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Research Article

Aeromonas Spp. Infection in Farmed Nile Tilapia, Oreochromis Niloticus

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ABSTRACT

Nile tilapia is one of the major formed fish species worldwide. However, pathogenic bacteria such as Aeromonas spp. cause a high economic losses. Therefore, the present study was aimed at isolating and investigating the effects of Aeromonas spp on Nile tilapia and evaluating its pathogenicity change after experimental infection. For this purpose, naturally infected Nile tilapia collected form the Ambo University experimental fishpond were transported to the biology laboratory using aseptic plastic bags. Aeromonas spp. isolation and identification were made using morphological and biochemical characterizations. The pathogenicity test was carried out for Aeromonas spp using an intra-peritoneum injection along the caudal peduncle of the fish. Finally, sensitivity test of the isolate was performed using different antibiotics. The results showed that after 24 hours of artificial injections, all the fish decreased their feeding rate and exhibited erratic swimming behaviour. The fish also remained at the bottom and became darkened on their dorsal body part. The fish also showed high hyperaemia on the base of the fins, fin rot and erosion, Internally, they showed pale gills, high intestinal fluid accumulation, pale gonads, pale liver and enlarged gall bladder. The fish also showed an increased of white blood cells as they may migration from the spleen to the blood circulation system. The results also showed that the Aeromonas isolate was highly sensitive to erythromycin and tetracycline. In conclusion, the Aeromonas isolate exhibited a serious effects on external and internal organs of Nile tilapia reared under pond culture system. Additionally, the isolate might affect the immune system of the fish by influencing the nature of blood cells. As control mechanism of the isolate, erythromycin, and tetracycline were more effective and thus these antibiotics can be used for treating the isolate by mixing with fish feed. However, as antibiotics have cumulative effect, it is important to find an alternative controlling methods such as plant based antibiotics and improve production management of the aquaculture systems.

Keywords: Aeromonas spp; antibiotic sensitivity test; Nile tilapia; pathogenicity

INTRODUCTION

World fish production from the aquaculture sector is increasing rapidly and become the main protein source in many developing and developed countries [1]. However, the production of Ethiopian aquaculture is limited, even though the physical and socio-economic conditions of the country are suitable for its development [2]. In recent years, Ethiopia aquaculture got a particular focus by the government and considered an important sector in enhancing the livelihood of rural farmers in the country. In this context potential factors that affect aquaculture production has been identified. Among those factors, bacterial diseases are one of the most limiting factors that affect particularly semi intensive and intensive aquaculture production systems.

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Bacterial disease is known to be occurred due to environmental pressure [3-5]. The incidence of microbial pathogens, especially those of bacterial origin is one of the most significant factors affecting fish culture [6] and considered a major cause of mortality among cultivable fishes leading to a great economic loss [4]. Aeromonas spp. are opportunistic gram-negative pathogen, rod-shaped bacterium, naturally occurring in the aquatic environment and causes high mortality in many fish species including Nile tilapia [3, 7, 8]. Recently, Aeromonas spp. disease outbreak of tilapia was observed in Thailand [9]. Aeromonas spp has been isolated from infected fishes with hemorrhagic septicemia, epizootic ulcerative syndrome, and tail and fin rot [8].

However, Aeromonas spp. frequently cause problems to both natural and cultured fishes [7] leading to heavy mortality and deterioration of product quality [4]. In Ethiopia, there are only a few literature available on Aeromonas spp. infection in cultivable fish species, therefore, the main objective of this study was to isolate and investigate the effects of Aeromonas spp. from naturally infected Nile tilapia collected from the Ambo University experimental fish pond and evaluate its pathogenicity change through experimental infection of Nile tilapia.

MATERIALS AND METHODS

Sample collection

Naturally infected Nile tilapia fingerlings collected from the Ambo University experimental fish pond was transported to the biology laboratory using clean and aseptic plastic bags with ice for clinical and bacteriological isolation. For clinical examination, surface lesions on body surfaces were observed. For bacteriological isolation, naturally infected fish with surface lesions were used for sample collection. In this regard, samples from the gills, intestine, kidney, and skin were taken separately for bacterial enumeration. For each sample, serial dilution was made and then cultured on nutrient and Glutamate Starch Phenol Red (GSPR) agar media and incubated at 250C incubator for 48 hrs. The bacterial load in different organs was counted under a dark field colony counter from nutrient agar. From GSPR media, random bacterial colonies were picked up and re-streaked into agar media for purification. The pure bacterial isolates were then exposed to colony morphology and biochemical identification following the procedure described by Abiola and Oyetayo [10].

Experimental infection

A total of thirty healthy Nile tilapia fingerlings with a mean weight of 15.03 g and mean length of 9.6 cm were equally divided into three groups: non-treated group, treated with saline solution (0.85%) and bacterial infected group. For the second and third groups, 0.1 ml sterile saline solution 0.85% and 0.1 ml of 1.4x104CFU ml-1 of the isolated Aeromonas spp. were injected through intra-peritoneum using 21/gauge sterile needle at ventral part of the body of the experimental fish. Later, all three groups were reared in rectangular glass aquaria (0.91 m x 0.30 m x 0.38 m) filled with 60 L de-chlorinated water. Before the experiment, the fish were acclimatized for one week in aquaria at 25+10C temperature. During the experimental period, the fish were fed with pelleted feed having 25% crude protein at a rate of 3 % of the bodyweight of the fish per day.

Antibiotic sensitivity test

The drug sensitivity testing of the isolated bacteria was performed by the disk diffusion techniques. The recommended antibacterial drugs such as Amoxicillin(25 µg), Ampicillin(10 µg), Erythromycin (15 µg), Chloramphenicol (30 µg) and Tetracycline (30 µg) were applied to the nutrient agar surface using disk dispenser. The sensitive patterns of the isolates were determined by measuring the zone of inhibition diameter for each antibiotic disc and the interpretation of the zone of inhibition were estimated and recorded [11].

Data collection and analysis

Visual observation of external and internal parts of infected fish was carried out. For isolation and identification, morphological and clinical characterizations were performed. For the hematological study, blood samples were drawn by the severing of the dorsal aorta of fish in the way of physical stunning and then evaluate blood parameters. Haematocrit (Hct) values were obtained using a blood capillary tube, blood glucose using a touch screen glucose reader, and a total count of Red Blood Cells (RBC) and white blood cells with a hemocytometer [12]. Differential counts of leucocytes like neutrophils, lymphocytes, and monocytes were determined through stained smears with Giemsa stain under the microscope. Hemoglobin, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) were calculated using the following formula [13].

The Number of Colony Forming Units (NCFU) was calculated by using the following formula :

NCFU = Nc x Df

Where; Nc= number of colonies ranged 30-300 per petri dish, Df=dilution factor of the petri dish

Later, the means of bacterial load were compared by using ANOVA followed by Tukey's post hoc for multiple comparisons using SPSS software version 20 windows.

RESULTS AND DISCUSSION

The distribution of heterotrophic bacteria in different organs of Nile tilapia is presented in Table 1. The results showed that the bacterial population in fish gill ranged from 1.06 ± 1.10 x 104 to 1.74 ± 10.8 x 104 CFU g-1; in intestine ranged from 1.62 ± 11.2 x 104 to 2.82 ± 13.0 x 104 CFU g−1; in kidney ranged from 0.82 ± 5.9 x 104 to 1.27 ± 22.6 x 104 CFU g-1, and in skin ranged from 0.48 ± 5.0 x104 to 0.77 ± 4.1x104 CFUg-1. The distribution showed no significant difference ($P > 0.05$) during November and December but significantly increased in February ($P < 0.05$). This may be related to changes in water quality parameters. Hardi. [3], and Sebastiao [6] stated that the environment could influence the microflora in fish pond (Table 1).

	Gill	Intestine	Kidney	Skin
	(x104 CFU g-1)	(x104 CFU g-1)	(x104 CFU g-1)	x104 CFU g-1
November	1.06 ± 1.10a	1.62 ± 11.2a	1.06 ± 1.5a	0.76 ± 2.6a
December	1.13 ± 10.6a	2.03 ± 3.40a	1.07 ± 1.3a	0.68 ± 4.9a
January	1.14 ± 2.10a	1.67 ± 9.80a	0.82 ± 5.9a	0.48 ± 5.0a
February	1.49 ± 12.8b	2.82 ± 13.0b	1.60 ± 12.1b	0.77 ± 4.1b
March	1.74 ± 10.8b	2.36 ± 14.5b	1.27 ± 22.6b	0.75 ± 4.0b

Table 1. Mean heterotrophic bacterial population in different organs of fish with their standard error Month

When the bacterial count of different organs was compared, the count in the month of February was significantly increased ($P < 0.05$) in the intestine and gills. The presence of high bacterial population in the gills and intestine of fish might be due to the high metabolic activity of fish associated with increased feeding rates at higher water temperatures. The bacterial population observed in fish samples was highest in the intestine followed by gills. This might be due to the voracious feeding behaviour of the fish and filtering microbes [2, 14]. Evidence from recent studies of feeding in tilapia suggests that small particles are entrapped among the gill apparatus in a mucous film [14]. Next to the intestine and gills, the bacterial count was more in the kidney. Kidney being an excretory organ, the bacterial population might have trapped inside the kidney in the process of excretion. The skin showed the minimum bacterial load. However, there was no significant difference among the different body parts. Beyond the microbial load, colony and cell morphology as well as biochemical characteristics of the isolates were determined and presented in Table 2. For Aeromonas spp. isolation, yellowish and rounded colonies from GSPR were transferred on nutrient agar for purification. Following this cell morphology such as straight rod shape with gram-negative (-ve) and motile cells were observed. Later biochemical characterization such as oxidase positive test (-ve), catalase test (-ve) of the isolates were observed (Table 2). Beyond the microbial load, different clinical signs such as hyperaemia of ventral and inflammation of the vent; extensive haemorrhage of fins and ulceration; darkness of skin and extensive haemorrhage, and erosion of the caudal fin on the different external body and pale colours of gills; intense fluid in the intestine; pale colours of gonads containing matured eggs; pale liver and enlarged gall bladder filled with emerald-black secretion internal body of the fish were observed.

Test	Results
Colony color	Yellowish with opaque
Shape	Straight, rod
Motility test	Motile
Hydrogen sulfide test	+ve/ -ve
Oxidase test	+ve
Catalase test	+ve
Starch hydrolysis test	-ve
Gas production	+ve
Lactose/sucrose/glucose fermented	+ve
Acid production	+ve
Triple sugar Iron Agar	Acid butt with gas

Table 2. Cell morphology and biochemical characteristics of Aeromonas spp.

Clinical symptom of natural and experimental infection in fish

Both external and internal clinical symptoms of both naturally and artificially infected fish were reported and presented in Figures 1 and 2. The symptoms observed on naturally infected fish were darkened body, mild hyperaemia on the caudal fins, ventral fins, margins of the vent, and surface lesion on the ventral part of the body (Figure 1). These symptoms were the basis for the selection of fish for the isolation and identification of Aeromonas spp. Such results agreed well with the report of Bekele et al. For artificial infection, after 12-16 hours following the injections of bacteria, all fish fingerlings became less active and mortality of a single fish was recorded. The rate of feeding decreased. After 24 hours, the dorsal part of the body became darkened, and mild hyperaemia of the pectoral and ventral fin bases was observed. Later, hyperaemia extended to the pectoral and ventral fin bases as well as the caudal fin. Fin rot developed especially on the tips of the caudal fin which was more obvious. Moreover, the injected fish developed extensive haemorrhages at the ventral body part and the fish stopped eating and showed erratic swimming behaviour, and remained at the bottom of the aquarium. Significant hyperaemia on the base of the fins, severe fin rot, and fin erosion were observed (Figure 1). The results of the current study are in agreement with the report of.

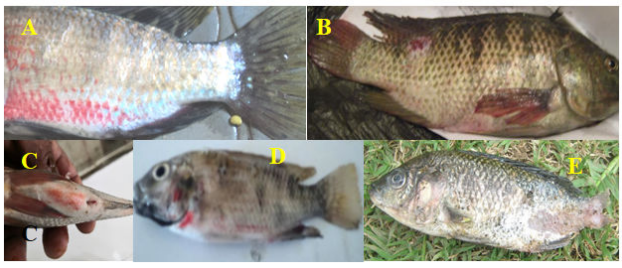

Figure 1. Photograph showing clinical symptoms on the external part fish injected bacteria: A and C) Hyperaemia of ventral and inflammation of the vent; B) Extensive hemorrhage of fins and ulceration; D) Darkness of skin and extensive hemorrhage on

different body part, scale detachment with excess mucus; E) Erosion of caudal fin.

The results further indicated that fish developed apparent clinical symptoms of internal organs such as pale gills, gonads and liver, intense fluid-filled intestine, and enlarged gall bladder filled with emerald-black secretion (Figure 2). Such observation was also reported by El-Son et al. [15]. The increased intensity of infection of Aeromonas spp. noticed during the present could be due to stress factors like local irritation and disturbances in the aquaria, handling, and crowding of fishes. It is reported that fish disease related to Aeromonas spp. infections can cause losses seldom exceed 50%, however, mortality is strongly influenced by the health status of the fish, stress conditions, and virulence of infecting bacterial strain.

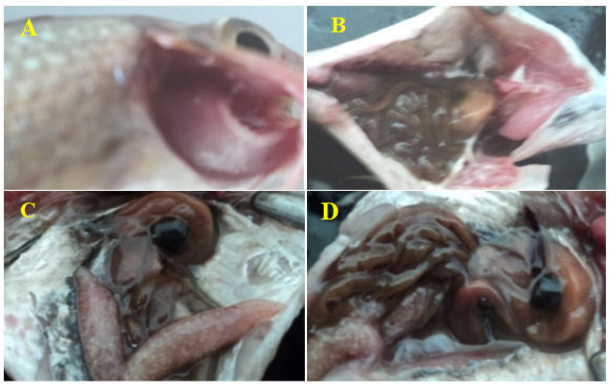

Figure 2. Photograph showing clinical symptoms of internal organs while post mortem examination: A) Pale colours of gills; B) Intense fluid in intestine; C) Pale colours of gonads containing matured eggs; D) Pale liver and enlarged gall bladder filled with emerald-black secretion.

Similar results have also been reported by Sebastiao et al. and Widiyati et al. Who reported that abrupt temperature change, handling, hauling, crowding, high stocking density, inadequate food consumption, and oxygen are known to be the predisposing factors which contribute to the infection of Aeromonas spp. Such stressors are most commonly associated with environmental and physiological parameters that adversely affect fish under intensive culture. In conformity with the findings of earlier reports, motile Aeromonas spp. appropriately receive much attention as pathogens of fish, and also as part of the normal intestinal microflora of healthy fish. Therefore, the presence of these bacteria, by itself, is not indicative of disease and, consequently, stress is often considered to be a contributing factor in the outbreaks of diseases caused by these bacteria. As also reported by El-Son et al. in the present study several clinical symptoms such as excess mucus secretion, scale protrusion, edema within scale pockets, skin ulceration, and abdominal distension was observed.

Hematological changes

The values of different hematological parameters such as glucose concentration and blood cells are presented in Table 3. The present results showed that glucose concentration was not significantly different among the treatments. White blood cells were significantly increased in fish injected with Aeromonas isolate when compared with the control groups. The hematocrit percentage, Red Blood Cells (RBC), and hemoglobin (Hgb) decreased in fish injected with Aeromonas isolate and injected with saline solution. This could be due to the effect of bacterial isolates and salt concentration on cell function. Variation in hematological response is one of most important indicators showing the status of fish health. However, it haematological response might be affected by environmental stresses stimulus and stocking density.

Treatment	PCV	RBC	WBC	Hgb	Glucose
	(%)	(x106.µl-1)	(x103.µl-1)	(mg.dl-1)	(mg.dl-1)
NI	26.4 ± 0.65a	4.40 ± 0.1a	1.36 ± 0.03a	8.80 ± 0.21a	49.8 ± 0.69a
ISS	23.8 ± 0.62a	3.96 ± 0.1a	1.38 ± 0.07a	7.94 ± 0.21a	47.1 ± 0.56a
IAI	14.0 ± 0.70b	2.32 ± 0.1b	2.46 ± 0.05b	4.68 ± 0.22b	45.9 ± 0.45a

Table 3. Mean haematological parameters with its standard error in Nile tilapia non-injected (NI), injected with saline solution (ISS), and injected with of Aeromonas isolate (IAI). Packed cell volume (PCV), Red blood cell (RBC), White blood cell (WBC), Hemoglobin (Hgb) and Glucose

Differential leukocyte counts were characterized by neutrophil predominance followed by lymphocytes and monocytes and were identified in fish blood (Table 4). Mean corpuscular value also calculated from circulating blood. The number of lymphocytes in the artificially infected fish was significantly ($P < 0.05$) higher than found in other groups fish. This implied that stressors such as pathogenic bacteria and stocking density of fish are the main cause to increase of lymphocytes. There was a significant increase in the number of neutrophils in saline injected fish when compared to bacterial injection. On the other hand, a significantly reduced number of monocytes were found after injection with Aeromonas spp. Similar results were reported by Parvez and Mudarris MCV, MCH and MCHC counts were not significantly different among the treatments.

Treatment	Lymphocyte	Neutrophils	Monocytes	MCV	MCH	MCHC
	(%)	(%)	(%)	(cµ)	(µg)	(%)
NI	10.0 ± 0.25a	86.7 ± 0.33ab	2.3 ± 0.21 a	60.05 ± 0.08a	20.06 ± 0.03a	33.3 ± 0.0a
ISS	10.4 ± 0.54a	87.5 ± 0.59b	1.7 ± 0.30ab	60.05 ± 0.05a	20.00 ± 0.00a	33.3 ± 0.0a

IAI	13.1 ± 0.48b	85.7 ± 0.42a	1.2 ± 0.32b	60.36 ± 0.22a	20.17 ± 0.11a	33.3 ± 0.0a

Table 4. Mean differential counts of leukocytes with its standard error in the blood of Nile tilapia Non-Injected (NI), Injected with Saline Solution (ISS) and injected with of Aeromonas isolates (IAI). Lymphocytes, Neutrophils, Monocytes, Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC)

Several factors like sex, age, and size, environmental and physiological conditions also have been reported to affect hematological responses in fish. In this study, the values of hematocrit percentage, RBC and Hgb of fish injected with Aeromonas spp. were lower than the control. The results indicated that the total numbers of RBC, Hgb, and hematocrit percentages were affected by the bacterial injection. The reduction in these parameters is an indication of severe anemia caused by bacterial infection in the exposed fish. The anemic response could be a result of disruption in erythrocyte production. Similar studies have also confirmed that Aeromonas infection decreased RBC, hemoglobin, and hematocrit in different fish species infected with Aeromonas spp.

On the other hand, fish injected with Aeromonas spp. showed increased WBC which was believed to be caused by migration of white blood cells from the spleen to the blood circulation and cause leucocytosis. This fact shows more production of leucocytes in fish injected with bacteria and this enhances the defence mechanism of the fish. Changes in white blood cells and the differential counts, neutrophils, lymphocytes and monocytes indicated a stress condition in Nile tilapia. The distribution of differential leucocytes was also affected. Fish injected with Aeromonas spp. were found to have the highest values of lymphocytes when compared with other treatments. Leucocytes belong to an important cell involved in the immune response, in which high white blood cells produced under severe infection. It can be added that lymphocytes have been reported as immune-competent cells. Fish injected with saline solution was responsible for an increased number of neutrophils, possibly due to the effect of stress as also suggested by Martins et al. Another result observed was a decreased number of monocytes in fish injected with bacteria. This is because the cells are being recruited to the lesion site. The results also showed Aeromonas spp. is highly sensitive to tetracycline (30mm) and erythromycin (23mm) and less sensitive to Chloramphenicol (10mm clear zonation), while the isolate was resistant to amoxicillin and ampicillin (Table 5), which agreed with the reports of Yu et al. However, the use of antibiotics is inappropriate as they caused bacterial resistance. Therefore, new natural antibacterial compounds such as plant extract, probiotics have better antibacterial activities against many bacterial pathogens that are still needed to face such problems.

Antimicrobial agent	Sym.	Zone of inhibition (mm)	Sensitivity test
Amoxicillin	Am	-	R
Ampicillin	Ap	-	R
Chloramphenicol	CF	10	I
Erythromycin	E	23	S
Tetracycline	TTC	30	S

Note: R-Resistance, I- Intermediate and S- Sensitive

Table 5. Antimicrobial sensitivity test of Aeromonas bacteria

CONCLUSION

The Aeromonas the isolate showed serious effects both on external and internal organs of the Nile tilapia reared under pond culture system. Additionally, the isolate might be influenced the immune system of the fish by affecting the level and function of blood cells. As the control mechanism of the isolate, Erythromycin, and Tetracycline was more effective and thus these antibiotics can be used for treating this isolate by mixing the antibiotics with fish feed. However, as antibiotics have cumulative effect, it is important to find plant-based antibiotics to replace synthetic antibiotics and also improve production management of the aquaculture systems.

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