FISHERIES & AQUATIC LIFE (2025) 33: 1 - 18
DOI 10.2478/aopf-2025-0001

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RESEARCH ARTICLE

Oxolinic acid therapy: An effective way to reduce *Aeromonas veronii* infection in *Oreochromis niloticus* and improve biochemical and hematological parameters and histopathological lesions

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Received – 02 September 2024/Accepted – 06 March 2025. Published online: 31 March 2025; ©National Inland Fisheries Research Institute in Olsztyn, Poland

Citation: Sinha, P., Abraham, T. J., Sen, A., Sharon, J., Das, R., Ghorai, A., Boda, S., Patil, P. K. (2025). Oxolinic acid therapy: An effective way to reduce *Aeromonas veronii* infection in *Oreochromis niloticus* and improve biochemical and hematological parameters and histopathological lesions. Fisheries & Aquatic Life 33, 1-18

Abstract. Bacterial diseases such as motile *Aeromonas* septicemia are major constraints on aquaculture. This study evaluated the efficacy of oral oxolinic acid (OA) therapy at 12 mg kg biomass^{-1} day^{-1} for seven days to treat *Aeromonas veronii*-Av-F (*AV*) infection in *Oreochromis niloticus*. The lethal dose of *AV* for 50% mortality was determined to be 1.81 × 10^{8} cells fish^{-1}. Following intramuscular *AV* infection at 2.47 × 10^{8} cells fish^{-1}, OA treatment significantly reduced fish mortality and accelerated wound healing. *AV* infection caused notable changes in biochemical, and hematological parameters, erythrocyte morphology, and histopathological damage to liver and kidney tissues. However, OA therapy facilitated normalizing these parameters more rapidly than in untreated fish, including erythrocyte morphology and histopathological alterations. The study highlights the effectiveness of OA in treating *AV* infection in *O. niloticus*. However, available evidence cautions against its overuse and violations of regulations because of its critical importance in human medicine.

Keywords: antibiotic treatment, aquaculture, bacterial disease, hemato-biochemistry, tissue architecture, wound progression

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Introduction

Global fisheries and aquaculture production reached approximately 185.4 million tonnes in 2022 (FAO 2024). In India, total fish production in 2021–2022 was 16.2 million tonnes contributing 7.56% to global fish production (DOF 2022). Tilapia, highly adaptable and cost-effective cichlid species, contributed 10.6% of global aquaculture yield in 2024, ranking third in production (FAO 2024). However, intensification in aquaculture has led to several diseases in

tilapia, notably bacterial infections such as streptococcosis, motile *Aeromonas* septicemia (MAS), vibriosis, columnaris, and others (Haenen et al. 2023). Motile aeromonads are Gram-negative, rod-shaped facultative bacteria that impact many farmed fishes and the global aquaculture industry (Haenen et al. 2023). These bacteria are proven to be zoonotic and cause diseases such as sepsis, diarrhea, and wound infections in humans (Pessoa et al. 2022). *Aeromonas* species, particularly *A. hydrophila* and *A. veronii*, are significant pathogens causing up to 100% mortality during outbreaks (Dien et al. 2022). In India, outbreaks of MAS in cage-cultured catfish (Khan and Panda 2023), Indian major carps (Bardhan et al. 2021), and Nile tilapia farms with 40–60% mortalities (Raj et al. 2019) have been documented. Additionally, Saharia et al. (2021) reported the prevalence of MAS (31.05%) in fish culture systems of the Central Brahmaputra Valley zone. *Aeromonas veronii* plays a key role in MAS, a disease that results in major economic losses globally (Saharia et al. 2021). Studies revealed that *A. veronii* by itself (El-Latif et al. 2019, Reda et al. 2022) or in co-infection with other bacteria (Dong et al. 2017) or viruses (Suresh et al. 2023) can cause mortalities and other disorders in tilapia.

Antimicrobials, notably quinolones, tetracyclines, and amphenicols, are used extensively to manage these infections, though this practice raises concerns about antimicrobial resistance (WHO 2024). China, India, and other Asian countries are leading consumers of these antimicrobials (Ferri et al. 2022). Among these, oxolinic acid (OA), a quinolone-based broad-spectrum antibiotic, is effective but poses risks of resistance and toxicity when misused (Thomassen et al. 2022). OA has shown efficacy in treating human urinary tract infections, with reported success rates of 86% in bacteriuria eradication (Kalowski et al. 1979). Globally, the most frequently used class of antimicrobial is quinolones at 27% followed by tetracyclines at 20%. Among various fish groups, tilapia accounted for 3.4% of global antimicrobial consumption (Bortolotte et al. 2020). Quinolones like OA are employed widely in aquaculture to combat bacterial infections (Quesada et al. 2013). However, the abuse of OA in aquaculture, such as prolonged treatment durations, excessive dosages, and unregulated use can lead to drug residues in fish and antimicrobial resistance in pathogens that pose health risks to fish and humans (Rigos et al. 2002, Quesada et al. 2013, Okocha et al. 2018, Lulijwa et al. 2020). Resistance mechanisms include mutations in topoisomerase genes, reduced membrane permeability, and active drug efflux (Bearden and Danziger 2001). High resistance rates have been reported in pathogens such as *Flavobacteriaceae* (52%), *Yersinia ruckeri* (54.9%), and *Aeromonas* spp. (57.8%) in a previous study (Delalay et al. 2020). Considering the negative impacts, its use in aquaculture is restricted in the United States of America (USFDA 2023). However, it is recommended and used in various European and Asian nations to prevent bacterial infections in several aquatic species (EMEA 2005, Quesada et al. 2013). While the efficiency and safety of OA in temperate fish have been proved (Samuelsen and Bergh 2004), there has been little research on tropical fish species (Abraham et al. 2024, Patel et al. 2024). Thus, there is a need for detailed studies in tropical aquaculture to evaluate the efficacy of OA and its impact on various biological responses of fish to ensure sustainable and safe aquaculture practices.

Materials and Methods

Experimental fish and bacterial strain

Healthy juvenile Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), from a government-registered farm in Sonarpur, West Bengal, (Lat: 22°27’50.2158” N; Long: 88°23’7.4004” E) were selected irrespective of gender and acclimatized for 15 days in well-aerated borehole water before the experiment (Patel et al. 2024). During the experimental period, the photoperiod was 11 h 34 min – 12 h 30 min (mean 11 h 55 min ± 19 min). *Aeromonas veronii* Av-F (*AV*) [WBUAFS-BGK6: NCBI accession number KP997198] was from the collections of the

Department of Aquatic Animal Health, Faculty of Fishery Sciences, West Bengal University of Animal and Fishery Sciences. For bacterial revival, an aliquot of glycerol stock of *A. veronii* Av-F was cultured in 5 mL brain heart infusion broth at 30±2°C for 18 h and confirmed using the KB002 HiAssorted™ biochemical test kit. A ten-fold serial dilution of the bacterial cell suspension was prepared, estimated cell counts were done by spread plating (Collins et al. 2004), and pathogenicity was tested with intramuscular (IM) injection (Julinta et al. 2017). The lethal dose (LD_{50}) was determined from mortality data following the Reed and Muench (1938) method. The minimal inhibitory concentration (MIC) of OA was estimated with the broth dilution method (CLSI 2024).

Efficacy of oxolinic acid (OA) against *Aeromonas veronii* infection

The recommended OA dose is 12 mg kg^{-1} $biomass^{-1}$ day^{-1} for seven consecutive days (EMEA 2005). The medicated feed for feeding *O. niloticus* juveniles at 2% bodyweight (BW) was prepared by top-dressing OA powder (Sigma-Aldrich, India: O0877-25G; CAS-No: 14698-29-4). A measured quantity of OA (0.60 g) was emulsified in 5 mL of soybean oil and mixed with 1 kg of basal floating pellet feed (32% protein, 6% fat, 12% moisture, and 8% fiber) made from plant and animal byproducts, rice grain, marine protein, essential nutrients, and additives. Similarly, a control feed without OA was prepared. The mixed feeds were spread out evenly, dried under a fan for 24 hours, and stored in sealed plastic containers in the dark at room temperature (Abraham et al. 2024). The efficacy study involved young *O. niloticus*, weighing 21.20 ± 1.49 g (n = 225). For the experiment, nine polypropylene tanks (L58 × H45 × B45 cm) were thoroughly cleaned, sanitized with 200 ppm chlorinated water, rinsed repeatedly with fresh water, and dried for three days. Each tank was filled with 80 l of water, conditioned for three days, and stocked with 25 acclimatized fish. The tanks were covered with nylon netting and labelled. Daily 50% water exchange was done to remove unconsumed feed and fecal wastes. The experimental fish were divided into three groups, each in triplicate. Group 1 was labelled the unchallenged control, Group 2 was labelled *AV*-challenged and fed OA feed (OA treated). Group 3 was labelled *AV*-challenged and fed control feed (untreated). After acclimatization, group 1 was injected IM at the base of the dorsal fin with 0.1 ml of sterile saline for the unchallenged control. The fish of groups 2 and 3 were injected IM with 0.1 ml of *AV* cell suspension to get a lethal dose of 2.48×10^8 cells $fish^{-1}$. Post-injection, the fish were transferred to their respective tanks and fed appropriate feeds at 2% BW three times daily in equal rations. Groups 1 and 3 were fed control feed consistently. Group 2 received OA feed for seven days post-injection (DPI), then they received the control feed. The water quality parameters were monitored and maintained at optimal levels throughout the experiment. Unconsumed feed was scooped from the water surface manually after each feeding, pooled for each tank separately, air-dried, and weighed daily to assess feed intake (Patel et al. 2024). Observations on weight gain, behavior, signs of disease, and mortality were recorded daily for up to 21 DPI.

Body discoloration, wound progression, and healing

Wounds at the IM injection site were digitally photographed daily for 30 days. Tissue damage was qualitatively scored on an ordinal scale of 0–6 (Roy et al. 2019). The intensity and severity of wound progression, healing, and discoloration were categorized and scored as 0: No damage/discoloration with no pathological importance, 0.5: Very mild damage/ discoloration with or no pathological importance, 1: Very mild damage/ discoloration with minimal pathological importance, 2: Mild damage/discoloration with minimal pathological importance, 4: Moderate damage/discoloration with moderate pathological importance, and 6: Severe damage/discoloration with marked pathological importance.

Collection of blood and plasma

The blood samples from all groups were collected on days 0, 1 and 7 of OA therapy and days 7 and 14 of post-OA therapy, i.e., DPI 0, 1, 7, 14, and 21. Two fish from each tank were sedated with clove oil at 40 µl l^{-1} of water and blood was drawn by caudal puncture using a sterile 2 ml syringe (Roberts 2012). As an anticoagulant, a 5% ethylenediaminetetraacetic acid solution was used to avoid clotting. Blood samples from the same tanks were transferred to 2 ml Eppendorf tubes that had been washed with anticoagulant. Plasma was collected by centrifugation at 4500 rpm for 15 min and stored at -20°C until analyzed.

Biochemical parameters

The plasma glucose, creatinine, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), calcium and chloride were quantified by using commercial kits as mentioned in Table 1. All commercial kits were used as per the manufacturer's instructions.

Hematology

The total counts of erythrocytes (TECs), leukocytes (TLCs), and thrombocytes (TCs) from anticoagulant-treated fish blood samples were measured using a Neubauer counting chamber. Hayem's and Turk's white blood cell diluting fluids were used for TECs and TLCs, respectively. For TCs, Rees and Ecker diluting fluid, modified by Wintrobe (1946), was used. The cell counting at 40 × magnification was executed using a trinocular research microscope (Olympus Model BX51, Japan) connected to a computer and SCO-LUX camera with a resolution of 16 MP.

Erythrocyte morphology

Giemsa-stained smears of blood without anticoagulant were studied using an oil immersion lens (100 ×) in a research microscope and photographs were made with a 16MP SCO-LUX camera. Descriptive data were obtained by observing any shape alterations to the cells and nuclei, as per descriptions in Bardhan et al. (2022).

Histopathology

On 0, 1, 7, 14, and 21 DPI, kidney and liver samples were taken from control, OA-treated, and untreated fish, in triplicate, without pooling after blood collection and euthanasia with clove oil at 100 µl l^{-1} water. The fish were dissected to carefully expose the internal organs and liver (≈0.75 g) and kidney (≈0.40 g) tissues were collected from each fish. The organ tissues were preserved in Bouin's fixative for 24 hours. The fixed samples were conventionally processed and embedded in paraffin wax. Thin slices of approximately 5 µm were prepared and stained with hematoxylin and eosin (Roberts 2012). To detect

Table 1
Commercial test kits used for to analyze plasma biochemical parameters

Parameters	Kits	Methods	References
Glucose	Glucose GOD FS kit[a]	Glucose dehydrogenase photometric method	Schmidt et al. 1961
Calcium	Calcium AS FS kit[a]	Arsenazo III technique	Michaylova and Ilkova 1971
Chloride	Chloride kit[b]	Thiocyanate technique	Schoenfeld et al. 1964
Creatinine	Creatinine test kit[c]	Jaffe's reaction and the initial rate assay	Junge et al. 2004
Alanine aminotransferase	ALT test kit[d]	Modified UV (IFCC) and kinetic assay	Wolf et al. 1972
Aspartate aminotransferase	AST test kit[d]	Modified UV (IFCC) and kinetic assay	Wolf et al. 1972
Alkaline phosphatase	ALP FS test kit[d]	Kinetic photometric method	Tietz 1994

[a]: DiaSys Diagnostic Systems, Germany; [b]: Coral Clinical Systems, India; [c]: Span Diagnostics Ltd., India; [d]: Erba Diagnostics Ltd., Germany; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; GOD: Glucose oxidase; FS: Fasting; AS: Arsenazo III; UV: Ultra-violet; IFCC: International Federation of Clinical Chemistry and Laboratory Medicine

anomalies, the sections were scanned and photomicrographs were taken at 20 × magnification under a microscope (Olympus Model BX51, Japan). The histopathological evaluation involved identifying and qualitatively scoring tissue alterations based on the percentage change from normal tissue architecture. An ordinal scale by Bowker et al. (2013) was used based on the extent of tissue damage with score 0: No change, 1: Normal (≤5% affected), 2: Mild (5–15% affected), 3: Moderate (15–25% affected), 4: Marked (25–50% affected), and 5: Severe (> 50% affected).

Statistical analyses

The results were presented as means ± standard deviation. Mortality, biomass, feed intake, hematological and plasma biochemical parameters were analyzed using one-way ANOVA. Significant differences among treatments and DPI were identified with Tukey's post-hoc test. Wound progression, healing, and discoloration were assessed with Friedman ANOVA for related samples and the Mann-Whitney U test for independent samples. Histopathological scores were analyzed using the non-parametric Kruskal-Wallis test with pair-wise comparisons. All statistical analyses were conducted using IBM-SPSS version 22.0, with a significance level set at 0.05.

Results

Pathogenicity of *Aeromonas veronii* Av-F on *Oreochromis niloticus*

The clinical signs observed in the infected fish were lethargy, abnormal behavior, self-isolation, bottom resting, and vertical swimming. Some fish showed bilateral exophthalmia, pale gills, opercular hemorrhages, tail rot, and body darkening. The injection sites had inflammation, scale loss, skin peeling, and hemorrhagic lesions. *AV* caused 100% mortality at 9.60×10^{10} cells fish^{-1}, with lower dosages (10^9, 10^8, and 10^7 cells fish^{-1}) resulting in 90–100%, 90%, and 40% mortalities. Saline-injected control had no mortality. The lethal dose (LD_{50}) of *AV* was determined to be 1.81×10^8 cells fish^{-1}. OA displayed a MIC of 6.25 μg ml^{-1} against *AV*.

Efficacy of oxolinic acid (OA) against *Aeromonas veronii* infection

The mortalities recorded on DPI 7 were 3.4-fold higher in the untreated group than in the OA-treated group. It significantly ($P < 0.05$) increased further in both groups on DPI 21. Significant differences ($P < 0.05$) in mortalities existed between these groups. No mortalities occurred in the saline-injected control. Following the *AV* infection, feed consumption was significantly decreased ($P < 0.05$) in both

Table 2
Mortality, feed intake, and weight gain in *Oreochromis niloticus* juveniles challenged with *Aeromonas veronii* Av-F at 2.47×10^8 cells fish^{-1} and administered oxolinic acid (OA) at 12 mg kg biomass^{-1} day^{-1} for seven consecutive days in comparison to the control. Control: unchallenged fish fed control feed. OA treated: fish challenged with *A. veronii* Av-F and fed OA feed. Untreated: fish challenged with *A. veronii* Av-F and fed control feed. a-c: Values with the same letter superscript in rows among treatment groups on specific days did not differ significantly ($P > 0.05$). 1-2: Values with the same numerical superscript in columns for specific parameters did not differ significantly ($P > 0.05$).

		Treatment groups		
Parameters	Day post-injection (DPI)	Control	OA treated	Untreated
Mortality (%)	DPI 7	0.00 ± 0.00^{1a}	$20.00 \pm 6.93^{1, 2b}$	68.00 ± 10.58^{1c}
	DPI 21	0.00 ± 0.00^{1a}	28.00 ± 8.00^{2b}	80.00 ± 12.00^{2c}
Feed intake (%)	DPI 7	100.00 ± 0.00^{1a}	78.64 ± 8.67^{1b}	49.42 ± 4.45^{1c}
	DPI 21	100.00 ± 0.00^{1a}	97.21 ± 3.14^{2b}	74.79 ± 15.01^{2c}
Weight gain (g/10 fish)	DPI 7	5.33 ± 0.58^{1a}	3.00 ± 1.00^{1b}	2.00 ± 0.02^{1b}
	DPI 21	20.00 ± 1.00^{2a}	13.67 ± 1.53^{2b}	9.50 ± 0.71^{2c}

groups, with the highest reduction in the untreated group. After 7 DPI, there was a substantial increase ($P < 0.05$) in feed intake, close to normalcy in the treated group at the end of the experiment. The saline-injected control group consumed all the rations offered. Between the *AV*-challenged groups, weight gain was significantly more ($P < 0.05$) in the OA-treated than in the untreated group (Table 2).

Wound progression, healing, and discoloration

Within 12–24 hours of injection, inflammatory reactions emerged, including redness, swelling, and skin peeling at the injection site. The infected area expanded and turned red, followed by scale loss and the formation of black scars along the ulcerated region (Fig. 1). On DPI 2, the untreated group's discoloration peaked, significantly higher than the OA-treated group. Healing involved the gradual disappearance of a black scar, with a centripetal regrowth of dermal fibrous tissue leading to skin and scale development at the wound site. Discoloration was reduced significantly ($P < 0.05$) by DPI 22 for the OA-treated group and DPI 26 for the untreated group. The experiment showed two peaks in wound and ulceration, indicating reinfection. The first peak was on DPI 6, and the second peak was on DPI 12, scoring significantly higher ($P < 0.05$) in the untreated group than in the OA-treated group. In the OA-treated group, the wounds healed significantly ($P < 0.05$) faster by DPI 20 compared to the untreated group by DPI 26 (Table 3).

Effects on plasma biochemistry

On DPI 1, the plasma glucose and creatinine levels in the challenged groups increased significantly four-fold compared to the control, while the plasma AST, ALT, and ALP levels increased more than two-fold ($P < 0.05$). On 21 DPI, the plasma glucose, creatinine, AST, ALT, and ALP decreased to near-normal levels. A significantly faster reduction ($P < 0.05$) was observed in the OA-treated group compared to the untreated group (Figs. 2a-e). Within 24 hours of the challenge,

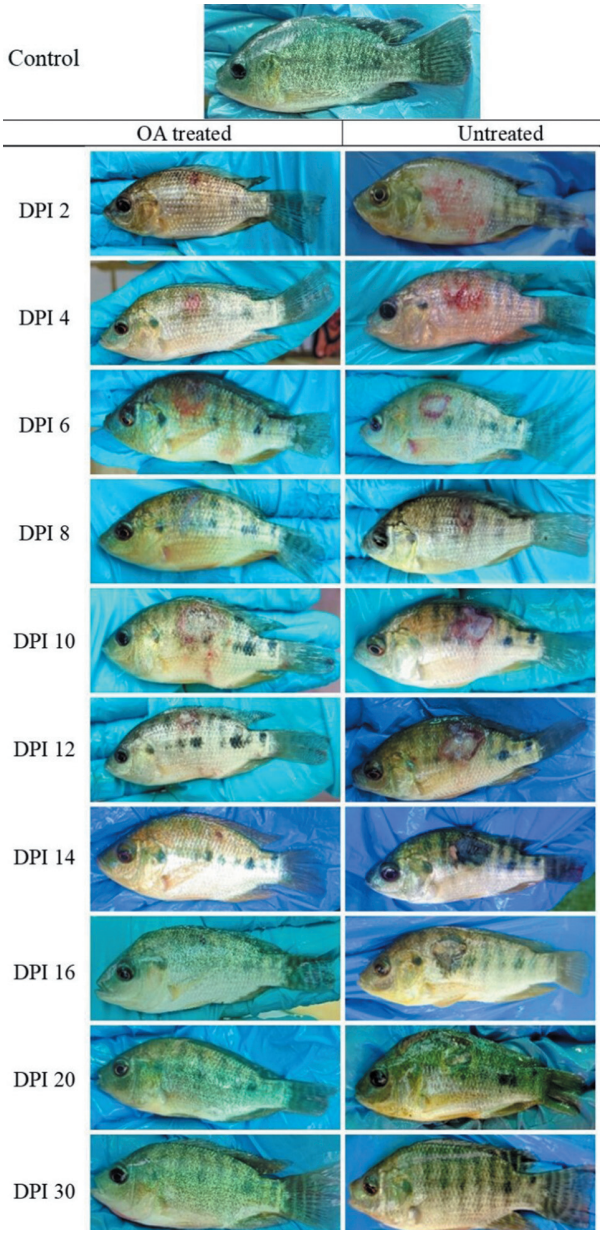

Figure 1. Gross and clinical changes, wound progression and healing, and discoloration in *Oreochromis niloticus* juveniles challenged with *Aeromonas veronii* Av-F at 2.47×10^8 cells fish^{-1} and administered oxolinic acid (OA) at 12 mg kg biomass^{-1} day^{-1} for seven consecutive days in comparison to the negative control. OA treated: fish challenged with *A. veronii* Av-F and fed OA feed. Untreated: fish challenged with *A. veronii* Av-F and fed control feed.

plasma calcium and chloride levels dropped significantly ($P < 0.05$). On and from 7 DPI, they progressively recovered to near normalcy. Their levels in the untreated group were significantly ($P<0.05$) lower compared to the OA-treated group (Figs. 2f, g).

Table 3
Qualitative scores on changes in discoloration and wound progression and healing in *Oreochromis niloticus* juveniles challenged with *Aeromonas veronii* Av-F at 2.47×10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg $kg\ biomass^{-1}\ day^{-1}$ for seven consecutive days in comparison to the control. DPI: Day post-injection; Values with symbols * and # in rows for specific changes did not differ significantly (P > 0.01). a-h: Values with the same letter superscript in columns did not differ significantly (P > 0.01). DPI 1-7: OA treatment period. OA treated: fish challenged with *A. veronii* Av-F and fed OA feed. Untreated: fish challenged with *A. veronii* Av-F and fed control feed. Only data from even sampling days appear in the table

	Discolouration		Wound progression and healing	
Days	OA treated	Untreated	OA treated	Untreated
Day 0	$0.25 \pm 0.16^{*a}$	$0.25 \pm 0.16^{*a}$	$0.00 \pm 0.00^{\#a}$	$0.00 \pm 0.00^{\#a}$
DPI 2	1.13 ± 0.40^{b}	4.41 ± 0.75^{b}	$0.08 \pm 0.12^{\#b}$	$0.43 \pm 0.35^{\#b}$
DPI 4	1.53 ± 0.67^{c}	2.30 ± 0.31^{c}	$0.14 \pm 0.19^{\#c}$	$0.29 \pm 0.29^{\#c}$
DPI 6	$1.71 \pm 0.45^{*c}$	$2.33 \pm 0.59^{*d}$	$0.63 \pm 0.44^{\#c}$	$1.63 \pm 0.86^{\#d}$
DPI 8	$1.77 \pm 0.50^{*c}$	$2.18 \pm 0.75^{*e}$	0.13 ± 0.14^{c}	1.28 ± 0.48^{e}
DPI 10	$1.48 \pm 1.01^{*c}$	$2.04 \pm 0.51^{*c}$	$0.79 \pm 0.94^{\#c}$	$2.10 \pm 0.80^{\#f}$
DPI 12	0.57 ± 0.29^{d}	1.33 ± 0.40^{c}	0.64 ± 0.20^{d}	2.48 ± 0.54^{g}
DPI 14	0.20 ± 0.18^{a}	1.42 ± 0.91^{c}	0.20 ± 0.27^{c}	1.90 ± 0.46^{d}
DPI 16	0.11 ± 0.14^{e}	0.98 ± 0.59^{f}	0.10 ± 0.16^{b}	1.53 ± 0.22^{d}
DPI 18	0.05 ± 0.08^{f}	1.20 ± 0.24^{g}	0.10 ± 0.20^{c}	0.90 ± 0.73^{h}
DPI 20	$0.06 \pm 0.10^{*f}$	$0.25 \pm 0.16^{*a}$	$0.00 \pm 0.00^{\#a}$	$0.25 \pm 0.16^{\#i}$
DPI 22	$0.01 \pm 0.02^{*f}$	$0.08 \pm 0.13^{*h}$	$0.00 \pm 0.00^{\#a}$	$0.13 \pm 0.14^{\#j}$
DPI 24	$0.00 \pm 0.00^{*f}$	$0.00 \pm 0.00^{*h}$	$0.00 \pm 0.00^{\#a}$	$0.08 \pm 0.08^{\#j}$
DPI 26-30	$0.00 \pm 0.00^{*f}$	$0.00 \pm 0.00^{*h}$	$0.00 \pm 0.00^{\#a}$	$0.00 \pm 0.00^{\#a}$

Effects on hematology

Within 24 hours of *AV* infection, TECs decreased significantly (P < 0.05). On DPI 21, TECs increased significantly (P < 0.05) close to the control in the OA-treated group, but the untreated group had continually lower counts (Fig. 3a). TLCs increased significantly (P < 0.05) in the *AV*-infected groups with a peak on DPI 7. The OA-treated group experienced a two-fold increase in TLCs, whereas in the untreated group, a three-fold increase was observed. TLC levels gradually decreased in both groups on DPI 21, with a significant (P < 0.05) and rapid reduction in the OA-treated group compared to the untreated group (Fig. 3b). On DPI 1, the *AV*-infected groups showed significantly (P < 0.05) higher TCs than the control, which thereafter, decreased significantly (P < 0.05) faster in the OA-treated group (Fig. 3c).

Effects on erythrocyte morphology

The damage observed in erythrocytes of *AV*-challenged groups were elongated, irregularly shaped, teardrop-like, echinocyte-like, and hypochromic cells, vacuolation, and hypertrophied, eccentric, notched, karyolitic nuclei. These changes increased from DPI 1 to 7 and gradually decreased from DPI 14 in both groups. The degree of changes was higher in the untreated group than in the OA-treated group (Fig. 4).

Effects on histopathology

The histopathological changes observed in the kidney of *AV*-challenged groups were the degeneration of renal tubular epithelium and tubules, inflamed renal tubules, vacuolation within the renal tubules, widened lumen, constricted renal tubules, dilated Bowman's space, fibrosis, hemocyte infiltration, necrotized areas, nephrocalcinosis, necrotized hematopoietic area, fragmented glomerulus, vacuolation, and melanomacrophage aggregation (Fig. 5). The changes observed in the liver of *AV*-challenged groups were glycogen-type vacuolation, cytoplasmic degeneration and vacuolation, cellular hypertrophy, necrotized areas, loose parenchyma,

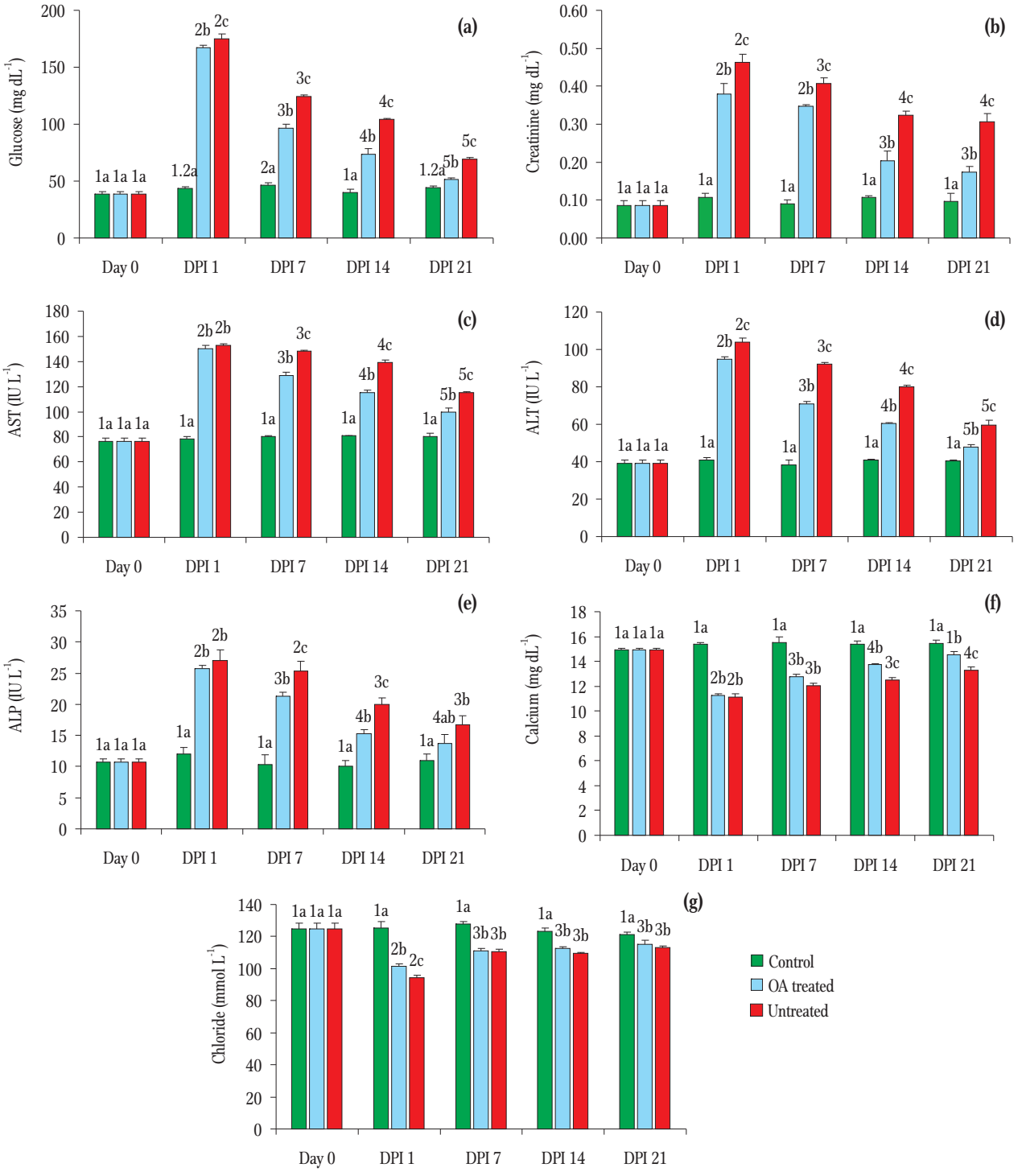

Figure 2. Plasma (a) glucose, (b) creatinine, (c) aspartate transaminase (AST), (d) alanine transaminase (ALT), (e) alkaline phosphatase (ALP), (f) calcium, and (g) chloride levels at different time points in *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47 × 10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg kg $biomass^{-1}$ day^{-1} for seven consecutive days. DPI: day post-injection. a-c: bars with the same letter superscript at particular time points did not differ significantly ($P > 0.05$). 1–5: bars with the same numerical superscript for particular treatments did not differ significantly ($P > 0.05$). Control: unchallenged fish fed control feed. OA treated: fish challenged with *A. veronii* Av-F and fed OA feed. Untreated: fish challenged with *A. veronii* Av-F and fed control feed.

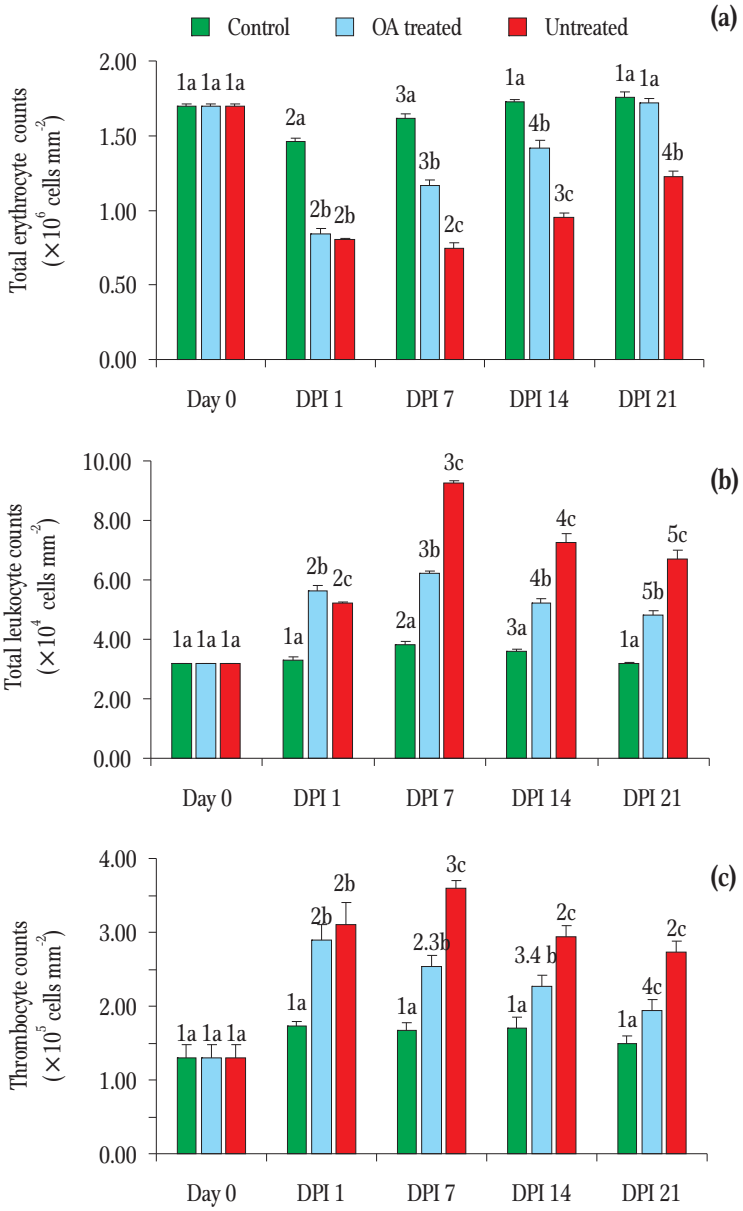

Figure 3. Counts of total (a) erythrocytes, (b) leukocytes, and (c) thrombocytes at different time points in *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47×10^8 cells fish^{-1} and administered oxolinic acid (OA) at 12 mg kg biomass^{-1} day^{-1} for seven consecutive days. DPI: day post-injection. a-c: bars with the same letter superscript at particular times did not differ significantly ($P > 0.05$). 1–5: bars with the same numerical superscript for particular treatments did not differ significantly ($P > 0.05$). Control: unchallenged fish fed control feed. OA treated: fish challenged with *A. veronii* Av-F and fed OA feed. Untreated: fish challenged with *A. veronii* Av-F and fed control feed.

and melanomacrophage aggregation (Fig. 6). The changes observed in the kidney and liver tissues significantly ($P < 0.05$) increased from DPI 1 to 7 and then gradually decreased from DPI 14 in both groups. The extent of tissue damage in the untreated group was, however, significantly ($P < 0.05$) higher compared to the OA-treated group (Table 4).

Discussion

MAS is a bacterial infection that commonly affects fish, and *A. hydrophila* and *A. veronii* are the major causes of disease outbreaks and deaths that can lead to substantial economic losses (Reda et al. 2021). The mortality rate varies based on fish health and

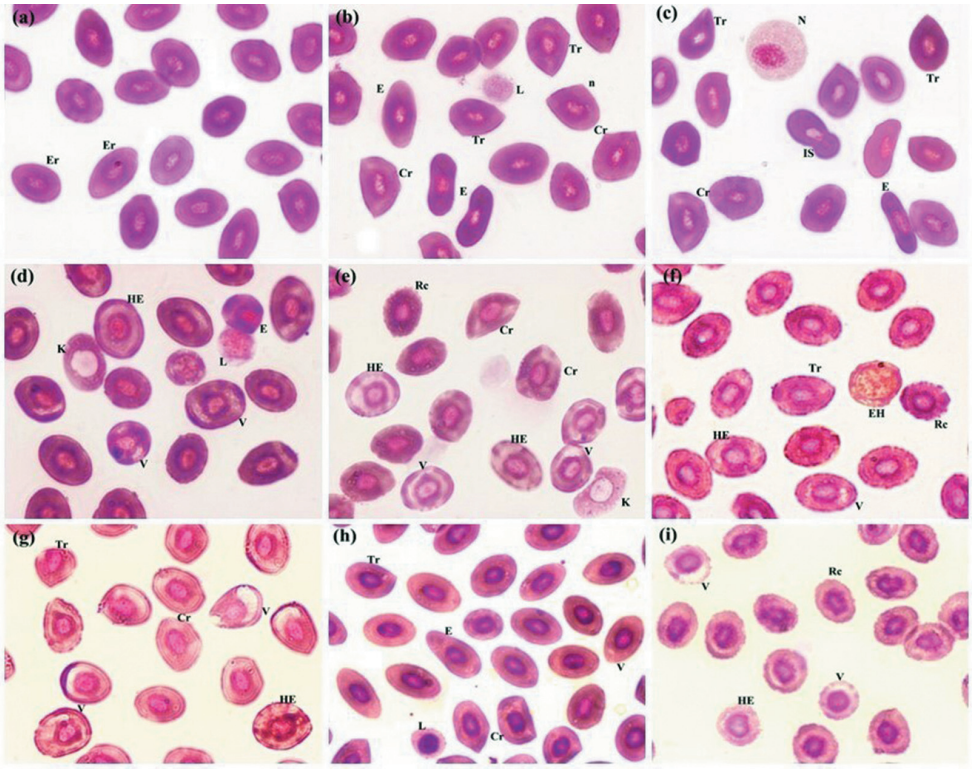

Figure 4. Morphological alterations in the Giemsa-stained erythrocytes of *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47 × 10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg kg $biomass^{-1}$ day^{-1} for seven consecutive days in comparison to untreated. (a) Control, (b) Day post-injection (DPI) 1 OA treated, (c) untreated; (d) DPI 7 OA treated, (e) untreated; (f) DPI 14 OA treated, (g) untreated; (h) DPI 21 OA treated, (i) untreated. Er: erythrocyte; L: lymphocyte; N: neutrophil; E: elongated cell; IS: irregular-shaped cell; Tr: teardrop-like cell; Ec: echinocyte-like cell; HE: hypochromic cell; V: vacuolation; Ehn: hypertrophied nucleus; En: eccentric nucleus; n: notched nucleus and K: karyolytic nucleus; ×1000 Giemsa staining.

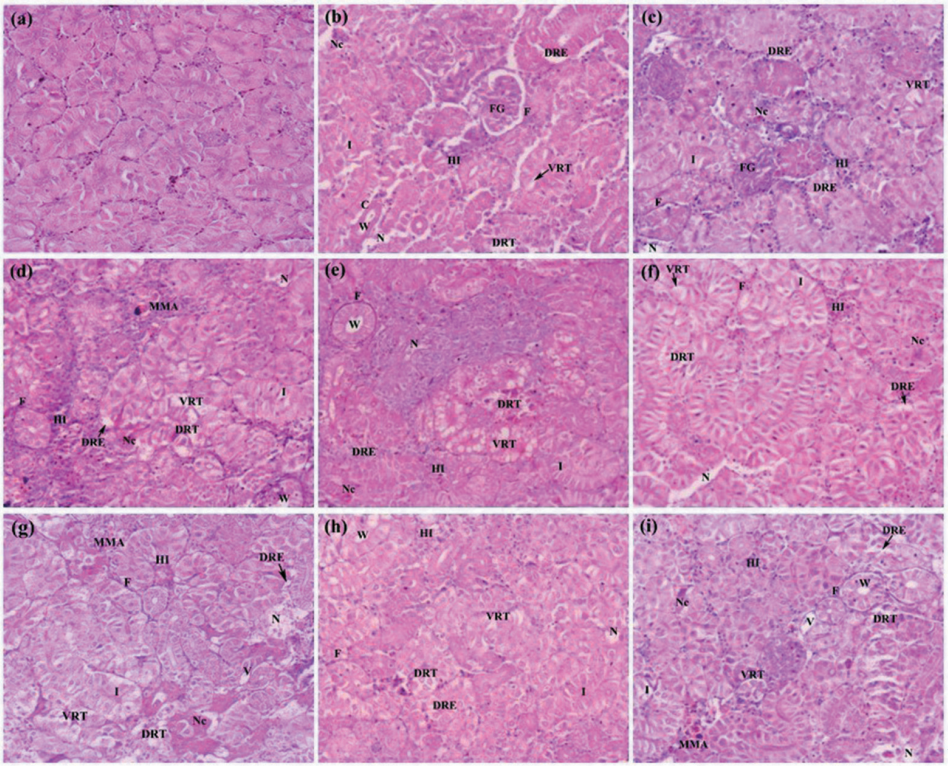

Figure 5. Histopathological changes in the kidney of *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47 × 10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg kg $biomass^{-1}$ day^{-1} for seven consecutive days in comparison to untreated fish. (a) Control, (b) Day post-injection (DPI) 1 OA treated, (c) untreated; (d) DPI 7 OA treated, (e) untreated; (f) DPI 14 OA treated, (g) untreated; (h) DPI 21 OA treated, (i) untreated. DRE: degeneration of renal tubular epithelium; DRT: degeneration of renal tubules; I: inflamed renal tubules; VRT: vacuolation within the renal tubules; W: widened lumen; C: constricted renal tubules DBS: dilated Bowman's space; F: fibrosis; HI: hemocyte infiltration; N: necrotized areas; Nc: nephrocalcinosis; NH: Necrotized hematopoietic area; FG: fragmented glomerulus; V: vacuolation; MMA: melanomacrophage aggregation; ×200 H and E staining.

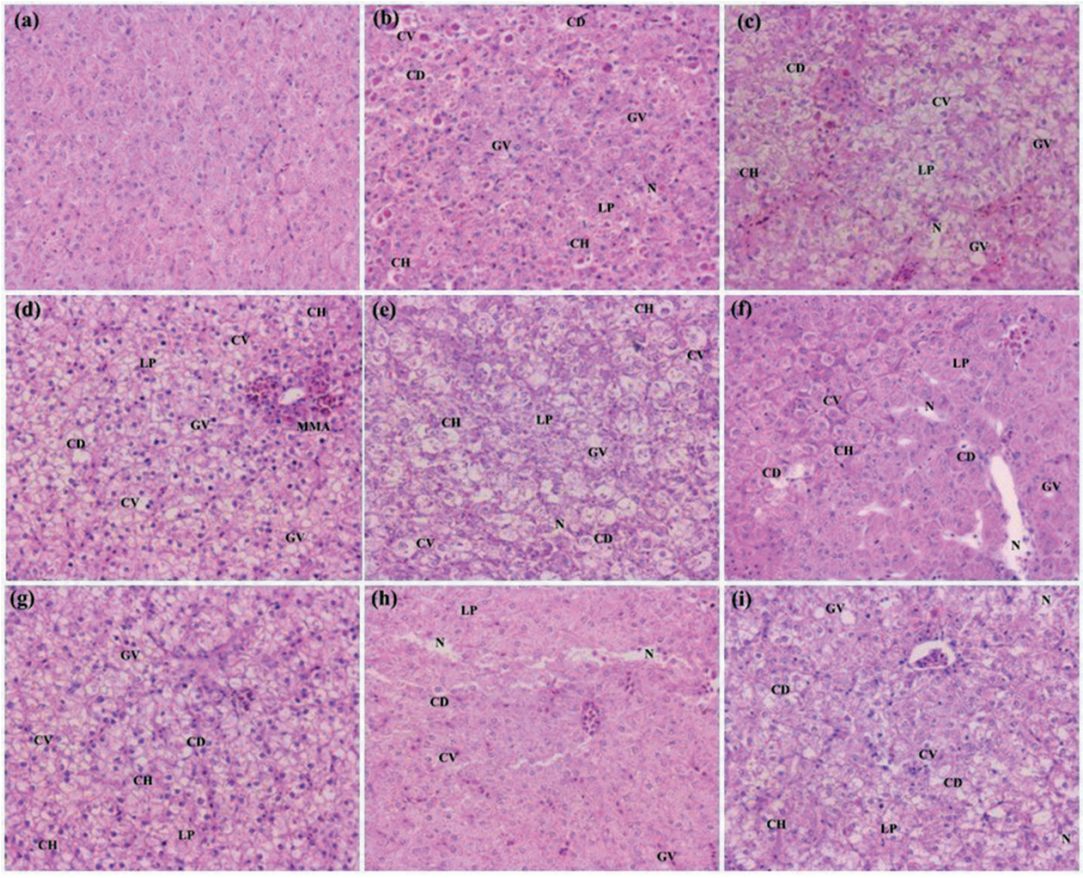

Figure 6. Histopathological changes in the liver of *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47 × 10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg kg $biomass^{-1}$ day^{-1} for seven consecutive days in comparison to untreated fish. (a) Control, (b) Day post-injection (DPI) 1 OA treated, (c) untreated; (d) DPI 7 OA treated, (e) untreated; (f) DPI 14 OA treated, (g) untreated; (h) DPI 21 OA treated, (i) untreated. CD: cytoplasmic degeneration; CH: cellular hypertrophy; CV: cytoplasmic vacuolation; GV: glycogen-type vacuolation; N: necrotized area; LP: loose parenchyma and MMA: Melanomacrophage aggregation; × 200 H and E staining.

stress levels and bacterial virulence (Sreedharan et al. 2013). Determining the LD_{50} was essential before testing the treatment efficacy (Rey et al. 2009). The LD_{50} of *A. veronii* Av-F was determined to be 1.18 × 10^8 cells $fish^{-1}$, which was consistent with the earlier findings (El-Latif et al. 2019). The MIC of OA was 6.25 μg ml^{-1} against *AV*, signifying that the strain tested was susceptible to OA. In the treatment trial, OA therapy significantly reduced mortalities of fish infected with *AV*. Both in-vitro and in-vivo studies demonstrated the effectiveness of OA and supported earlier research with different fish pathogens (Samuelsen and Bergh 2004). All infected fish initially reduced their feed intake, but they recovered over time. On DPI 21, the OA-treated group had a 23% higher feed intake compared to the untreated group, corroborating several earlier studies (Dong et al. 2017, Patel et al. 2024). Bacterial infections generally reduce fish appetite and lead to weight loss, which was noted in the *AV*-challenged *O. niloticus* in the present study that was likely due to stress and reduced feeding. In this study, *AV* induced severe skin ulcers, characterized by rapid inflammatory responses such as redness, and swelling within 24 h post-injection, which intensified by DPI 3 with subsequent scale loss, culminating in the formation of black scars and ulcers within DPI 5–6, which was similar to reports in Patel et al. (2024). These responses indicated high immune cell influx and melanocyte accumulation, facilitating phagocytosis

Table 4

Qualitative assessment of the major histopathological changes in *Oreochromis niloticus* challenged with *Aeromonas veronii* Av-F at 2.47×10^8 cells $fish^{-1}$ and administered oxolinic acid (OA) at 12 mg kg $biomass^{-1}$ day^{-1} for seven consecutive days in comparison to the control. No changes were noted in the control group. 1-2: Values with the same numerical superscript in rows among treatment groups on a specific day did not differ significantly ($P > 0.05$). a-d: values with the same letter superscript in rows for specific treatment groups did not differ significantly ($P > 0.05$); DPI: day post-injection. Glomerulopathy includes changes such as fragmented glomerulus (FG) and dilated Bowman's space (DBS); nephropathy includes changes such as degeneration of the renal tubular epithelium (DRE), and renal tubule (DRT), vacuolation in the renal tubule (VRT), and widened lumen (W)

	Qualitative assessment on an ordinal scale							
	DPI 1		DPI 7		DPI 14		DPI 21	
Histopathological changesž	OA treated	Untreated	OA treated	Untreated	OA treated	Untreated	OA treated	Untreated
Kidney								
Haemocyte infiltration	1.23 ± 0.07^{1a}	1.41 ± 0.18^{2a}	1.78 ± 0.49^{1a}	1.91 ± 0.32^{1ab}	1.62 ± 0.23^{1a}	2.15 ± 0.43^{2b}	1.19 ± 0.62^{1a}	1.62 ± 0.44^{1ab}
Glomerulopathy	0.95 ± 0.50^{1a}	0.91 ± 0.31^{1a}	0.40 ± 0.19^{1b}	0.91 ± 0.38^{1b}	1.08 ± 0.45^{1b}	1.51 ± 0.42^{1c}	0.49 ± 0.24^{1ab}	1.02 ± 0.42^{2d}
Inflammation	1.68 ± 0.75^{1a}	2.00 ± 0.34^{1a}	2.20 ± 0.42^{1a}	2.59 ± 0.55^{1a}	2.07 ± 0.34^{1a}	2.96 ± 0.47^{2a}	1.83 ± 0.36^{1a}	2.70 ± 0.71^{2a}
Nephropathy	1.42 ± 0.48^{1a}	1.33 ± 0.17^{1a}	1.49 ± 0.37^{1b}	2.34 ± 0.43^{2b}	1.50 ± 0.18^{1ab}	2.09 ± 0.24^{2c}	1.17 ± 0.12^{1ab}	1.55 ± 0.11^{2bc}
Vacuolation	1.39 ± 0.37^{1a}	1.59 ± 0.61^{2a}	0.94 ± 0.10^{1b}	1.19 ± 0.11^{1a}	1.10 ± 0.29^{1bc}	1.93 ± 0.56^{1a}	1.14 ± 0.15^{1ac}	1.78 ± 0.34^{2a}
Necrosis	0.66 ± 0.14^{1a}	1.22 ± 0.33^{1a}	1.92 ± 0.73^{1ab}	1.35 ± 0.34^{2b}	1.28 ± 0.44^{1b}	1.63 ± 0.83^{1b}	0.96 ± 0.44^{1ab}	1.68 ± 0.69^{1ab}
Nephro-calcinosis	0.81 ± 0.23^{1a}	0.90 ± 0.29^{1ab}	1.03 ± 0.16^{1b}	1.69 ± 0.45^{2a}	1.51 ± 0.57^{1ab}	1.87 ± 0.46^{2b}	1.14 ± 0.17^{1ab}	1.63 ± 0.57^{2ab}
Fibrosis	0.44 ± 0.37^{1a}	0.73 ± 0.62^{1a}	1.38 ± 0.74^{1b}	1.65 ± 0.57^{1b}	1.10 ± 0.43^{1ab}	1.63 ± 0.47^{1b}	0.81 ± 0.70^{1ab}	1.23 ± 0.59^{1ab}
Liver								
Glycogen-type vacuolation	1.63 ± 0.18^{1a}	1.92 ± 0.24^{1a}	2.95 ± 0.09^{1b}	3.10 ± 0.29^{1b}	1.98 ± 0.12^{1c}	1.95 ± 0.15^{2c}	2.40 ± 0.17^{1d}	1.92 ± 0.42^{2a}
Cytoplasmic vacuolation	0.59 ± 0.38^{1a}	0.72 ± 0.87^{1a}	1.83 ± 0.10^{1b}	2.08 ± 0.43^{1b}	1.03 ± 0.34^{1a}	1.42 ± 0.28^{1c}	0.92 ± 0.33^{1a}	1.32 ± 0.29^{1c}
Cytoplasmic degeneration	0.69 ± 0.46^{1a}	0.89 ± 0.21^{1a}	1.06 ± 0.55^{1a}	1.29 ± 0.17^{1a}	0.72 ± 0.44^{1a}	1.08 ± 0.41^{1a}	0.50 ± 0.27^{1a}	0.90 ± 0.84^{1a}
Cellular hypertrophy	0.82 ± 0.63^{1a}	1.40 ± 0.54^{1ab}	1.32 ± 0.75^{1b}	1.98 ± 0.53^{1b}	1.27 ± 0.60^{1b}	1.56 ± 0.60^{1ab}	0.43 ± 0.29^{1a}	0.96 ± 0.36^{2a}
Necrosis	0.97 ± 0.53^{1a}	1.07 ± 0.46^{1a}	0.90 ± 0.33^{1a}	2.50 ± 1.02^{2b}	1.47 ± 1.02^{1a}	2.07 ± 0.51^{1bc}	1.02 ± 0.60^{1a}	1.27 ± 0.66^{1ac}

of pathogen and infected cells, which are crucial for tissue repair (Richardson et al. 2013). Melanin granules released from the skin during severe wounds altered pigmentation, suggesting neural regulation in healing (Sveen et al. 2019). The healing process involved gradual scar fading with dermal fibrous tissue regrowth and subsequent skin and scale development from the wound edges toward the centre, similar to the stages observed in fish muscle wound healing (Vinoth et al. 2018). In the present study, OA treatment significantly accelerated the healing process compared to the untreated group and supported the findings noted in *A. hydrophila*-infected *O. niloticus* (Patel et al. 2024). Thus, OA emerged as a promising therapeutic for enhancing wound healing in *AV*-infected fish.

The study observed a sharp increase in plasma glucose in the challenged fish, indicating *AV*-induced stress, corroborating earlier findings (Julinta et al. 2017, Patel et al. 2024). The four- and two-fold increase in creatinine and AST and ALT levels on DPI 1 suggested renal dysfunction and hepatic damage, respectively, which resembled the observations reported in Elgendy et al. (2022) and Said et al. (2023). The histopathological alterations in the liver and kidney tissues supported these observations. An 2.5-fold increase in ALP levels confirmed altered immune responses, which was also justified by the rise in leucocytes. Additionally, a significant drop in plasma calcium and chloride levels after the challenge highlighted ionic imbalance, aligning with Wei et al. (2016). The gradual normalization of their levels indicated recovery from and re-establishment of homeostasis. Nevertheless, OA therapy resulted in a quicker normalization of all parameters measured compared to the untreated group, demonstrating the efficiency of OA in lowering pathogenic bacterial burden and supporting findings noted in *Cyprinus carpio* (Harikrishnan et al. 2003) and *O. niloticus* (Patel et al. 2024).

In the present study, a reduction in TECs in *AV*-challenged groups was noted, with counts decreasing drastically within 24 hours, supporting recent findings in *AV*-infected *O. niloticus* (Elgendy et al. 2022, Said et al. 2023). This decrease could have been attributed to hemolysin-induced congestion and hemorrhages in hematopoietic organs (Liu et al. 2022). However, the fish subjected to OA therapy were able to mount adaptive responses and increase the TEC levels similarly to the responses Husien and Younis (2000) observed in *O. niloticus* infected with enteric red mouth disease. The present research noted a three-fold increase in TLCs, the principal non-specific defence against infections in fishes, in the untreated group, and a two-fold increase in the OA-treated group on DPI 7. In contrast, Elgendy et al. (2022) and Said et al. (2023) reported that *O. niloticus* exhibited decreased TLCs when injected with *AV,* most likely because of cell lysis. However, TLCs gradually decreased from DPI 14 to 21 but remained high, indicating persistent infection, with greater reductions in the OA-treated group. The TCs, which have critical roles in hemostatic and inflammatory responses to microbial stimuli (Ferdous and Scott 2015), were increased in *AV*-challenged fish on DPI 1, corroborating findings on *A. salmonicida*-infected *Oncorhynchus mykiss* (Bektas and Ayik 2022). Subsequently, TCs dropped in both treated and untreated groups, with the OA-treated group showing a faster reduction, potentially due to the OA. This study, thus, emphasized the effectiveness of OA in reducing *AV*-infection and subsequent thrombocyte responses. Erythrocytes alter significantly in response to various stimuli, resulting in erythrocytic cellular abnormalities (ECA) and erythrocytic nuclear abnormalities (ENA) (Bardhan et al. 2022). The extracellular toxic enzymes of *AV* reportedly affect erythrocytes significantly (Pessoa et al. 2019). Besides, *AV* pathogenicity is associated with virulence genes such as *act*, *alt*, *aerA*, and *hlyA*, which encode enterotoxins and hemolytic toxins (Sreedharan et al. 2013), specifically *hlyA* expression caused erythrolysis and cytotoxicity (Gao et al. 2013). In the present study, the morphological abnormalities in *AV*-challenged fish were intensified from DPI 1 to 7, which then gradually decreased from DPI 14 with mild abnormalities persisting even on DPI 21. The untreated group had more ECAs from the cytotoxic effect of *AV*, while the OA-treated group had more

ENAs, possibly from the nucleotoxic effect of OA (Abraham et al. 2024).

The liver and kidney are the major target organs for *AV*-infection, which in severe cases causes widespread necrosis along with renal hypertrophy (Smyrli et al. 2017). In the present study, the kidney histopathology revealed severe damage in the renal tubules. The infiltration of blood cells near the renal capillaries indicated the inflammatory responses. Nephrocalcinosis can be linked to a decrease in plasma calcium. In a similar study, Hassan et al. (2017) found that the kidneys of *AV*-infected *O. niloticus* exhibited interstitial tissue hemorrhages, melanophore aggregation, hemosiderin deposition, and eosinophilic hyaline droplets in the epithelial lining of certain renal tubules. Further, the *AV*-infected kidney of *O. niloticus* showed tubular degeneration and hemorrhage within the renal tubules (El-Latif et al. 2019), which confirmed the findings of the present study. The renal tissue damage in *AV*-challenged groups increased on DPI 7 and then gradually decreased on DPI 21. However, the damage was significantly lower in the OA-treated group. The observed reduction in plasma creatinine from DPI 7 to 21 indicated an improvement in kidney function of the OA-treated group and was comparable to the findings of Hal and El-barbary (2021). These findings, which have been confirmed in several antibiotic studies (Limbu et al. 2021, Patel et al. 2024), demonstrated the beneficial effects of OA on renal tissue architecture within tolerable toxicity levels. The liver tissues of *AV*-challenged groups showed glycogen-type vacuolation and other anomalies that corresponded to previous studies (Hassan et al. 2017, El-Gohary et al. 2020). The damage increased on DPI 7, but decreased progressively from DPI 14. Our results corroborate a previous study (Dong et al. 2017) that noted hemosiderin depositions around liver cells and arteries, tissue disintegration, and blood congestion in the liver of *Aeromonas*-infected fish. The treated group showed steady recovery indicating the ability of OA to combat *AV*-infection. Yet, even on DPI 21, anomalies were still visible in both groups, indicating the persistent effects of *AV*-infection and minor drug toxicity. Quinolones, such as OA, have been associated with structural abnormalities in the liver, such as glycogen-type vacuolation, in healthy *O. niloticus* (Abraham et al. 2024). The results of the present study suggested that OA, though mildly toxic, can help improve feed intake and biomass and control *AV*-infection in *O. niloticus* by reducing mortalities.

Conclusions

Overall, administering OA in the feed at the proper dose facilitated faster wound healing and was effective in improving the survival, hematological, erythrocyte, biochemical, and histopathological anomalies of *AV*-challenged fish. The results further highlighted OA as a promising therapeutic agent against bacterial infection, particularly effective in managing *AV* infection in *O. niloticus*. The present study's results on the ability of OA to enhance wound healing, normalize biochemical and hematological parameters, and mitigate organ damage underscore its multifaceted therapeutic benefits. Nevertheless, concerns over the use of OA, alongside other quinolones like enrofloxacin and ciprofloxacin, in global aquaculture underscore regulatory gaps, as these antibiotics lack specific guidelines for their application. Globally recognized as critically important for human and veterinary medicine by the World Health Organization (WHO 2024) and the World Organisation for Animal Health (WOAH 2021), the restrained use of antimicrobials including OA is crucial to avert the development of antibiotic resistance. Following WOAH guidelines, OA should only be used as a secondary treatment option in food-producing animals when no alternatives exist, aiming to uphold sustainable aquaculture practices and safeguard public health against antibiotic resistance.

Ethical Statement. The present investigations adhered to the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, for conducting experiments on fish. The experimental protocols received approval from ICAR, Government of India, New

Delhi, as part of the All-India Network Project on Fish Health (No. CIBA/AINP-FH/2015-16 dated July 16, 2015).

Acknowledgments. The authors would like to thank the Vice-Chancellor of West Bengal University of Animal and Fishery Sciences, Kolkata for providing the necessary facilities for carrying out this work.

Conflicts of interest. The authors have no conflicts of interest to disclose.

Author contributions. P.S., A.S., J.S., R.D., A.G. and S.B.: Laboratory investigations, formal analysis, data generation, data curation, statistical analyses, and drafting the manuscript. T.J.A.: Conceptualization, experimental design, data interpretation, writing - review and editing the manuscript. P.K.P.: Resources, project administration, and funding acquisition.

Funding. Indian Council of Agricultural Research (ICAR), Government of India, New Delhi under the All-India Network Project on Fish Health (Grant F.No. CIBA/AINP-FH/2015-16 dated 02.06.2015).

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