

Protective effect of squilla chitosan–silver nanoparticles for *Dicentrarchus labrax* larvae infected with *Vibrio anguillarum*

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Abstract Antimicrobial nanoparticle therapy was proposed as an alternative strategy to reduce the use of antibiotics in larval-rearing systems. Antibacterial potential of the prepared squilla chitosan–silver nanoparticles and its protective effect on *Dicentrarchus labrax* (sea bass) larvae in the early stages were studied against *Vibrio anguillarum*. Different concentrations of squilla chitosan (Csq) and squilla chitosan–silver nanoparticles (Csq–AgNps) (1, 2, 5, 10, and 20 %) were, in vitro, tested against *V.anguillarum* and expressed as a role of Log₁₀ mean. Sea bass larvae were treated using: 10 % Csq and 5 % Csq–AgNps as effective inhibitory concentrations against the pathogen either encapsulated during the feeding regime or added directly to the model system via the water from the onset of 4 weeks. The long-term administration of Csq–AgNps through enriched food for both non-infected and infected systems had survival % of 74.5 ± 1.5 and 72.5 ± 2.5 , respectively. Larval clinical observations using Csq–AgNps were studied compared with the two controls. The current study found that 5 % encapsulated Csq–AgNps was enough to suppress infection and considered as an alternative to antibiotics in controlling virulent fish pathogens.

Keywords Sea bass larvae · *Vibrio anguillarum* · Squilla chitosan · Silver nanoparticles · Encapsulation

Introduction

Aquaculture is the fastest growing sector of agriculture in the world. However, infectious disease is a major impediment to the development of aquaculture and often a menacing problem in farming worldwide (Mohapatra et al. 2013). Sea bass (*Dicentrarchus labrax*) is one of the most economically important fish species in the marine Mediterranean aquaculture (Terova et al. 2011) and the most sensitive species to *Vibrio anguillarum* (Frans et al. 2011). *V. anguillarum* is the causative agent of vibriosis, a fatal haemorrhagic septicaemia, causing significant economic losses in the aquaculture industry (Austin and Austin, 2012). It was previously

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reported that homogenized animals (either fish or brine shrimp larvae) increase the mortality of sea bass larvae infected with different *V. anguillarum* strains (Li et al. 2014a).

Antibiotics, drugs, and chemicals have been utilized for many years in the treatment of vibriosis diseases (Stoskopf 1993). Improper administration of antibiotics against *V. anguillarum* outbreaks could result in the development of resistant bacteria strains; accordingly, there is growing interest in finding ways to formulate new natural types of safe and cost-effective biocidal materials (Plant and LaPatra 2011). Recently, the impact of many effective active agents on the virulence of *V. anguillarum* in a highly controlled model system to reduce mortality of sea bass larvae was investigated (Li et al. 2014b, 2015; Meloni et al. 2015).

Squilla chitosan (natural polysaccharide isolated from crustaceans), being cheaper, is one among several compounds which has received a great deal of attention for the preparation of micro and nanoparticles (Chun et al. 2007). Studies have shown that it is non-toxic and biodegradable (Kumar et al. 2008). It has unique properties, such as biocompatibility, biodegradability, low-immunogenicity, and nontoxicity (Thanou et al. 2001). Chitosan has the advantage of mucoadhesion to increase the transcellular and paracellular transport of macromolecules across the intestinal epithelial monolayer (Borchard 2001). Researchers have addressed the possibility of oral delivery of a drug by chitosan particles in fish (Gómez-Estaca et al. 2010; Li et al. 2013; Chen et al. 2015).

Silver or its compounds have been recognized for their broad-spectrum of antimicrobial activities, because they provide non-toxic carriers for drug applications (Liu et al. 2015). The antimicrobial properties of silver nanoparticles (AgNps) are well established, and several mechanisms for their bactericidal effects have been recently proposed (Swathy et al. 2014; Rahisuddin et al. 2015; Ajitha et al. 2015). Fabrication of chitosan/nanosilver film and its potential for antibacterial application were studied (Thomas et al. 2009). During the past few years, chitosan nanoparticle has a variety of aquaculture applications (Rajeshkumar et al. 2009; Vimal et al. 2013; Rivas-Aravena et al. 2015; Zhenga et al. 2016).

Accordingly, the present study was focused to determine the *in vitro* antimicrobial activity of prepared squilla chitosan Csq and Csq–AgNps against the pathogenic *Vibrio anguillarum* and their protective effects on *Dicentrarchus labrax* larvae under *in vivo* conditions.

Materials and methods

Bacterial strain

Vibrio anguillarum was collected from the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University. *V. anguillarum* was grown on Thiosulfate Citrate Bile salts sucrose (TCBS, DM218D) agar and the bacterial suspension was performed in nutrient broth with 2 % NaCl medium at 28 °C over-night up to the log phase of growth. The broth culture was transferred in sterilized falcon tubes and immediately centrifuged for 15 min at 5000g. Later, the pellet was scraped and resuspended in phosphate-buffered saline (PBS; pH 7.4) and bacterial turbidity, expressed as absorbance (A), was measured by a spectrophotometer (UNICO UV-2000) at OD₆₁₀, assuming 1×10^7 cfu ml⁻¹.

Preparation of chitosan nanoparticles

Chitosan preparation

Squilla mantis (squilla) chitosan was prepared by chitins de-acetylation according to Ramasamy et al. (2012). One kg squilla shell was treated using acid removal of calcium carbonate (demineralization), by hot reaction using 1.25-N HCl at room temperature for 1 h. Deproteinization was done by boiling the shells in 3 % NaOH for 20 min. The alkaline-treated shell (chitin) was then washed with water to completely remove the alkali. Drying at 30 °C and then grinded to powder in mortar. De-acetylation step was done using NaOH (5:1 w/w) at 200 °C for 3 h. The obtained chitosan was washed with distilled water. Chitosan was left to dry at 30 °C (Fig. 1), then dissolved in 1 % lactic acid for bacterial inhibitory effect determination and larval experimental designs.





Fig. 1 Prepared chitosan from *Squilla* shell

Synthesis of chitosan-based silver nanoparticles

Chitosan-based silver nanoparticles was prepared by dissolving 10 ml of 52.0-mM AgNO_3 solution in 10 mg ml^{-1} of the prepared chitosan solution (2 % v/v in acetic acid), then stirred until homogenous. The mentioned mixture allowed standing for 12 h at 95 °C. The color of the solution progressed from colorless to light yellow, and finally to yellowish brown which contains chitosan-based silver nanoparticles. The nanoparticles formed spontaneously were then incubated at room temperature for 30 min prior to the further analysis. The resultant nanoparticles were then collected by centrifugation (Centurion Scientific K3 Series) at 25,000 rpm for 30 min. The supernatants were discarded, and the nanoparticles were redispersed in distilled water. The obtained Csq–AgNPs solution was further used for inhibitory effect and larval treatment.

Characterization of chitosan nanoparticles

Characterization of Csq–AgNPs solution was evaluated for size through scanning electron microscopy (SEM) (JSM-5300 scanning microscope, JEOL at the Electronic Microscope Unit, Faculty of Science, Alexandria University, Egypt); and used to record Csq and Csq–AgNps morphology and size. The samples were treated first using ultrasonic radiation of the two examined samples, then, subsequently, gold coating performance before transferring them to the microscope (McMullan 2006).

Inhibitory effect of different Csq and Csq–AgNps concentrations

Different Csq and Csq–AgNps levels were used to examine the effective inhibitory concentration on *V. anguillarum*. Approximately 1×10^7 cfu ml^{-1} of tested bacterium were inoculated in 10 ml of phosphate-buffered saline (PBS; pH 7.4) supplemented with 1, 2, 5, 10, and 20 % for both Csq, Csq–AgNps corresponding to 0.1, 0.2, 0.5, 1.0, and 2.0 mg ml^{-1} of Csq and Csq–AgNps. Each concentration was applied on TCBS agar, incubated at 30 °C for 24 h and expressed as a role of Log_{10} mean colony forming units per ml.

The disc diffusion method was used to study the bactericidal activity of the appropriate concentrations Csq and Csq–AgNps (NCCLS 2002). Twenty-five ml of sterilized nutrient agar medium were mixed with 500 μL of over-night culture of *V. anguillarum*, then poured into two sterile petri-dish, as replicates, and allowed to solidify. Approximately 10 μL of Csq and Csq–AgNps were loaded on sterilized disc. The plates were incubated at 30 °C for 24 h, and the zone of inhibition (ZI) was measured.

Larvae acclimation and feeding regime

Eggs were obtained from induced spawning of sea bass broodstock at the marine hatchery of the National Institute of Oceanography and Fisheries (NIOF) Alexandria, Egypt. Clear water technique was carried out using the newly hatched larvae (0–4 day post hatch, dph). During the incubation period, eggs were placed in 1 m^3

incubation tanks in a flow-through system with filtered sea water (20 μm). Temperature, salinity, and oxygen concentration were 18 ± 1 °C, 39 ± 1.0 gm⁻¹, and 8 mg⁻¹, respectively. Before the challenge starts, the hatched larvae of sea bass were acclimatized for 15 days. Thirty-five larvae l⁻¹ were stocked in 200 L cylindroconical fiber glass tanks. Water was maintained by continuous flow (1 L min⁻¹) and gradually increased to 2 L min⁻¹ of filtered aerated sea water. The larvae were reared under dark condition for first 10 days of the acclimation conditions.

During the acclimation period, larvae were first fed with rotifers, *Brachionus plicatiles* from day 4–15 dph with density 10–25 rotifers ml⁻¹, followed by *Artemia nauplii* (A. F. Great salt Lake, USA), from day 10 to 45 dph (overlapping period between rotifers and *Artemia* diet is to allow the post-larvae acclimatize to the new live prey diet before removing the rotifer). Enriched metanauplii were maintained at 1–5 *Artemia* ml⁻¹, and gradually increased with increasing age of larvae. Health status of larval rearing was monitored for a month.

Bio-encapsulation process

Once the optimal concentrations were assessed, considering safe dose for larval rearing. Treatment levels of Csq and Csq–AgNps were added to the enrichment bucket (10 L) filled with filtered sea water and provided with continuous aeration at 28 ± 1 °C. *Artemia* were added at density of 200 nauplii ml⁻¹. After 24 h, the enriched *Artemia* were harvested and washed with sea water before distributed to the fiber glass tanks.

Experimental design and infection

The time course study has begun on ten tanks (each in triplicate), containing sea bass larvae. Four tanks were of Csq and Csq–AgNps exposure; two orally using encapsulated Csq and Csq–AgNps and two directly in larval-rearing water. Additional four tanks, with the same levels of exposure as above, were infected with 0.1 ml of *V. anguillarum* life cells, assuming 1×10^7 cfu ml⁻¹. Two control tanks were hosted by group of non-infected larvae (control 1) and infected larvae (control 2) at the ambient conditions.

These tanks were used for larval-rearing period in the post-larvae stage to study their effects on survival percentage, and their protective effect throughout the infection with *V. anguillarum*.

Larval-rearing condition and bacterial enumeration

The feeding schedule for the post-larvae was conventional by adding enriched *A. metanauplii* (ranged from 1 to 5 individuals' ml⁻¹) increasing according to grading age as mentioned above ended up day 45 dph. The light intensity was moderate along the experiment (800 lx). The aeration was appropriate and was supplied with air blower. The daily work was siphoning bottom dirt during the experiment except for the control (formal work of exchanging water was done for control tanks as usual).

To enumerate total *V. anguillarum* collected from infected tanks, samples were directly inoculated onto TCBs agar plates, incubated at 28 °C for 24 h, and then the number of *V. anguillarum* was expressed as a role of Log₁₀ mean. Bacterial enumeration on TCB medium was carried out to understand the impact of using Csq and Csq–AgNps tanks in the reduction or cease the virulence effect of this pathogen.

Clinical signs

Clinical signs demonstrated on sea bass for all tanks were observed under microscopic examination (Olympus SZ 61) to emphasize the protective effect of squilla chitosan–silver nanoparticles (Csq–AgNps) on the larvae of choice.

Statistical analysis

The experimental data were subjected to the statistical analysis, where the difference between the treatment means and within the treatment means was done by the two-way analysis of variance (ANOVA) test followed

by Post Hoc test (Duncan's method) using statistical package (SPSS 14.0) to find out the significant difference at 5 % level ($P < 0.05$) of significance.

Results

SEM of chitosan-based nanoparticles

The SEM micrograph revealed that the chitosan particles are dispersed as individual with a spherical to short rod shape and are homogeneously distributed around 2.10–2.92 μm in diameter (Fig. 2a), while silver nanoform is found with average sizes 22.73–39.77 nm (Fig. 2b).

Csq and Csq–AgNps inhibitory effect

The number of *V. anguillarum* colonies grown on TCB plates was directly proportional to the concentration of Csq and Csq–AgNps when 1×10^7 cfu ml^{-1} were applied. The presence of Csq and Csq–AgNps at a concentration of 0.5 and 0.2 mg ml^{-1} inhibited bacterial growth by 83 and 93 %, respectively. The effective inhibitory concentrations of Csq (1.0 mg ml^{-1}) and Csq–AgNps (0.5 mg ml^{-1}) were significantly ceased bacterial growth (Fig. 3). Number of *V. anguillarum* colonies as a function of the concentration expressed as a role of Log_{10} mean colony forming units per ml compared with Csq and Csq–AgNps-free control plates. The antibacterial activities of potent Csq and Csq–AgNps concentrations were tested against *V. anguillarum*. The result was presented in Fig. 4. Csq–AgNps recorded the highest IZ value at 32 and 24.4 mm for Csq. No activity was observed for dissolved solution (control).

Experimental treatment of *Dicentrarchus labrax*

The comparative survival % of *Dicentrarchus labrax* in the ten tanks (treated with Csq and Csq–AgNps), along with the two control groups, after being not infected or infected with using enriched life food or direct effect administration is shown in Table 1. After 45 dph, at the end of the experiment, the treated post-larvae with 5 % Csq–AgNps via encapsulation showed a significant high survival % at 74.5 ± 1.5 ($P < 0.05$) in the non-infected group. This is followed by 72.5 ± 2.5 % survival of the infected group, using the same level of encapsulated Csq–AgNps. At the same time, both non-infected and infected treated groups with 10 % encapsulated chitosan (Csq) showed survival by 66 ± 6.0 and 52.5 ± 2.5 %, respectively. No significance difference was observed using Csq and Csq–AgNps within the direct effect groups. Lower survival % was

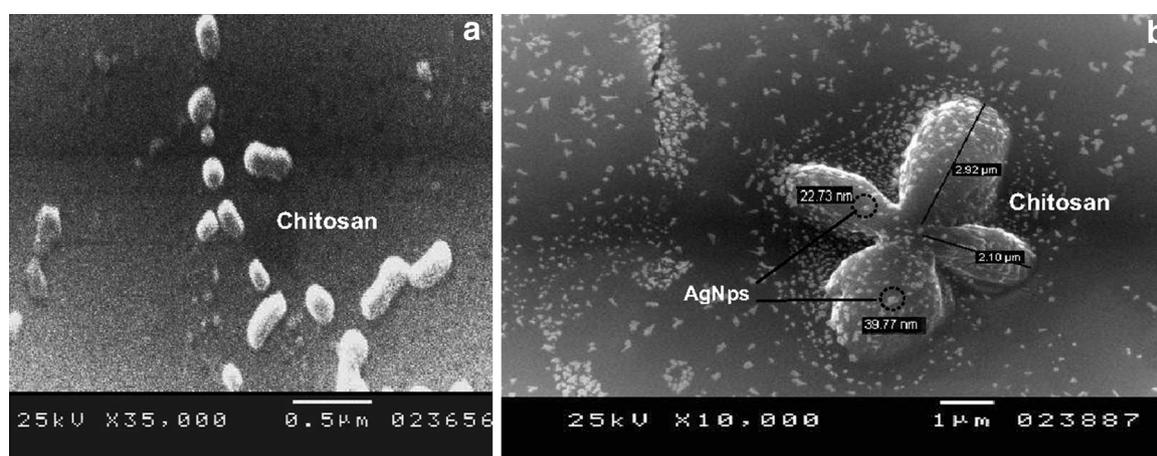


Fig. 2 Scanning electron microscope (SEM) showing squilla chitosan particles (Csq) (a) and chitosan-based silver nanoparticles (Csq–AgNps) (b)

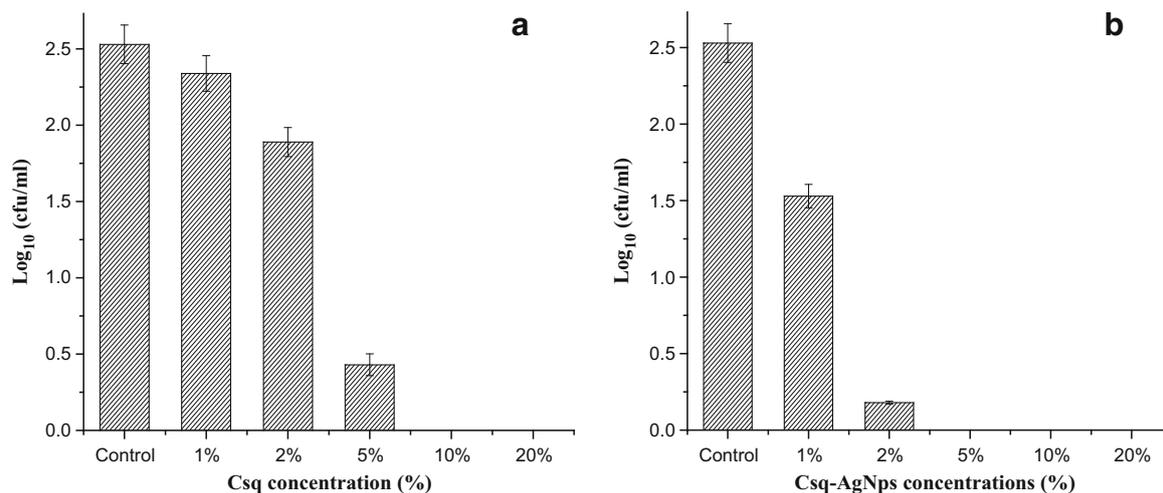


Fig. 3 Number of *Vibrio anguillarum* expressed as a role of Log₁₀ mean colony forming units per ml using different concentration of squilla chitosan (Csq) (a) and squilla chitosan–silver nanoparticles (Csq–AgNps) (b). Bars are expressed as mean \pm SE; $n = 5$, significantly different from the control ($P < 0.05$)

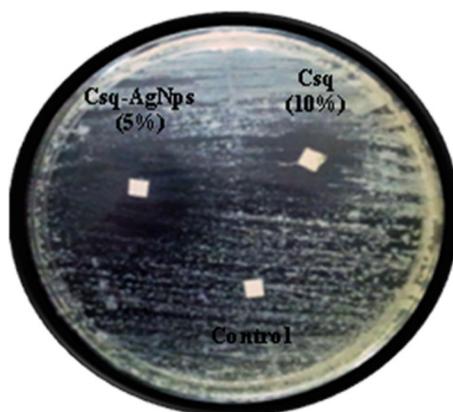


Fig. 4 Bactericidal test of 5 % Csq–AgNps and 10 % Csq showing the inhibition zones around the loaded disc against *V. anguillarum*

recorded at 25.5 ± 1.5 and 29.5 ± 0.5 for the infected larvae using direct effect of Csq and Csq–AgNps, respectively.

Quantification of *V. anguillarum*

Water samples from all infected groups were collected and applied on TCBS agar for *V. anguillarum* enumeration, as shown in Table 2. The Log₁₀ mean *V. anguillarum* (cfu ml⁻¹) were significantly decreased ($P < 0.05$) resulting in a density of 0.3 ± 0.02 and 0.81 ± 0.04 for infected sea bass larvae fed with encapsulated Csq–AgNps (5 %) and Csq (10 %), respectively, at the end of the experiment. No significance difference was observed using the direct effect of 5 % Csq–AgNps, where, density was recorded at 2.18 ± 0.11 cfu ml⁻¹ compared with control.

Microscopic examination and clinical observation

Sea bass morphometry and clinical signs were observed during the 30th days of the encapsulated experiment using Csq–AgNps (5 %) for both non-infected and infected condition. Microscopic examination was carried out to show the morphometric growth phases from hatchery till post-larvae stages (Fig. 5). After 45 dph, full



Table 1 Survival (%) of *Dicentrarchus labrax* larvae using eight Csq and Csq–AgNPs trials of the two major categories: encapsulated live food and direct supplement via sea bass-rearing water, from 15th till 45th DPH refer to control 1 (non-infected larvae) and control 2 (infected larvae)

| Exposure time (day) | Control 1 | | Control 2 | | Encapsulated | | | Direct effect | | |
|---------------------|-----------------------|-----------------------|-------------------------|-------------------------|-------------------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|
| | Csq | Csq + infected | Csq–AgNPs | Csq–AgNPs + infected | Csq | Csq + infected | Csq–AgNPs + infected | Csq | Csq + infected | Csq–AgNPs + infected |
| 15 th | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |
| 18 th | 75 ± 2.5 ^a | 68 ± 6.0 ^a | 94 ± 4.0 ^b | 94 ± 1.0 ^b | 96.5 ± 1.5 ^b | 96 ± 1.0 ^b | 96 ± 1.0 ^b | 96 ± 1.0 ^b | 74 ± 1.0 ^a | 94 ± 1.0 ^b |
| 21 st | 70 ± 1.0 ^a | 56 ± 2.5 ^b | 91 ± 5.0 ^c | 90 ± 0.0 ^c | 92.5 ± 3.5 ^c | 89 ± 4.0 ^c | 89.5 ± 4.5 ^c | 89 ± 4.0 ^c | 68 ± 2.0 ^{ab} | 89 ± 1.0 ^c |
| 24 th | 68 ± 2.5 ^a | 52 ± 0.5 ^b | 86 ± 4.0 ^c | 86 ± 1.0 ^c | 88.5 ± 3.5 ^c | 81.5 ± 0.5 ^c | 81.5 ± 0.5 ^c | 84 ± 1.0 ^c | 66.5 ± 2.5 ^a | 82.5 ± 1.5 ^c |
| 27 th | 62 ± 0.0 ^a | 48 ± 2.0 ^b | 80.5 ± 4.5 ^c | 80.5 ± 0.5 ^c | 86.5 ± 2.5 ^c | 79.5 ± 0.5 ^c | 79.5 ± 0.5 ^c | 77.5 ± 2.5 ^c | 64 ± 3.0 ^a | 77.5 ± 4.5 ^c |
| 30 th | 61 ± 0.0 ^a | 44 ± 1.0 ^b | 76.5 ± 3.5 ^c | 76 ± 1.0 ^c | 84 ± 4.0 ^c | 79.5 ± 0.5 ^c | 79.5 ± 0.5 ^c | 72 ± 3.0 ^c | 58 ± 5.0 ^{ab} | 72.5 ± 4.5 ^c |
| 33 rd | 57 ± 2.0 ^a | 40 ± 4.0 ^b | 74 ± 5.0 ^c | 63 ± 3.0 ^{ac} | 78.5 ± 0.5 ^c | 78 ± 1.0 ^c | 78 ± 1.0 ^c | 67.5 ± 2.5 ^{ac} | 52.5 ± 2.5 ^{ab} | 66.5 ± 4.5 ^{ac} |
| 36 th | 54 ± 1.0 ^a | 36 ± 2.5 ^b | 72 ± 5.0 ^c | 56 ± 1.0 ^a | 76.5 ± 0.5 ^c | 75.5 ± 0.5 ^c | 75.5 ± 0.5 ^c | 58 ± 8.0 ^a | 45 ± 5.0 ^{ab} | 62 ± 2.0 ^a |
| 39 th | 50 ± 0.5 ^a | 11 ± 2.5 ^b | 69.5 ± 5.5 ^c | 50 ± 0.0 ^a | 75.5 ± 0.5 ^c | 73 ± 3.0 ^c | 73 ± 3.0 ^c | 55 ± 0.0 ^a | 42 ± 3.0 ^{ad} | 51.5 ± 0.5 ^a |
| 42 nd | 43 ± 2.5 ^a | 5 ± 1.0 ^b | 68 ± 6.0 ^c | 47 ± 2.0 ^a | 75 ± 1.0 ^c | 73 ± 3.0 ^c | 73 ± 3.0 ^c | 47 ± 2.0 ^a | 32.5 ± 2.5 ^d | 46 ± 2.0 ^a |
| 45 th | 40 ± 4.0 ^a | 0 ± 0.0 ^b | 66 ± 6.0 ^c | 52.5 ± 2.5 ^c | 74.5 ± 1.5 ^c | 72.5 ± 2.5 ^c | 72.5 ± 2.5 ^c | 42.5 ± 2.5 ^a | 25.5 ± 1.5 ^d | 41.5 ± 0.5 ^a |

No significant difference ($P < 0.05$) for values in row with the same superscript

Table 2 Log₁₀ mean count of *Vibrio anguillarum* in rearing water collected from infected Csq and Csq–AgNPs tanks for both categories encapsulated live food and direct effect, from 15th till 45th DPA of *Dicentrarchus labrax* larvae

| Days | Control | Csq | | Csq–AgNPs | |
|------------------|--------------------------|---------------------------|---------------------------|--------------------------|--------------------------|
| | | Encapsulated | Direct effect | Encapsulated | Direct effect |
| 15 th | 0.48 ± 0.02 ^a | 0.93 ± 0.05 ^b | 0.88 ± 0.04 ^b | 0.81 ± 0.04 ^b | 1.24 ± 0.06 ^c |
| 18 th | 0.78 ± 0.04 ^a | 1.19 ± 0.06 ^b | 1.26 ± 0.06 ^b | 1.19 ± 0.06 ^b | 1.61 ± 0.08 ^b |
| 22 nd | 1.4 ± 0.07 ^a | 1.361 ± 0.07 ^a | 1.75 ± 0.09 ^{ab} | 1.38 ± 0.07 ^a | 1.96 ± 0.10 ^b |
| 27 th | 1.78 ± 0.09 ^a | 1.43 ± 0.07 ^{ad} | 1.82 ± 0.09 ^c | 1.2 ± 0.06 ^d | 2.15 ± 0.11 ^c |
| 33 rd | 1.99 ± 0.10 ^a | 1.34 ± 0.07 ^b | 1.73 ± 0.09 ^a | 1.08 ± 0.05 ^b | 2.21 ± 0.11 ^a |
| 36 th | 2.17 ± 0.11 ^a | 1.22 ± 0.06 ^b | 1.7 ± 0.09 ^{ab} | 1.04 ± 0.05 ^b | 2.24 ± 0.11 ^a |
| 41 st | 2.18 ± 0.11 ^a | 1.06 ± 0.05 ^{bc} | 1.63 ± 0.08 ^b | 0.88 ± 0.04 ^c | 2.22 ± 0.11 ^d |
| 45 th | 2.2 ± 0.11 ^a | 0.81 ± 0.04 ^b | 1.57 ± 0.08 ^c | 0.3 ± 0.02 ^d | 2.18 ± 0.11 ^a |

No significant difference ($P > 0.05$) for values in row with the same superscript

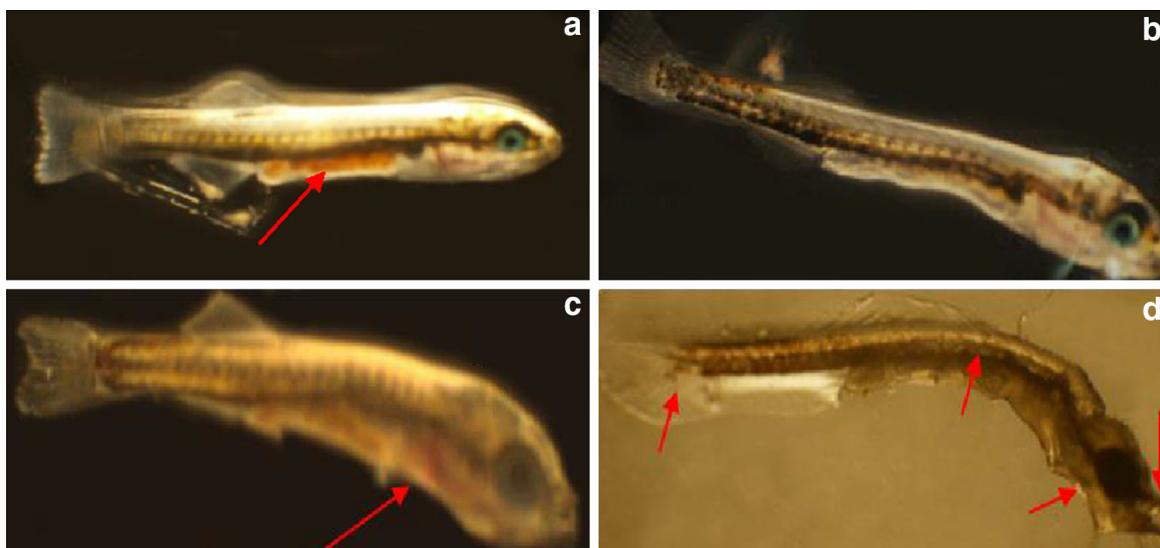


Fig. 5 Full healthy post-larva (a) after 45 DPH fed on encapsulated Csq–AgNPs compared with control 1 (b). The healthy larva with the gut containing the digested enriched live-feed (*Artemia metanauplii*), well developed dorsal, ventral, and tail fin. Control larvae showed delaying in the development, the dorsal, and ventral fins not full formed. Slight healthy post-larva (c) after 45 dph infected with *V. anguillarum* and fed on encapsulated Csq–AgNPs compared with control 2 (d). The health larva with the digested enriched live-feed (*Artemia metanauplii*) gut and well developed dorsal, ventral, and tail fin. Infected control larva appeared with severe deformation in the lower and upper jaw, delaying in the development, tail fin got erosion, the dorsal and ventral fins not full formed, and deformed vertebral column

healthy post-larva fed on encapsulated Csq–AgNPs showed a well developed dorsal, ventral, and tail fin; however, post-larva infected using the same enriched live food showed slight healthy larvae with good growth development. The two controls non-infected and infected larva using the same feeding regime appeared with severe deformation in the lower and upper jaw, delaying in the development, tail fin got erosion, the dorsal, and ventral fins not full formed and deformed vertebral column. Encapsulated Csq–AgNPs reduced clinical signs showed healthy treated larvae with the appearance of asymmetrical pigmentation in the post larval stage.

Discussion

Most of marine fish larvae reared in aquaculture hatch with immature digestive and immune system being extremely susceptible to diseases. Thus, sub-optimal rearing conditions are often associated with high larval



mortalities, leading to high production losses (Santos et al. 2012). This work aimed to improve immunity, survival percentage and development of sea bass larvae via chitosan, and silver nanoparticles in different ways of applications with and without infection.

Chitosan offer a better, safe, environment-friendly, and efficient way to control infectious pathogens in aquaculture (Rodrigues et al. 2006). Researchers have continued using chitosan to attempt and develop novel methods of control fish larvae from its opportunistic pathogens in the hope that it will prove protective (Kotzamanis et al. 2007; Kumar et al. 2008; Tian et al. 2008). Synthesis based on the biotransformation of silver salts into AgNps has been recently reported using prepared chitosan (Rivas-Aravena et al. 2013). It has been well documented that the bactericidal properties of chitosan films loaded with silver nanoparticles to achieve complete elimination of antibiotic resistant were studied (Regiel et al. 2013). In addition, silver nanoparticles were synthesized via chitosan as both reducing and stabilizing agent showed excellent antimicrobial activities (Abdel-Fattah et al. 2014; Hebeisha et al. 2014).

In the present study, squilla chitosan-based silver nanoparticles were prepared and explored its application on *Dicentrarchus labrax* larvae to control *V. angularium* infections. This chitosan nanoform was able to give full levels of protection to experimental challenge with *V. anguillarum*, which indicated by the reduction in the number of fish with no or slight morphometric alterations compared with control.

Synthesized nanoparticles dispersed in a spherical shape are homogeneously distributed with average sizes range from 22.73 to 39.77 nm using SEM suggesting that the chitosan silver nanoparticles were appropriate for fish oral administration. In comparison, data reported by Li et al. (2013) showed the diameters of most chitosan-based nanoparticles obtained were around 200 nm using to protect black sea bream from *V. parahaemolyticus*.

Results obtained from the growth of *V. angularium* at different Csq–AgNps concentrations are directly proportional to the reduction in cfu of *V. angularium* on TCB agar plates. It was shown that more than 0.2 mg ml⁻¹ of Csq –AgNps effectively reduced the colony forming units when compared to the control. Vaseeharan et al. (2010) demonstrated that the use of AgNps synthesized by tea leaf extracts at the concentration of 0.075 mg ml⁻¹ significantly reduced *Vibrio harveyi* growth. In addition, the antibacterial activities of biocompatible dispersed AgNps in chitosan-derived solution using 0.0125 mg (Ag) per mg (Cs) were investigated by Trana et al. (2010). Synthesis of silver nanoparticles (0.1 mg ml⁻¹) using cashew nut shell liquid with its antibacterial activity against fish pathogens was studied by Velmurugan et al. (2014).

Application of Chitosan-metallic nanoparticles on controlling bacterial pathogens in aquatic animals still remains as an unexplored area of research, and the present study made a successful attempt to demonstrate the ability of encapsulated Csq–AgNps on controlling aquaculture diseases. The idea behind bioencapsulation is to use live-feed organisms as carriers of nutrients, or chemotherapeutants, offered to the larvae. Incorporating antibacterial agents in live-feed organisms is well-known, using *Artemia* as carrier for the delivery of various antibacterial agents to marine larvae (Figueiredo et al. 2009).

The survival percentage of non-infected and infected sea bass fed on 5 % encapsulated Csq AgNps showed its highest significant value at 74.5 ± 1.5 and 72.5 ± 2.5 % with a reduction in mortality by 25 %, and 28 %, respectively. Similar to our results, Vaseeharan et al. (2010) recorded the reduction into 29 % in shrimp mortality challenged by *V. harveyi* during the administration of AgNps synthesized by tea leaf extracts after the 14th day of experiment. A relative percent survival of 46 % was recorded using chitosan nanoparticles for oral delivery in Asian sea bass (*Lates calcarifer*) to protect from *Vibrio anguillarum* (Kumar et al. 2008). Black seabream was protected from *V. parahaemolyticus*, with 72.3 % relative percentage survival post-vaccination with chitosan nanoparticles (Li et al. 2013). This demonstrates that Csq–AgNps can be used as an alternative to antibiotic administration for the control of *V. angularium* infections in sea bass culture.

It was also observed that encapsulated Csq–AgNps has effective bactericidal activity by reducing *V. angularium* count from the collected water samples for the 30 days of the experiment upon the Log₁₀ mean from 2.5 to 0.03 cfu/ml. In comparison, the development and antibacterial activity of cashew gum-based silver nanoparticles showed a reduction in pathogens count in Log₁₀ cfu/ml from 3 to 2 to extremely no growth (Quelemes et al. 2013). Gradually decrease in spore-forming bacteria count Log₁₀ cfu ml⁻¹ from 5.5 to 4.5 using synthesized silver nanoparticles with a synergistic action of cinnamaldehyde was studied by Ghosh et al. (2013). Several mechanisms of growth inhibition by AgNps were postulated in bacteria, binding on the sulfur-rich proteins present in the membrane surfaces, DNA binding properties, and inhibition of cell cycle (Feng



et al. 2000). Interestingly, the amount of AgNPs was necessary for inhibition of metabolic pathways of the pathogenic bacteria (Pommerville and Alcamo 2006).

Conclusion

Sea bass is one of the commercial important fish that commonly used in the seed hatchery production system. The onset of the first 4–6 weeks larval feeding is a crucial step in the young life; therefore, it is important to overcome this delicate and key phase. Therefore, it was demonstrated that the effect of encapsulated Csq–AgNps on sea bass larval rearing showed promising results on controlling *V. anguillarum* infections and improve survival percentage for more economical and practical mass immunization in seed production.

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