology has the potential to reduce microbial load without forming drug residues in poultry products, thus improving performance and immune status of poultry birds (Anwar *et al.*, 2019). Chitosan nanoparticles are effective antimicrobial agent with antibiofilm activity through reducing biofilm bacteria and disrupt biofilm structure (Annie *et al.*, 2010; Divya *et al.*, 2017). In addition, nano-materials applied in embryonated chicken eggs can improve embryos development (Abd El-Ghany, 2019).

*In Ovo* feeding of nanominerals at the 18<sup>th</sup> day of incubation through amniotic route and chitosan- bacteriophage-loaded nanoparticle by a chorioallantoic membrane were non-toxic and not harmful to the developing embryos and did not influence the hatchability (Kaikabo *et al.* 2016; Joshua *et*

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al., 2016). As *E. coli* frequently isolated from omphalitis cases than other bacteria that showing resistance to available antimicrobial agents. This study was undertaken to determine the prevalence of biofilm forming *E. coli* strains by different methods and tried to experimentally evaluate Chitosan nanoparticles as alternative to antibiotics in controlling *E. coli* infection.

## Materials and methods

### Samples Collection and Bacterial Isolation

A total of 100 yolk sac samples were collected from diseased and dead chicks (1-10 days old) suffered from omphalitis, the gross lesions observed in chicks died of yolk sac infection included unabsorbed and discolored yolk sacs, distended abdomen, watery or cheesy consistency. Yolk sacs were collected from different farms of Assiut city, Egypt, during the period from March to December 2019. All samples were collected and handled aseptically to prevent cross contamination. Isolation and biochemical identification of *E. coli* isolates were carried out according to Quinn *et al.* (2002).

### Serological typing of *E. coli* isolates

The obtained 36 isolates that were preliminary identified biochemically as *E. coli* were subjected to serological identification according to Kok *et al.* (1996) using slide agglutination test, by using rapid diagnostic *E. coli* antisera, which included polyvalent and monovalent antisera sets (DENKA SEIKEN Co., Japan) for detection *E. coli* serotypes.

### Detection of biofilm formation in *E. coli* isolates

#### Congo red agar method

The medium composed of brain heart infusion agar (52 g/l); Congo red dye (0.8 g/l) and sucrose (36 g/l). Congo red stain was prepared as a concentrated aqueous solution and autoclaved at 121°C for 15 minutes, then it was added to previously autoclaved brain heart infusion agar and sucrose. Congo red agar plates were inoculated with tested organism and incubated at 37 °C for 24 h. aerobically. Black colonies with a dry crystalline consistency indicated biofilm production; non-biofilm producers usually remained pink (Nachammai *et al.*, 2016).

#### Crystal violet tube method

According to Christensen *et al.* (1982), trypticase soy broth with 1% glucose (10 mL) was inoculated with loopful of colonies from overnight nutrient agar culture plates and incubated for 24 h at 37°C, then tubes decanted and washed with phosphate buffer saline (PBS) (pH 7.3) and dried. Dried tubes stained with crystal violet (0.1%), tubes were washed with deionized water to remove excess stain, then dried in the inverted position and observed for biofilm formation. Biofilm production was considered positive when a visible film lined the wall and the bottom of the tube.

### Antibiotic Sensitivity test

The eleven biofilm forming *E. coli* isolates were subjected to different antimicrobial agents by the disc diffusion method and evaluated according to Finegold and Martin (1982) and Clinical and Laboratory Standard Institute (CLSI, 2014). The following antibacterial agents were used; amoxicillin/clavulanic acid (30 mg), ampicillin (10 mg), cephradine (30 mg), doxycy-

cline (30 mg); Enrofloxacin (5 mg); gentamicin (10 mg); Neomycin (30 mg); Flurophenicol (30 mg); Erythromycin (15 mg), Colistin (10 mg) and Vancomycin (30 mg). All antimicrobial discs were purchased from Oxoid, UK.

### Preparation of chitosan nanoparticle (ChNPs)

Chitosan powder (0.25 g) was dissolved in 230 ml distilled water contained 2.5 ml acetic acid 1% (v/v), the pH was adjusted to 4.6–4.8 by using sodium hydroxide (NaOH). Nano-chitosan formed spontaneously upon addition of 20 ml of an aqueous tripolyphosphate, it was dropped into the chitosan beaker at room temperature and under magnetic stirring for 45 minutes to get a white precipitate, which indicated nanoparticle synthesis. Nanoparticles were purified by centrifugation at 9000 g for 30 min., supernatant was discarded and the nano-chitosan were extensively rinsed with distilled water to remove any NaOH and then freeze-dried before further use or analysis (Qi *et al.*, 2004). Chitosan powder (low-molecular-weight, 161.16\ 93% deacetylated), and chemicals were obtained from Oxford Lab. Chem. India Estate.

### Characterization of chitosan nanoparticle(ChNPs)

A drop of colloidal solution was placed on copper grid, covered with an amorphous carbon film, then dried at room temperature and examined using Transmission Electron Microscopy (TEM) without being negatively stained (Hu *et al.*, 2002). TEM image was used to observe the morphology (shape and size) of ChNPs. this image was captured in The Electron Microscopy unit at Assiut University, Egypt (Fig.1).

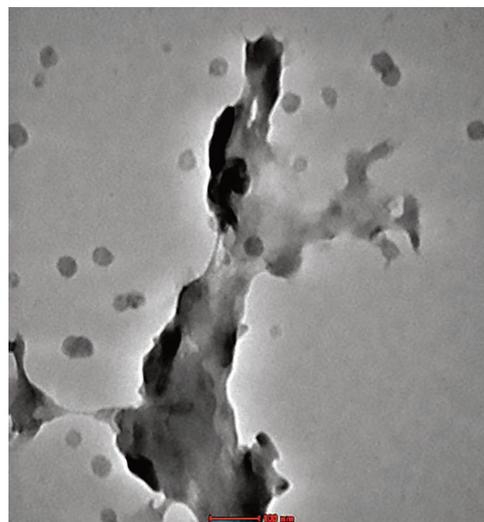


Fig. 1. Transmission Electron Microscope (TEM) image of synthesized ChNP size was less than 100 nm.

### Determination of the antibacterial activity of nano-chitosan

Agar well diffusion method (qualitative test) was used as described by Wolf and Gibbons (1996), wells were punched on the surface of Muller Hinton Agar plates that was previously spread with 1 ml of *E. coli* broth culture, 100 µl of chitosan nanoparticles, which were previously prepared were injected into the wells then the plates were left at 4-5°C for 2 h to allow diffusion of ChNPs and then incubated aerobically at 37°C for another 24h, after which the diameter of the inhibition zones were measured and recorded for each indicator organism.

Experimental design

Ethical approval

The research protocol was reviewed and approved by the Animal Health Research Institute, Dokki, Egypt.

On day 18 of incubation, 130 embryonated chicken eggs from a flock of 62-week-old fayoumi breeders (Assiut city farm) were submitted to egg candling to determine the presence of living embryos at the Animal Health Research Institute, Assiut branch. Ten eggs were subjected to bacteriological examination, which proved to be free from bacterial contamination. The remaining 120 eggs were grouped into four equal groups then set in incubation trays, and inoculated in the amniotic fluid, according to the following treatments;

Group 1: 30 embryonated chicken eggs that were kept as control negative (not infected) and put in a separate incubator.  
 Group 2: 30 embryonated chicken eggs were inoculated via amniotic fluid with 0.1 ml suspension of  $10^9$  CFU/ml of *E. coli* organism in brain heart infusion broth and 10 µl of 0.1% ChNPs.  
 Group 3: 30 embryonated chicken eggs were inoculated via amniotic fluid with 0.1 ml suspension of  $10^9$  CFU/ml of *E. coli* organism in brain heart infusion broth only.  
 Group 4: 30 embryonated chicken eggs were inoculated only with 10 µl of 0.1% ChNPs.

The eggs were incubated at 37°C with relative humidity of 80% and candled daily for embryo livability or mortality till hatch.

Microbial count

After hatching, chick's gut prior to feeding, caecal contents from four birds of each group were collected aseptically and homogenized, 10-fold serial dilutions were prepared in PBS, volumes of 0.1 ml of each dilution were spread on freshly prepared EMB agar media and incubated at 37°C for 24 h in an aerobic conditions and characteristic *E. coli* colonies were counted, results were expressed in CFU/ml (Pineda *et al.*, 2012).

Results

Bacterial isolation

Out of examined 100 yolk sac samples that were collected from diseased and freshly dead chicks, 36 *E. coli* isolates were recovered with an overall percentage of 36%.

Serological typing of *E. coli* isolates

The serotyping of 36 biochemically identified *E. coli* isolates, were belonged to 11 different serogroups. The most commonly isolated *E. coli* serogroups were O158 (6 isolates), O111:H4 and O44:H18 (5 isolates each), O124 and O86 (3 isolates each), O78, O2:H6, O142 and O26:H11 (3 isolates each), O26 and O125 (one isolate each).

Biofilm formation ability

All the tested *E. coli* serotypes (n.= 11) were confirmed to be biofilm producers by two assays (crystal violet tube method and congo red agar method). As shown in Figs. 2,3.

Antibiotic Sensitivity pattern of biofilm forming *E. coli* isolates

As shown in Table 1, all biofilm forming *E. coli* isolates were resistant to more than one antibacterial drug.

Antibacterial activity of nano-chitosan

Nano-chitosan showed an inhibitory effect on the biofilm forming *E. coli* isolates and the results are given in Table 2. The results were substantiated with Fig. 4.



Fig. 2. Congo red agar black dry crystalline colonies indicated biofilm formation

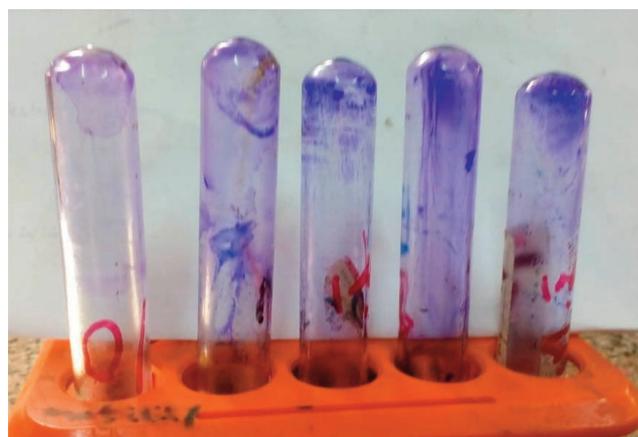


Fig. 3. Crystal violet tube assay, visible film lined the walls, and the bottom of the tube indicates biofilm formation of *E. coli*.

Table 1. Antibiotic Sensitivity pattern of 11 biofilm forming *E. coli* isolates

Antibiotics	Sensitive (%)	Resistant (%)
Ampicillin	0	100%
Amoxycillin	18.20%	81.80%
Neomycin	27.20%	72.80%
Enrofloxacin	63.60%	36.40%
Gentamicin	45.5	54.5
Colistin	54.5	45.5
Erythromycin	36.3	63.7
Doxycyclin	36.3	63.7
Cephradin	18.2	81.8
Vancomycin	54.5	45.5
Flurophencol	27.2	72.8

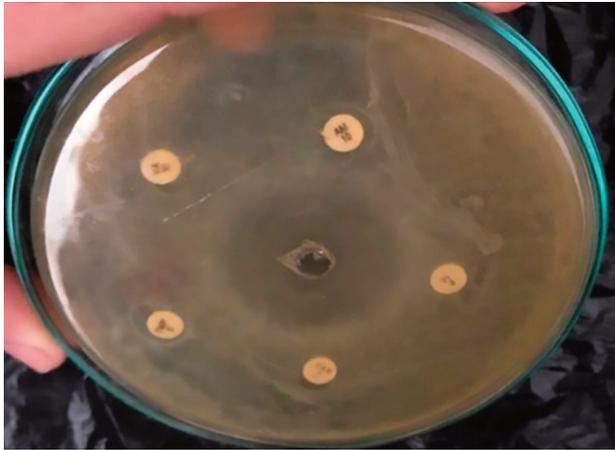
Table 2. Antimicrobial activity of nano-chitosan using agar well diffusion assay.

Biofilm forming <i>E. coli</i> strains	1	2	3	4	5	6	7	8	9	10	11
Inhibition zone diameter (mm) of ChNPs 0.1% Conc.	32	24	26	30	25	11	14	11	28	18	16

ChNPs: chitosan nanoparticles

Table 3. Effect of chitosan-nanoparticles on embryonated chicken eggs and *E. coli* count (CFU).

Chicken embryo inoculated groups	Colony forming unit (CFU)	Mortality		Morbidity	
		NO	%	NO	%
Group 1	$7.8 \times 10^6$	0/30	0	0/30	0
Group 2	$5.3 \times 10^8$	6/30	20	4/30	13.3
Group 3	$2.7 \times 10^{10}$	15/30	50	9/30	30
Group 4	$2.9 \times 10^5$	0/30	0	0/30	0

Fig. 4. Agar well diffusion test showing antimicrobial activity of Nano-chitosan against biofilm forming *E. coli* isolates.

## Discussion

The normal gut flora of chicks is highly complex and not yet fully understood (Shang *et al.*, 2018). Although the alimentary tract of the healthy newly hatched chick is usually sterile it rapidly becomes colonized by facultative anaerobic bacteria, particularly coliforms and streptococci (Board and Fuller, 1994). The percentage of *E. coli* isolated in chicks suffering from omphalitis in the present study was 36%, which agreed to some extent with that obtained by Waleed *et al.* (2019), who isolated *E. coli* at 34%, and also partially agreed with that reported by Amare *et al.* (2013), who reported that *E. coli* is the most common contaminant of yolk sacs in chickens and approximately 51.17% of chicks with omphalitis had such species in their yolk sacs.

Serological identification of *E. coli* isolates (n.=36) recovered from diseased chicks revealed that they were belonged to the eleven different serogroups. The most commonly detected *E. coli* serogroups were O158 (6 isolates), O111: H4 and O44:H18 (5 isolates each), O124 and O86 (3 isolates each), O78, O2:H6, O142 and O26:H11 (3 isolates each). O26 and O125 *E. coli* serotypes had been previously isolated from newly hatched chicks in Egypt as reported by Ashraf *et al.* (2014) and Waleed *et al.* (2019). While, Abd al-salam and Irfan (2013) recorded *E. coli* serotypes were O114 with 17.86% of the total isolates, O125 and O55 with 14.29% each, O111 and O26 with 10.71%.

In the present study, biofilm production was seen to be positive in all *E. coli* serotypes detected by crystal violet method and congo red method, these findings come in accordance with Khalil and El-Shamy (2012) and Karigoudar *et al.* (2019), who recorded that 100% of the tested *E. coli* strains were biofilm producer that exhibited multiple drug resistance to more classes of antibiotics. Even more, *E. coli* strains from chicken have been reported to form biofilm and can persist during food processing and consequently lead to greater risks of food contamination (Pavlickova *et al.*, 2017).

The obtained results revealed that biofilm forming strains showed maximum resistance to Ampicillin (100%), followed

by cephradine and amoxicillin (81.8%), gentamicin (54.5%), neomycin and florphenicol (72.8% each), doxycycline and erythromycin (63.7%), vancomycin (45.5%) and enrofloxacin (36.4%), these results are comparable to Wasyl *et al.* (2013), who recorded that *E. coli* strains isolated from chicken showed 64% resistance to amoxicillin, ampicillin, doxycycline and gentamicin. Resistance to antibiotics were higher among all biofilm producer *E. coli* isolates, confirming positive association between them. In the same context, Sevanan *et al.* (2011); Mohamad and Shalakany (2015) and Gharajalar (2017) concluded that *E. coli* biofilm producers from avian and human sources had multidrug resistance patterns. While results from this study disagree with Pavlickova *et al.* (2017), who recorded the highest antibiotic resistance in weak biofilm producers, the antibiotic resistance was present in 28% of non-biofilm producers. On characterization of nano-chitosan the obtained chitosan nanoparticles had small particle size (less than 100 nm) and spherical in shape, which completely agree with Parida *et al.* (2013) and Divya *et al.* (2017), who reported that ChNPs were found to have higher antimicrobial activity than chitosan and chitin. This is due to the spherical character of a nanoparticle, which gives it a low particle size and high specific surface and efficient antibacterial activity.

Concerning antibacterial activity of nano-chitosan against biofilm forming *E. coli*, ChNPs showed higher degree of inhibition than that done by antibiotics, maximum inhibition zone diameter recorded was 32 mm at concentration of 0.1% and the lowest with *E. coli* was 11 mm at the same concentration. More specifically, ChNPs represented the highest susceptibility to all tested biofilm forming *E. coli* strains (100%) with inhibition rate ranging from 11- 32 mm. As a result, the polycationic ChNPs interact with the negatively charged surface of bacteria (Qi *et al.*, 2004). Similarly, Abdeltwab *et al.* (2019) recorded that the maximum inhibition zone diameter recorded was 23 mm at concentration of 23 µg/ml and the lowest with *E. coli* was 21 mm at the same concentration. In the same context, ChNPs produced a zone of inhibition of 9.6-14 mm against *E. coli* and inhibited 85% of *E. coli* at a concentration of 40 mg/ml in a study done by Divya *et al.* (2017). Moreover, ChNPs were found to have antibiofilm activity, with an inhibition rate of up to 97% with 500 mg/ml.

With regard to bacterial count of *E. coli* in hatched chicks *E. coli* isolated from the intestine of newly hatched chicks in control group with average number  $7.8 \times 10^6$  CFU/g, with no mortality, as Kizerwetter-Swida and Binek (2008) assumed that bacteria including *E. coli* isolated from cecal content of newly hatched chickens 3 h after hatching with  $1.16 \times 10^6$  CFU/g. In addition, in chickens, *E. coli* is the main pathogenic microorganism that colonize the intestinal tract. These colonizers do not cause serious illness in birds and protection against pathogenic bacteria (Roto *et al.*, 2016).

In current study, under challenged condition as shown in Table 3, the CFU/g of *E. coli* in the intestine of challenged embryonated eggs in the group 2 (inoculated with 10 µl ChNPs and challenged with *E. coli*), recorded  $5.3 \times 10^8$  with 20% mortality rate; however, it demonstrated a noticeable decrease in CFU/g of *E. coli* of the experiment, when compared to group 3 (*E. coli* challenged group only), CFU of *E. coli* recorded

$2.7 \times 10^{10}$  with mortality rate 50%. As, Wang *et al.* (2003) described a reduction in *E. coli* recovered from the ceca of chicks treated with 0.1% of oligochitosan in the feed. In addition, *In Ovo* feeding of nanomaterial at the 18<sup>th</sup> day of incubation through amniotic route did not harm the developing embryo and did not affect the hatchability (Joshua *et al.*, 2016).

In the present study, the chicks in group 4 inoculated with 10  $\mu$ l ChNPs only showed marked reduction in the CFU/g of *E. coli* in the intestines to  $2.9 \times 10^5$  CFU *E. coli* /g with improvement of mortality rate to zero. So, *E. coli* populations were markedly affected by treatments with ChNPs. Subsequently, ChNPs could inhibit the growth and reproduction of *E. coli* and showed high antibacterial activity towards *E. coli*.

*In Ovo* administration of nanoparticles, acting as bioactive agents and as carriers of nutrients may be seen as a new method of nano-nutrition, providing embryos with bioactive compounds and/or with an additional quantity of nutrients or energy (Joshua *et al.*, 2016). Data from this study are in harmony with Huang *et al.* (2007), who suggested that the prebiotic effect of chitosan could be related to a chitosan attachment to the bacteria, leading to an immune response to this antigen, or by direct stimulation of the immune system. In addition, chitosan possesses several unique chemical and biofunctional properties useful for various applications in many fields including food and biomedicine (Jeon *et al.*, 2002). Furthermore, nanotechnology has the potential to reduce microbial load without resulting drug residues in poultry products, thus improving performance and immune status of poultry birds (Anwar *et al.*, 2019).

## Conclusion

Chitosan nanoparticles has the highest inhibitory effect on biofilm forming *E. coli* strains so this nanoparticle can be used as an effective antimicrobial agent. *In Ovo* inoculation with chitosan-nanoparticles reduce *E. coli* count without any adverse effect on hatchability.

## Conflict of interest

No conflict of interest has been declared by the authors.

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