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Full Length Research Paper

In Vitro Evaluation the Disinfectant Efficacy of Silver nanoparticles and Envirolyte-Anolyte for the Control of *Flavobacterium columnare*

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Abstract
An experiment was performed to evaluate the in vitro efficacy of silver nanoparticles (Ag NP) and Envirolyte-Anolyte (Env-1/1000) against Flavobacterium columnare. F. columnare was isolated from naturally infected Tilapia fish (Oreochromus niloticus). In vitro, F. columnare treated with Ag NP at 0.1 mg/L for 1 h exhibited a 90% reduction in colony-forming units (CFU).While; Env required half an h at 1/1000 to redue 80 % of colony-forming units (CFU). The results suggest that Ag NPs and Env are beneficial for reducing F. columnare load in the water column and possibly on fish. Further research is warranted to investigate the value of Ag NPs and Env as a therapeutic agent for fish with a columnare infection and as a treatment to prevent further spread of columnare in a fish population.

Keywords: *Envirolyte-Anolyte, Nanoparticles, F. columnare, Oreochromus niloticus, Disinfection.*

Introduction

Flavobacterium columnare, a Gram-negative gliding bacterium, is the causative agent of columnarae disease. Flavobacterium columnare is an important bacterial pathogen of freshwater fish), it is a dermotropic disease that affect a wide range of cultured and wild ranging freshwater fishes around the globe (Decostere et al., 1999; Figueiredo et al., 2005). It is believed that the infection has a worldwide distribution, with economic losses associated with skin lesions and mortality (Bader et al., 2003).

It is one of the main causes of mortality in tilapia rearing and is responsible for large economic losses worldwide (Sebastião *et al.*, 2011). It affects many cool- and warm water fish species, typically in warm waters at 20–25 ◦C and above; however, it is not unusual to diagnose columnarae disease in many cultured and free-ranging fish species are considered at risk for infection and possible disease (Austin and Austin ,2007 ;Starliper ,2010).
Columnarae is a contagious disease that can be transmitted horizontally through direct contact, skin wounds as well as through oro-fecal route (Bullock et al.1986; Welker et al., 2005). Due to the ubiquitous nature of the *F. columnare* in the freshwater, an injury to the skin or gills of fish with elevation of water temperature may quickly initiate the co-lumnarae infection.

The health of the fish depends mainly on water with its chemical, physical and microbial characters . In fact, no wild or cultured freshwater fish, including ornamental fish in tanks, is known to be totally resistant to columnarae (Plumb 1999).

Clinical signs of columnarae include frayed necrotic fins with grayish to white margins and depigmented and necrotic skin. The bacteria attack the fins, skin, and gills of fish. The gill lesions have white to brown necrotic areas; viscera, however, exhibit little or no pathology, even when the infection is systemic (Plumb 1999).

In vitro, *F. columnare* treated with KMnO4 at 2 mg/L for 8 h exhibited a 70% reduction in colony-forming units (CFU). A minimum KMnO4 concentration of 10 mg/L was needed to inhibit bacterial growth (Darwish et al.,2008)

There is a pressing demand to discover novel strategies and identify new antimicrobial agents from natural and inorganic substances to develop the next generation of drugs or agents to control microbial infections (Rai and Bai, 2011).

Nanoparticles usually ranging in dimension from 1-100 nanometers (nm) have properties unique from their bulk equivalent. With the decrease in the dimensions of the materials to the atomic level, their properties change. The nanoparticles possess unique physico-chemical, optical and biological properties which can be manipulated suitably for desired applications (Feynman, 1991). Moreover, as the biological processes also occur at the nanoscale and due to their amenability to biological functionalization, the

nanoparticles are finding important applications in the field of medicine (Parak et al., 2003).

Our objectives were to evaluate the in vitro disinfectant efficacy of silver nanoparticles (Ag NP) and desanol-711 (Des) against *Flavobacterium columnare*, and to assess their antibacterial activity.

Material and Methods

Bacteria.—Virulent *F. columnare* (isolate from infected Tilapia fish, *O. niloticus*) was cultured on Hsu–Shotts medium at 25^{8}C. The isolate was presumptively identified by the biochemical method of Griffin (1992) using both cultural characteristics and conventional biochemical tests

In vitro sensitivity of *F. columnare*.—Two in vitro experiments were conducted. The first experiment was used to determine the reduction of the colony-forming units (CFU) of *F. columnare* treated with Ag NP at 0.5 and 1 mg/L for half and 1 h. The second experiment was to determine the reduction of the colony-forming units (CFU) of *F. columnare* treated with Env at 0.5 and 1 mL/L for half and 1 h.

Flavobacterium columnare was inoculated into *F. columnare* growth medium (FCGM) and incubated at 28^{8}C for 24 h (Farmer 2004). The bacteria were centrifuged and suspended in sterile physiological saline (0.85% NaCl). Optical density was used to standardize the bacterial suspension inoculated into 96 microwell plates (Darwish et al. 2000). The bacteria treated were inoculated into Ordal's agar; bacterial colonies were visually reported as present or absent.

Disinfectants

Silver oxide-nanoparticles - (Ag Ns) stock solution was prepared by adding 0.5 and 1 mg of 15 nanometer diameter Ag NPs powder (IBU-tec / Nano technology, 15 nm in diamter) in 100 mL of sterile saline solution. The stock solution was diluted and inoculated into the wells containing the bacteria. The test wells contained a final bacterial concentration of 6.5 3 10^{8} CFU/mL and Ag NP concentration of 0.5 and 1 mg/L. The positive control wells contained sterile saline with bacteria but no Ag NP, whereas the negative control wells had sterile saline but no bacteria or Ag NP. The treatments were incubated at room temperature (22–24°C) for half and 1 h; three replicate wells were used for each treatment. After the incubation period, we serially diluted 100 µL from each well and determined CFU on Ordal's agar after 48 h of incubation at 28°C (Herigstad et al. 2001).

Envirolyte-Anolyte (Env) It contains various mixed oxidants predominantly hypochlorous acid and sodium hypochlorite (HClO, ClO_2, $HClO_3$, $HClO_4$, H_2O_2, O_2, ClO^-, ClO_2^-, ClO_3^-, O^-, HO_2^-, OH^- - working substances, pH from 2.0 to 8.5, 1\500 =:2 mg /L active chlorine, 1\1000=:1mg /L active chlorine.

The second in vitro experiment employed the methods of the first experiment, but the Env concentrations used ranged at 0.5 and 1 mL/L for half and 1 h.

Results and Conclusion

F. columnare infected tilapia showed marked skin ulcerations at the head region with marked liquefaction of the underlying (Fig.1$_a$) , marked signs of fin rot (Fig.1$_b$) , and on the mouth, the lesions looked moldy or cottony (Fig.1$_c$) with severe corneal opacity (Fig.2) The gill lesions were typically necrotic and the filaments disintegrate as the bacteria invade them (Fig.3).

Colony morphology on Hsu-Shotts agar plates yellow, round with entire margins, convex, rhizoid, and strongly adherent to agar (Fig.4$_a$). Gram stained smear made from the yellow colonies of *F. columnare* showed the presence of long (5-8 µm) pleomorphic Gram negative bacilli (Fig.4$_b$). *F. columnare* isolated from infected tilapia showed the following biochemical reactions: Gelatin hydrolysis ,catalase , Growth at 25º;C30ºC and37ºC , Growth with 0.5% NaCl, Growth in the presence of Neomycin sulfate, Growth in the presence of Polymyxin B are positive while: Growth on Trypticase Soy broth (TSB) and Growth with 1% NaCl are negative.

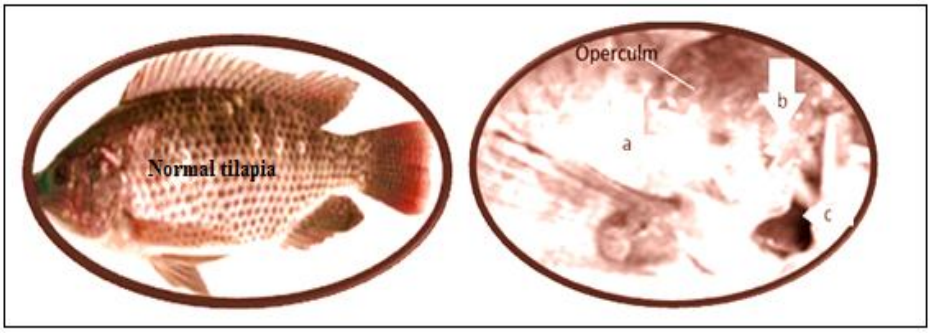


Fig.1$_{a, b, c}$: *F. columnare* infected tilapia showed marked skin ulcerations (a) at the head region with marked liquefaction of the underlying frontal tissues (b),

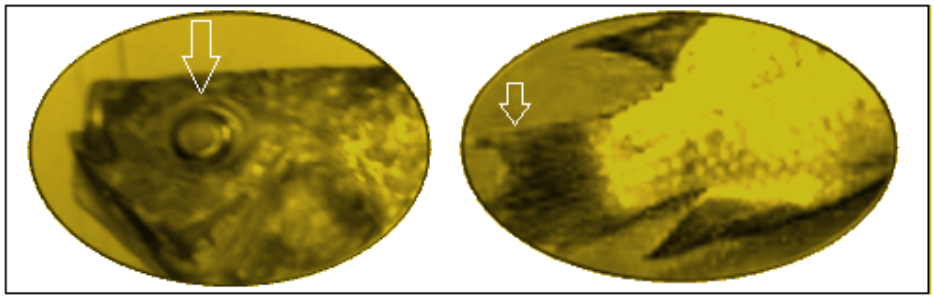
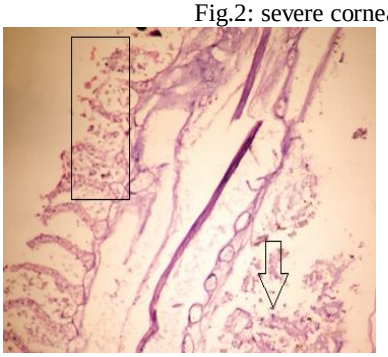
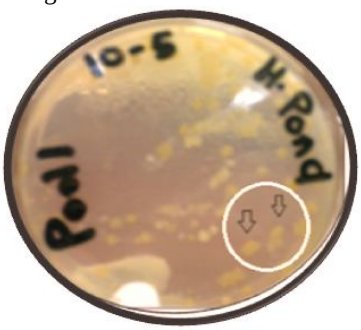


Fig.2: severe corneal opacity and marked signs of fin rot.





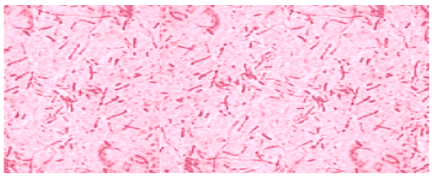


Fig.3: Photomicrograph of tilapia gills showing degeneration and necrosis of the gills lamellae H&E X 400). The gill lesions were typically necrotic and the filaments disintegrate as the bacteria invade them (arrow &the rectangular).

Fig.4$_a$: Primary cultured *Flavobacterium* colonies are on Hsu-Shotts agar showing the rhizoid yellow pigmented colonies. Colony morphology on Hsu-Shotts agar plates Yellow, round with entire margins, convex, rhizoid, and strongly adherent to agar.

Fig.4$_b$: Gram stained smear made from the yellow colonies of *F. column are* showed the presence of long (5-8 µm) pleomorphic Gram negative bacilli.

Ag NPs

Bacterial exposure to Ag NPs concentration of 0.5 mg/L for half h resulted in a count of 2.4 10^6 CFU/mL, and 2 10^5 CFU/mL at concentration of 0.5 mg/L for 1 h. *F. columnare* treated with Ag NPs at 1 mg/L for half and 1 h. resulted in a count of 2.6 10^2 CFU/mL, and 0.4 10^2 CFU/mL, respectively (Table 1). Whereas the count was 2 10^7 CFU/mL for untreated bacteria suspended in saline.

This difference was statistically significant and represents a huge reduction in CFU count *(P<0.05)*. Ag NPs concentration of 1 mg/L applied for 1 h was required to prevent all bacterial growth on Ordal's medium (Table 1).

Table.1: Efficacy of Silver oxide-nanoparticles (Ag Ns) and Envirolyte-Anolyte (Evn) on *F. columnare*

Disinfectants / Time	CFU/mL after disinfection: Half hour	One hour
Ag NPs		
0.5 mg/L	$2.4X10^6$	$2X10^5$
1 mg/L	$2.6X10^{2}$ *	$0.4 X 10^{2}$*
Env		
0.5 mL/L	$2.6 X10^6$	$2.1X 10^5$
1 mL/L	$1.6 X 10^{2}$*	0 *

The count was $2X10^7$ CFU/mL for untreated bacteria suspended in saline. * : Statistically significant and represents a huge reduction in CFU count. *(P<005)*.

The mechanism of antibacterial effect of silver nanoparticles has been reported in the literature (Sondi and Salopek-Sondi, 2004), which suggests that the particles are bactericidal.

The bactericidal effect of Ag NPs typically ranging from 2 to 5 nm has been investigated using green fluorescent protein (GFP)-expressing recombinant *Escherichia coli* (Alexander et al., 2008). Apart from the conventional viability tests, the morphological changes of the fluorescent bacteria and electrophoretic analysis of cellular DNA and protein migration profiles were performed to establish the effect of silver nanoparticles on GFP bacteria. The silver nanoparticles of less than 10 nm diameters attached to the bacterial cell wall causes perforation of the cell wall, which leads to the cell death. This study suggests that the mode of action of silver nanoparticles is that the NPs get attached to the sulfur-containing proteins on the bacterial cell wall, leading to increased permeability of the membrane, finally causing cell death (Alexander et al., 2008).

There are also studies reporting that metal ions induce generation of intracellular reactive oxygen species in bacterial cells (Stohs and Bagchi,1995) Ag ions released by active surfaces of silver nanoparticles and silver oxide present on the surfaces of these nanoparticles are reported to be the actual biocidal agents (Sondi et al.,2004). The silver ions enter the bacterial cells, where they are reduced as the cell attempts to remove them from the cell interior, eventually leading to cell destruction (Morones et al., 2005). It has been recently demonstrated that silver nanoparticles of less than 10 nm diameter make pores on the bacterial cell walls. The cytoplasmic content is released to the medium, which leads to cell death without affecting the intracellular and extracellular proteins and nucleic acids of the bacterium (Sondi et al., 2004).

The antibacterial properties of zinc oxide nanoparticles were investigated using both Gram-positive and Gram-negative microorganisms. These studies demonstrate that ZnO nanoparticles have a wide range of antibacterial activities toward various microorganisms that are commonly found in environmental settings. The antibacterial activity of the ZnO nanoparticles was inversely proportional to the size of the nanoparticles in *S. aureus*. Surprisingly, the antibacterial activity did not require specific UV activation using artificial lamps; rather activation was achieved under ambient lighting conditions. Northern analyses of various reactive oxygen species (ROS) specific genes and confocal microscopy suggest that the antibacterial activity of ZnO nanoparticles might involve both the production of reactive oxygen species and the accumulation of nanoparticles in the cytoplasm or on the outer membranes. Overall, the experimental results suggest that ZnO nanoparticles could be developed as antibacterial agents against a wide range of microorganisms to control and prevent the spreading and persistence of bacterial infections (Raghupathi et al., 2011).

Several mechanisms have been proposed to explain the inhibitory effect of silver nanoparticles on bacteria. It is assumed that the high affinity of silver towards sulfur and phosphorus is the key element of the antimicrobial effect. Due to the abundance of sulfur-containing proteins on the bacterial cell membrane, silver nanoparticles can react with sulfur-containing amino acids inside or outside the cell membrane, which in turn affects bacterial cell viability. It was also suggested that silver ions (particularly Ag+) released from silver nanoparticles can interact with phosphorus moieties in DNA, resulting in inactivation of DNA replication, or can react with sulfur-containing proteins, leading to the inhibition of enzyme functions (Gupta ,1998; Matsumura et al.,2003). The general understanding is that Ag nanoparticle of typically less than 20 nm diameters get attached to sulfur-containing proteins of bacterial cell membranes leading to greater permeability of the membrane, which causes the death of the bacteria (Morones et al., 2005).

The unique physiochemical properties of the nanoparticles combined with the growth inhibitory capacity against microbes has led to the upsurge in the research on nanoparticles and their potential application as antimicrobials.

Envirolyte-Anolyte (Evn)

Exposure of *F. columnare* to Envirolyte-Anolyte (Evn) at concentration 0.5 mL/L for half h resulted in a count of 2.6 10^6 CFU/mL, and 2.1 10^5 CFU/mL at concentration of 0.5 mL/L for 1 h. *F. columnare* treated with Evn at 1 mL/L for half and 1 h. resulted in a count of 1.6 10^2 CFU/mL, and 0 CFU/mL, respectively. Whereas the count was 2 10^6 CFU/mL for untreated bacteria suspended in saline. This difference was statistically significant and represents a huge reduction in CFU count (Table 1).

Ag NPs concentration of 1 mg/L or Evn 1 mL/L applied for 1 h were required to prevent all bacterial growth on Ordal's medium.

Ag NPs and Env reduced the number of *F. columnare* in the water and potentially reduced the number of bacteria on the surface of the fish. Both factors could be beneficial for reducing the number of bacteria capable of attaching to fish to cause disease or the number of superficial bacteria that attach to and colonize the host surface. Using Ag NP and Evn in a natural infection therefore could inhibit further spread of the infection or might allow mildly infected fish to recover.

The disease is induces by overcrowding the fish in a static system, which would result in increased bacterial load in the water column (reproducing bacteria were not flushed out) as well as creating stressful conditions for the fish (Jee and Plumb, 1981). Thomas-Jinu and Goodwin (2004) also used a static system and added the bacteria to the water. No clinical signs were reported in this model, and all the mortalities occurred within 48 h after the bacterial challenge started. The in vivo results of the current experiment indicate that $KMnO_4$ therapeutic value would probably be limited for an acute and systemic *columnare* infection.

In conclusion our results demonstrated that Ag NPs and Env reduced the number of *F. columnare* in the water column. Further research would be warranted to investigate the value of Ag NPs and Env as therapeutic agents for *columnare* infection and as a prophylactic treatment for *columnare*.

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