



Vitamin C Improves the Efficacy of Oxytetracycline in Treatment of *Aeromonas hydrophila*-infected Juvenile Catfish (*Clarias gariepinus*)

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Abstract

The recent global increase in demand for catfish products has led to intensive catfish farming, favoring *Aeromonas* infections. Therefore, there is a need to enhance the current management method of *Aeromonas* infection in catfish. This study evaluated the efficacy of adding vitamin C to Oxytetracycline in the treatment of *Aeromonas* infection through the development of skin lesions, mortality and serum antioxidant defense system. One hundred and five juvenile catfish were randomly assigned to seven groups (n=15). The first group served as control and was neither infected nor treated, while the other groups were infected with *A. hydrophila*. Seventy-two hours after inoculation, catfish in groups 2-7 were treated as follows: no treatment, Oxytetracycline through bath, Oxytetracycline through feed, Oxytetracycline through bath with 500 mg of vitamin C in water, Oxytetracycline through feed with 500 mg of vitamin C in water and 500 mg of vitamin C only in water, respectively. The treatment was done for five consecutive days. Behavioral changes, clinical signs and mortality were observed. Data were presented as Mean±SD and analyzed using descriptive statistics or One-way Analysis of Variance (ANOVA) and Tukey's Test. $p < 0.5$ were regarded as significant. The cumulative percentage mortality in groups (Groups 5 and 6) supplemented with Vitamin C was 26.7 and 33.3 compared to 90% and 46.6% in groups 2 and 4, respectively. The results showed that adding vitamin C to Oxytetracycline reduced the development of skin lesions and mortality. Therefore, Vitamin C is recommended to manage *A. hydrophila* infection in catfish.

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INTRODUCTION

The aquaculture business, which accounts for more than half of worldwide fish output, is the fastest-growing food-producing sector (Rocha *et al.*, 2022). Aquaculture has exploded in popularity during the last two decades, becoming a significant economic force. Compared to all other animal food manufacturing industries, the industry continues to grow at an annual pace of 8.8% globally (Onada and Ogunola, 2017). *A. hydrophila* is an aquatic bacterium that causes Motile Aeromonas Septicemia, a disease complex (Janda and Abbott, 2010). This illness causes ulcers, hemorrhages, and fin erosion in farmed and wild fish. Extracellular proteins such as aerolysin, lipase, chitinase, amylase, gelatinase, hemolysins, and enterotoxins play a role in pathogenicity. These proteins are responsible for the evasion of the host immune system (Doan *et al.*, 2013).

Vitamin C is one of the most potent antioxidant defense catalysts found in fish. Both inside and outside the cell, it suppresses the generation of reactive oxygen species. Vitamin C is abundant in the cytoplasm of phagocytes, which are important participants in the innate immune response of fish (Carr and Maggini, 2017). Vitamin C also plays a significant role in the immune response and resistance to infectious diseases of fish, probably through its antioxidant properties by the enhancement of complement activity in fish. Vitamin C affects immune functions in protecting cells from auto-oxidation, especially in the case of the initiation of the oxidative burst of macrophages (Verlhac *et al.*, 1993). Indeed, vitamin C helps to maintain the integrity of the immune cells through their protection from oxidation and within the cells (high amount of vitamin C stored in the immune cells).

There is a relationship between the ascorbic acid concentration (nmoles/10⁸ cells) in leukocytes and the dietary intake of vitamin C (Verlhac *et al.*, 1996). A combination of experimental data acquired in multiple controlled research

and field experiences anytime the immune system is tested has proved the positive effect of dietary supplementation of vitamin C above recommended for optimal growth. The recent global surge in catfish demand has resulted in intensive catfish farming, which is marked by high stocking density, high dissolved ammonia levels, overcrowding, and the use of antimicrobials, among other things (Ajani *et al.*, 2015). Several researchers (Dias *et al.*, 2012; Yu *et al.*, 2015) demonstrate that distinct *Aeromonas* spp. infections are also linked to environmental changes and stressors.

High stocking densities, overcrowding, a sudden change in water and air temperature, harsh handling, abrasive handling, poor nutritional status, hypoxia, fish transfer, mishandling, and transportation are traumatic and stressful occurrences for fish. *Aeromonas* infection is associated with mass mortality and economic loss to fish farmers. The current management method includes the use of antibiotics, probiotics, vaccination, and use of botanicals. There are, however, challenges with each of these management approaches. For example, the misuse of antibiotics in the fish industry has reduced the effectiveness of antibiotics in the management of bacterial diseases (Olatoye and Basiru, 2013). This is reported to be due to the developing resistance to available antibiotics (Mdegela *et al.*, 2021). More so, the vaccine against *Aeromonas* infection is not currently available in Nigeria and indeed most developing countries of the world. Therefore, there is a need to enhance the current management method of *Aeromonas* infection in catfish. This study evaluated the efficacy of adding vitamin C to Oxytetracycline in the treatment of *Aeromonas* infection through the development of skin lesions, mortality and serum antioxidant defense system.

METHODOLOGY

Place and Time

This study was carried out at the aquatic medicine laboratory of the Department of Veterinary Medicine, Faculty of Veterinary medicine, University of Ilorin, Nigeria, between January and March 2021.

Research Materials

A. hydrophila used in this study was from naturally infected catfish skin lesions in Oshogbo, South West, Nigeria, Oxytetracycline hydrochloride (Kepro® BV), Vitamin C, Electronic balance (Golden-Mettler, USA), Spectrophotometer (SP6100 model, Jenway England), Jenway pH meter (Barloworld Scientific Ltd, Dunmow, Essex, UK), Centrifuge (Axiom Medical, Ltd, UK) and plain bottles were used during the course of this research.

Research Design

The fish were grouped into seven groups of 15 juvenile catfish per group. The groupings are as follows: Group 1: Control, uninfected or treated. Group 2: infected but not treated. Group 3: infected and treated with oxytetracycline (20mg/L) through a bath. Group 4: infected and treated with oxytetracycline through feed (60mg/Kg of fish per day). Group 5: infected and treated with oxytetracycline (20mg/L) through bath supplemented with 500 mg of vitamin C in water. Group 6: infected and treated with oxytetracycline through feed (60mg/Kg of fish per day) supplemented with 500 mg of vitamin C in water. Group 7: infected and treated with 500 mg of vitamin C in water.

Work Procedure

Preliminary Study

Bacterial Cultures and Preparation of Inocula

The study used *A. hydrophila* stock cultures derived from naturally infected catfish skin lesions in Oshogbo, South West, Nigeria. The isolates were sub-

cultured on nutrient agar, incubated at 37 °C for 24 hours, and used biochemical features to validate their identity. Colonies were homogenized in sterile phosphate-buffered saline and the turbidity was adjusted to 1.5×10^8 and 2×10^8 CFU/mL, respectively, using McFarland's turbidity standards. Fifteen post-juvenile catfish were randomly divided into three groups (n=5). Group one was not infected and served as control, while groups two and three were inoculated with 0.5 and 1.0 McFarland's at 1 ml per liter of water. The fish were observed hourly for the first day and three times daily for five days (Anyanwu *et al.*, 2015).

Experimental Catfish

For this study, one hundred and five 10-week-old (10.65 ± 0.35 g) juvenile catfish were used. The fish were obtained from a reputable fish farm in Ilorin, Kwara State, Nigeria. The fish were acclimated to their new environment in a 1000L capacity plastic tank for two weeks. The fish were fed with AllerAqua® size 2.0 mm of 45% crude protein. Water parameters were monitored daily. All ethical procedures involving the use of animals for research were observed. The Ethical approval was obtained from the University of Ilorin Ethical Review Committee with UERC/FVM/023/2021.

Experimental Infection of Catfish

0.5 McFarland's turbidity standard equivalent to 1.5×10^8 was used (based on our preliminary study). One milliliter of the inoculum per liter of water was used for the experimental infection of catfish. The water was slightly mixed for the uniform distribution of the organism. The catfish were minimally fed throughout the experiment to ensure that the water was not further contaminated (Iqbal *et al.*, 1998), and 50% of the water was replaced at 24-hour intervals to ensure good water quality (Thomas *et al.*, 2013). Throughout the investigation, the water's ammonia, pH, and oxygen concentrations were kept below acceptable limits. Two times a day,

the experimentally infected fish were observed. Clinical signs, sores on the skin, deaths, and the color of the water were all documented and recorded.

Collection of Blood Samples

After the fish were anesthetized in a 5-L water bath with 0.2 mg benzocaine dissolved in 5ml acetone, blood samples were obtained at the end of the experiment, as described by Carrasco *et al.* (1984). Blood was drawn through posterior caudal venipuncture with a 22-G hypodermic needle and 2-ml syringes. Two milliliters of aspirated blood were gently poured into anticoagulant-containing polypropylene tubes containing the sodium salt of ethylenediaminetetraacetic acid (EDTA). Adedeji and Adegbile (2011) described staining whole blood for the enumeration of red blood cells. After that, the blood smears were air-dried for five minutes, fixed in absolute methanol, and stained with Giemsa stain for 60 seconds.

Determination of Serum Antioxidants

Diagnostic reagent kits (Randox® Laboratories, Crumlin, County Antrim, UK) were used to assess the antioxidant activity in fish sera. The ferricytochrome-C method was used to test superoxide dismutase (SOD) activity using xanthine/xanthine oxidase as the source of superoxide radicals. 50 mM potassium phosphate buffer (pH 7.8), 0.1 mM ethylenediaminetetraacetic acid, 0.1 mM xanthine, 0.013 mM cytochrome C, and 0.024 IU/mL xanthine oxidase made up the reaction mixture. One activity unit was defined as the amount of enzyme required to block the ferricytochrome-C reduction rate by 50% as measured at 550 nm (McCord and Fridovich, 1969). Following Aebi (1984), catalase activity was

evaluated by measuring the decrease in H₂O₂ concentration at 240 nm. The reaction mixture contained freshly prepared 50 mM potassium phosphate buffer (pH 7.0) and 10.6 mM H₂O₂.

The activity of glutathione S-transferases (GST) against 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 340 nm. 100 mM sodium phosphate buffer (pH 6.5), 60 mM glutathione (GSH), and 60 mM CDNB were used in the test (dissolved in ethanol). At 25 °C ($\epsilon_{340\text{nm}} = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$) one unit of GST activity was defined as the quantity of enzyme catalyzing the conjugation of 1 mol of CDNB with GSH per minute. The result is given in nmol/min/mg/protein units. Catalase (CAT) activity was determined based on the first order reaction of Catalase with H₂O₂ as described by Xu *et al.* (1997).

Data Analysis

The rates of skin lesions induction and death were calculated using descriptive statistics and expressed as percentages. Other data were presented as Mean \pm Standard Deviation (Mean \pm SD). GraphPad Prism® (Version 5.0) was used to perform the One-way Analysis of Variance (ANOVA) and Tukey's Multiple Comparison Test to determine differences between groups; p values less than or equal to 5% were regarded as significant.

RESULTS AND DISCUSSION

Result of the Preliminary Study

The result of the preliminary study is presented in Table 1. One hundred percent mortality was observed seventy-two hours after infection of the catfish juvenile with 1.0 McFarland's at the rate of 1 ml per liter of water while the group infected with 0.5 McFarland's (1.5×10^8) at the rate of 1 ml per liter of water showed 40% mortality.

Table 1. Results of the preliminary study.

	Control n=10	0.5 Mc Farlands	1.0 Mc Farlands
C/S 4 hours pi	No sign observed	No sign observed	Rubbing of body against plastic container,
C/S 24 hours pi	No sign observed	Sluggish movement, lethargy	Sluggish movement, increased respiration, lethargy
% Mortality 48 hours pi		30	60
% Mortality 72 hours pi	0	50	100

Note: C/S= Clinical signs, pi= Post-infection with *Aeromonas hydrophila*.

In this study, juvenile catfish were challenged with *A. hydrophila* through bath immersion. The higher mortality observed with 1.0 Mc Farlands' inoculum is attributed to a higher dose of *A. hydrophila*. By 12 hours after infection, the infected fishes were rubbing their bodies against the plastic container's walls, indicating that there was skin irritation as a result of the infective organisms' attachment and colonization (Ahamad *et al.*, 2013). This shows that the *Aeromonas* strain used in this study attached to the skin of juvenile catfish established infection within 24 hours of inoculation. The incubation period of *A. hydrophila* varies between 2-4 days for both natural and experimental infections (Yarim, 2011). The onset of clinical symptoms was found to be between 2-4 days in this investigation.

Development of Clinical Signs

Twenty-four hours post-infection (p.i), catfish in all the infected groups showed signs of sluggish, constant rubbing of the body against the tank and reduced appetite. Some of the infected fish became very pale with hyperemic areas at the base and tips of the fins (Figure 1B) with or without skin lesions in all of the infected groups. Skin ulcers with a wide distribution were found in groups 2-7. (Figure 1B). There were also severe hyperemic areas on the fins in these groups. On the head and fins, erosive lesions were discovered. None of these clinical signs were observed in fish in the control group (Figure 1A). The development of skin lesions in experimentally infected catfish is presented in Table 2. The skin lesion was reduced post-treatment in groups (5 and 6) treated with oxytetracycline and supplemented with vitamin C.

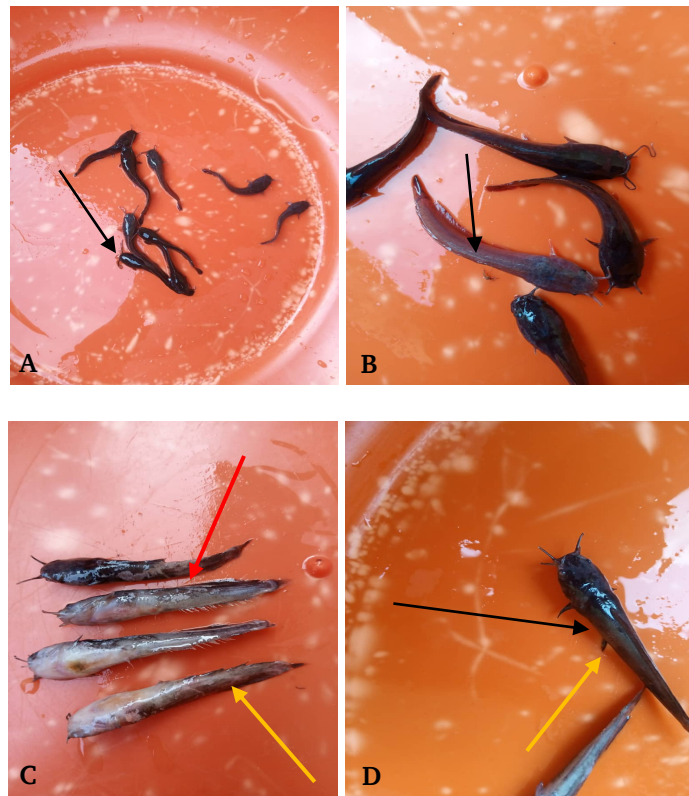


Figure 1. Catfish 24 hours post-infection. A. Control group with intact skin (black arrow); B. Shows of the pale skin and hyperemia of the fin (black arrow) in juvenile catfish experimentally infected with *A. hydrophila*; C. Erosion of the skin (red arrows) and loss of fins (yellow arrows); D. Swelling of the head (black arrow) and paleness of skin (yellow arrow) in juvenile catfish experimentally-infected with *A. hydrophila*.

Experimental studies using *Aeromonas* infection in catfish species in other regions of the world have also reported erosive and ulcerative skin lesions similar to those seen in the current study. These skin lesions appeared to be identical to those seen in naturally ill catfish (Thomas *et al.*, 2013). Bacterial adhesion to host tissues is a vital step in the early stages of many bacterial infections. Bacteria colonize host tissues and cells by attaching to them and modifying their defense mechanisms. The flagella, pili, capsule, Slayer, and lipopolysaccharides are the structural components of *Aeromonas* involved in colonization that have received the greatest attention (Beaz-Hidalgo and Figueras, 2013). A skin lesion covering as low as 10% of a fish's body surface area might result in substantial acute mortality (around 50%) (Bouck and Smith, 1979).

Several studies have shown that after an *Aeromonas* infection, the host expresses a variety of immune-related genes, including those involved in pathogen identification, cell-signaling proteins, and apoptosis (Srivastava *et al.*, 2017). The fish's homeostasis may have been disrupted as a result of the skin injury, resulting in death (Noga, 2000).

Cumulative Mortality Percentage

There was no mortality in the juvenile catfish in the control group (neither infected nor treated) while the group infected with *Aeromonas* but not treated showed 80% mortality. The juvenile catfish in the groups infected with *Aeromonas* and treated with oxytetracycline through feed, vitamin C with Oxytetracycline in feed, Oxytetracycline through bath with vitamin C showed 46.7%, 33.3% and 26.7%

cumulative mortalities respectively. The result is presented in Tables 2 and 3.

Table 2. Number of fish with skin lesions following experimental infection with *A. hydrophila* and treatments.

Group	∑ fish	D 1 pi	D 2 pi	D 3 pi	D 1 PT	D 2 PT	D 3 PT	D 4 PT	D 5 PT	% Cumulative with skin lesion
1	15	0	0	0	0	0	0	0	0	0 (0/15)
2	15	0	1	3	2	3	2	1	0	80 (12/15)
3	15	0	2	1	2	1	0	2	0	60 (9/15)
4	15	0	2	1	2	2	2	1	0	66.7 (10/15)
5	15	0	2	1	1	1	0	2	0	26.6 (4/15)
6	15	0	2	1	1	1	0	2	0	33.3 (5/15)
7	15	0	1	2	1	2	1	0	0	46.7 (7/15)

Note: D= Day, Pi = Post-infection, PT = Post treatment, 1= Neither infected nor treated, 2= infected not treated, 3= infected + Oxytetracycline via bath, 4= infected + Oxytetracycline via feed, 5= infected + Oxytetracycline via bath + Vitamin C, 6= infected + Oxytetracycline through feed + Vitamin C, 7= infected + Vitamin C.

Table 3. Mortality rate following experimental infection with *A. hydrophila* and treatment.

Group	∑fish	Mortality rate post-infection	Mortality rate post-treatment	% Cumulative mortality
1	15	0	0	0
2	15	12	0	80
3	15	3	3	40
4	15	3	4	46.6
5	15	3	1	26.7
6	15	3	2	33.3
7	15	2	6	53.3

The highest cumulative percentage mortality observed in the group infected but not treated (group 2) is indicative of the pathogenicity of the *Aeromonas* strain used in this study. The *Aeromonas* overcame the immune system of the juvenile catfish. More so, the treatment groups supplemented with vitamin C (groups 5 and 6) showed a lesser percentage of mortality (Table 3). This is due to the immune-enhancing potentials of vitamin C.

Serum Antioxidant Status Post-Treatment

The result of the serum level of superoxide dismutase, catalase and glutathione transferase of juvenile catfish infected with *A. hydrophila* after treatment is shown in Table 4. The serum levels of SOD, CAT and GST were significantly increased ($p < 0.001$) when the group infected and treated with oxytetracycline supplemented with vitamin C were compared with those infected with *A. hydrophila* but not treated.

Table 4. Serum antioxidant status of *Aeromonas* infected catfish after treatment.

Parameters	1 (n=5)	2 (n=3)	3 (n=5)	4 (n=5)	5 (n=5)	6 (n=5)	7 (n=5)
SOD (U/mL)	0.63±0.04	0.47±0.05	0.67±0.16	0.70±0.10	0.93±0.06 ^{a,b}	0.97±0.13 ^{a,b}	0.56±0.09
CAT (U/mL)	2.20±0.07	1.90±0.06	1.80±0.21	1.90±0.14	2.80±0.09 ^{a,b,c}	2.80±0.12 ^{a,b,c}	2.20±0.29
GST (U/mL)	1.4±0.02	1.7±0.02 ^a	1.6±0.13	1.6±0.13	1.8±0.03 ^b	1.8±0.06 ^a	1.7±0.06

Note: ^a significant difference at p<0.01 when compared with group 1, ^b significant difference at p<0.001 when compared with group 2, ^c significant difference at p<0.001 when compared with group 3.

Vitamin C is involved in many physiological processes, including growth, development, reproduction, wound healing, stress response, and maybe lipid metabolism through carnitine synthesis. Vitamin C has a key role in fish immune response and disease resistance, most likely because of its antioxidant properties (Anbarasu and Chandran, 2001). Despite the fact that vitamin C lacks coenzyme activity, it is a cofactor in a number of hydroxylating enzyme processes. Ascorbic acid-dependent hydroxylases speed up collagen formation (the hydroxylation of particular prolyl and lysyl residues in procollagen). The collagen triple helix is stiffened by hydroxyproline residues, which bind carbohydrates to form intramolecular cross-links that provide it structural rigidity. As a result, these tissues will be harmed if the body's collagen production is impeded by low vitamin C levels (Azad *et al.*, 2007). When ascorbate levels are low, so is complement activity in fish (the complement component C1q is rich in hydroxyproline and hydroxylysine).

Catecholamine production is the second function of vitamin C in fish. The endocrine system regulates stress responses primarily through cortisol and catecholamines, whose production is dependent on ascorbic acid-dependent hydroxylases. In stressful situations, the body's need for ascorbic acid rises. It can compensate for the immune system's downregulation caused by stress. It is therefore not surprising to see a reduced number of juvenile catfish with skin lesions during treatment with

oxytetracycline and addition to vitamin C (Table 2).

However, the cumulative percentage of mortality was high in the group treated with only vitamin C. This suggests that vitamin C lacks a therapeutic effect on *Aeromonas* infection in catfish despite its immunostimulatory effect. More so, the significant increase in serum level of antioxidants in the groups with vitamin C observed in this study confirms the immune stimulatory role of vitamin C administered through bath. Vitamin C influences immunological processes by shielding cells from auto-oxidation, particularly when macrophages initiate an oxidative burst (Misra *et al.*, 2007). Vitamin C helps to keep immune cells healthy by protecting them from oxidation and maintaining their integrity within the cells. Phagocytes, which are key players in fish's innate immune response, have a high concentration of vitamin C in their cytoplasm, which provides strong protection against the massive creation of reactive oxygen species that occurs when pathogens are attacked. Vitamin C boosts immunological responses such as macrophage activity, cell proliferation, natural killer cell activity, complement activity, lysozyme levels, leucocyte phagocytic activity, cytokine synthesis, and antibody formation (Lin and Shiau, 2004).

CONCLUSION

The *A. hydrophila* isolates used in this study induced skin lesions and caused mortalities. The addition of vitamin C to oxytetracycline used for the treatment of

experimentally infected juvenile catfish improved the treatment outcome when compared with the use of oxytetracycline only. This can be attributed to the antioxidant properties of vitamin C. It is recommended that vitamin C should be added to oxytetracycline in the treatment of *A. hydrophila* infection in juvenile catfish.

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