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REPORT

Haematological Analysis of Common Carp (*Cyprinus carpio*), Gold Fish (*Carassius auratus*), Tilapia (*Oreochromis mossambicus*) and Stinging Catfish (*Heteropneustes fossilis*) spontaneously infected with *Aeromonas hydrophila*

Dhanaraj M.[1*], **Haniffa M. A**[1]., **Muthu, R. C.**[1], **Arockiaraj, A. J.**[2]., **Raman, S. S.**[1] and **Singh, A. S. V.**[1]

[1] Centre for Aquaculture Research and Extension (CARE) St. Xavier's College (Autonomous), Palayamkottai, 627 002, Tamil Nadu, India
* dhana.micro@gmail.com (corresponding author)
[2] Bengis Centre for Desert Aquaculture, The Albert Katz Department of Dryland Biotechnologies, The Jacob Blaustein Institute for Desert Research, Ben-Gurion University of the Negev, Sede Boker Campus 84990, Israel
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ABSTRACT Gram negative ubiquitous bacterium *Aeromonas hydrophila* was injected into four freshwater fish species: common carp *Cyprinus carpio*, gold fish *Carassius auratus*, tilapia *Oreochromis mossambicus* and stinging catfish *Heteropneustes fossilis* at the rate of 10^6 cfu/ml intramuscularly. Dermomuscular lesions resulted in *H. fossilis* and *C. auratus* was severe where as in *C. carpio* and *O. mossambicus* it was less severe. The result of cumulative mortality was maximum (100%) in *H. fossilis* on day 21, followed by *C. auratus* (60%), *C. carpio* (45%) and *O. mossambicus* (10%) respectively. The hematological parameters *viz.*, white blood cells (WBC), red blood cells (RBC), haemoglobin (Hb) and phagocytic activity were monitored at days 0, 7, 14, and 21. WBC (10^4 mm^{-3}) showed a linear increase from day 0 to day 21 and it was statistically different at $p < 0.05$ level. RBC (10^6 mm^{-3}) exhibited a linear decrease from day 0 to 21 in *C. carpio, C. auratus* and *H. fossilis*, but in *O. mossambicus* it decreased from day 0 to day 7 and increased after 7 days and reached a high level on day 14 and then decreased again in day 21 reaching the count observed on Day 0. Hb content of *C. carpio, C. auratus* and *H. fossilis* were statistically significant ($p < 0.05$) but it was not statistically significant in *O. mossambicus*, since the measurement was not uniform like the other three species. The results of RBC measurement of *O. mossambicus* were also similar to the results of Hb. The phagocytic activity *of C. carpio, C. auratus* and *H. fossilis* increased significantly from day 0 to day 21 and it was statistically significant at $p < 0.05$ level, but in *O. mossambicus*, a slight fluctuation was noticed during the experimental period.

(Hematological parameters, *Heteropneustes fossilis, Cyprinus carpio, Oreochromis mossambicus, Carassius auratus* and *Aeromonas hydrophila*)

INTRODUCTION

Aeromonas hydrophila is one of the most common bacteria in the freshwater habitat throughout the world. It is a ubiquitous free living gram negative bacterium prevalent in aqueous environments such as freshwater lakes and streams, domestic tap water and sewage [12]. *A. hydrophila* is recognized as a scourge of freshwater fish farming world wide and considered to be a major economic problem. It causes hemorrhagic septicemia and Epizootic Ulcerative Syndrome (EUS) in freshwater fishes in all Asian countries [10, 21]. Outbreaks of EUS and hemorrhagic septicemia caused by *A. hydrophila* infection occur in both wild and

cultured fish such as snakehead (*Channa striatus*), walking catfish (*Clarius batrachus*), brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*), chinook salmon (*Oncorhynchus tshawytscha*), Japanese eel (*Anguilla japponica*), American eel (*Anguilla rostrata*), goldfish (*C. auratus*), golden shiner (*Natemigonus crysoleucas*) and tilapia (*Oreochromis mossambicus*) [2, 7, 9, 17]. The objective of this study was to assess the changes and compare certain blood parameter of *C. carpio, C. auratus, H. fossilis* and *O. mossambicus* spontaneously infected with *A. hydrophila.*

MATERIAL AND METHODS

Collection of fish

C. carpio (length 13 ± 2 cm and weight 35 ± 2.4g), *O. mossambicus* (9 ± 2 cm and 25 ± 0.5g), *H. fossilis* (10 ± 1cm and 30 ± 2g) and *C. auratus* (5.5 ± 0 cm and 17 ± 0.4g) were collected from river Thamiraparani, Tirunelveli (8.41° N, 77.44° E), India. They were transported to Centre for Aquaculture Research and Extension (CARE) in plastic containers filled with oxygenated water and acclimatized in the stocking pond (11x 5x 1.5m) for two weeks.

Growth of *A. hydrophila*

A. hydrophila was obtained from Microbial Type Collection Centre, Chandigar, India. Subcultures were prepared and maintained on tryptic soy agar slopes (Himedia, India) and stored at 5°C. Culture was harvested in tryptic soy broth (Himedia, India). The broth was incubated overnight in a shaker for 12 h at 20°C and centrifuged at 10,000 rpm for 20 min at 4°C. The suspension was washed twice with phosphate buffered saline (pH 7.2) and prepared to the concentration of 10^6 cfu/ml and counted in a Neubauer haemocytometer slide [27].

Experimental Design

The acclimatized fishes were collected from the stocking pond using drag net and the species groups were separated as assigned for the treatment. Four different treatments were conducted for four species. Each treatment consisted of 20 fishes. Three replicates were maintained for each treatment. They were fed *ad libitum* with commercial feed (Avanthi Feeds, India).

Experimental injection

Fishes were injected intramuscularly (IM) with 100µl of *A. hydrophila* at a concentration of 10^6 cfu/ml to induce ulcers [6]. Control group was injected with fish physiological saline. Mortality and development of lesion were observed and recorded regularly till the termination of the experiment criteria for grading lesions were graded followed by the methodology of Lio-Po *et al.* [18] as follows:

- \- Normal intact skin
- -/+ Intact but melanized skin at injection site
- \+ Blanching slide swelling of injection site
- ++ Blanching furuncle-like lesion with dermal erosion with or without haemorrhagic periphery
- +++ Extensive blanching lesion with furuncle like ulcerated core.
- ++++ Ulcerated lesion with underlying necrotic musculature.

Blood sample collection

Blood sample (0.05ml) was collected from randomly selected six individuals from each species group by vein puncture on days 0, 7, 14 and 21 at one week intervals. The blood was collected using vacutainer fitted needle (20 gauge). The blood was collected at 19.00 h after the last feeding done at 10.00 h. Before collecting the blood sample the needle were treated with either 0.5% EDTA or sodium heparin (5000IU/5ml; JBE limited, India) to avoid blood coagulation [24]. The samples were stored at 4°C for further examinations. During the experimental period temperature (30°C), dissolved oxygen (5.8mg O_2 ml^{-1}) and pH (7.4) were maintained.

Haematological indices

The following haematological parameters *viz.*, WBC count, total erythrocyte count (RBC), Hb and phagocytic activity were analyzed on days 0, 7, 14 and 21. RBC (10^6 mm^{-3}) was determined by 1:20 dilution ratio of the blood sample in Hayem's solution and WBC (10^4 mm^{-3}) with 1:200 dilution ratio of the blood sample in Turke's solution. The cells were counted in Neubauer haemocytometer. Hb (g/dl) was determined by cyanhaemoglobin method [13, 15, 16, 32]. Phagocytic activity was analysed on kidney samples. The percentage of phagocytic activity was calculated [25] as follows:

Phagocytic activity =

$$\frac{\text{No. of phagocytic cells}}{\text{Total no. of cells}} \times 100$$

Statistical analysis:
The data were subjected to mean, standard deviation and student "t" test using SPSS 7 software package.

RESULTS

Dermal lesions were induced by *A. hydrophila* at the concentration of 10^6 cfu/ml and the details of the results were presented in Table 1. Cumulative mortality is shown in Figure 1. After 24 hours post injection, extensive blanching ulcerative lesion was started in *H. fossilis* (+++) and dermal erosion was noticed in *C. auratus* (++) and *C. carpio* (++) on day 1. Eroded lesions developed at the beginning (day 0) in *O. mossambicus* and it disappeared on day 6. At the end of the trial (Day 21) the necrotic tissue eventually sloughed off, leaving a deep and severe ulcerative lesion in *H. fossilis* (++++) and *C. auratus* (+++). No symptoms were seen in control individuals injected with fish physiological saline. Among the four experimental fishes *H. fossilis* was easily susceptible to injection and expressed the symptoms within 24 hours. Cent percentage mortality was noticed first in *H. fossilis* on day 21 followed by 68% in *C. carpio* and 45% in *C. auratus*. The total mortality was comparatively less (10%) in *O. mossambicus* and also no mortality was noticed after day 7, showing the strong and hardy nature of the fish. No mortality was noticed in control groups.

Table 1. Lesion development in experimental fishes infected with *A. hydrophila*

EXPERIMENTAL FISH	CONCENTRATION OF *A. hydrophilla*	DAYS OF POST EXPOSURE							
		1	3	6	9	12	15	18	21
Control	10^6	-	-	-	-	-	-	-	-
H. fossilis	10^6	+++	+++	++++	++++	++++	++++	++++	++++
C. auratus	10^6	++	+++	+++	+++	++++	++++	+++	+++
C. carpio	10^6	++	++	++	++	+++	++	++	+/-
T. mossambicus	10^6	+	-/+	-/+	-	-	-	--	--

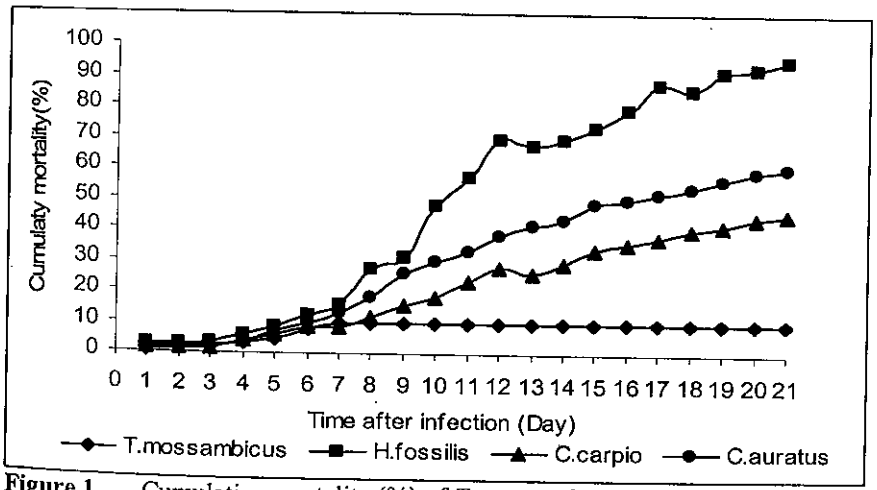

Figure 1. Cumulative mortality (%) of *T. mossambicus* (♦), *H. fossilis* (■), *C. carpio* (▲) and *C. auratus* (●) after injected with *A. hydrophila* at 10^6 cfu/ml

The haematological indices of fishes infected with *A. hydrophila* and control is presented in Figures 2a to 2d. *A. hydrophila* failed to induce dermal lesions in *O. mossambicus* but infection caused internally the variation in WBC, RBC, Hb and phagocytic activity. The WBC increased from day 1 to day 7 and it was 3.78± 0.75, 3.84 ± 0.71, 4.26 ± 1.08 and 4.09 ± 0.98 in *O. mossambicus, C. auratus, H. fossilis* and *C. carpio* respectively. Between day 14 and 21, WBC levels significantly increased to a maximum in infected *H. fossillis* (5.08 ± 1.52)

and *C. auratus* (4.51 ± 1.10). *O. mossambicus* showed a slight decrease in WBC on day 14 (3.24 ± 0.62) and day 21 (3.46 ± 0.51) respectively. In *O. mossambicus* the RBC count decreased from day 1 to day 7 (2.45 ± 0.35) and increased upto a maximum (3.38 ± 0.98) level on day 14. When compared to the initial level of RBC (2.79 ± 0.88) in *O. mossambicus*, it was not statistically significant, but in the other three species RBC count was statistically significant ($p < 0.05$). Hb content of all species declined from their initial level. The phagocytic activity was also decreased, but not in *O. mossambicus*. The phagocytic activity of *C. carpio, H. fossilis* and *C. auratus* increased from day 7 to day 14 and it was statistically significant at $p < 0.05$. But in *O. mossambicus* the phagocytic activity reached near the initial value (65.7 ± 3.5) at day 21.

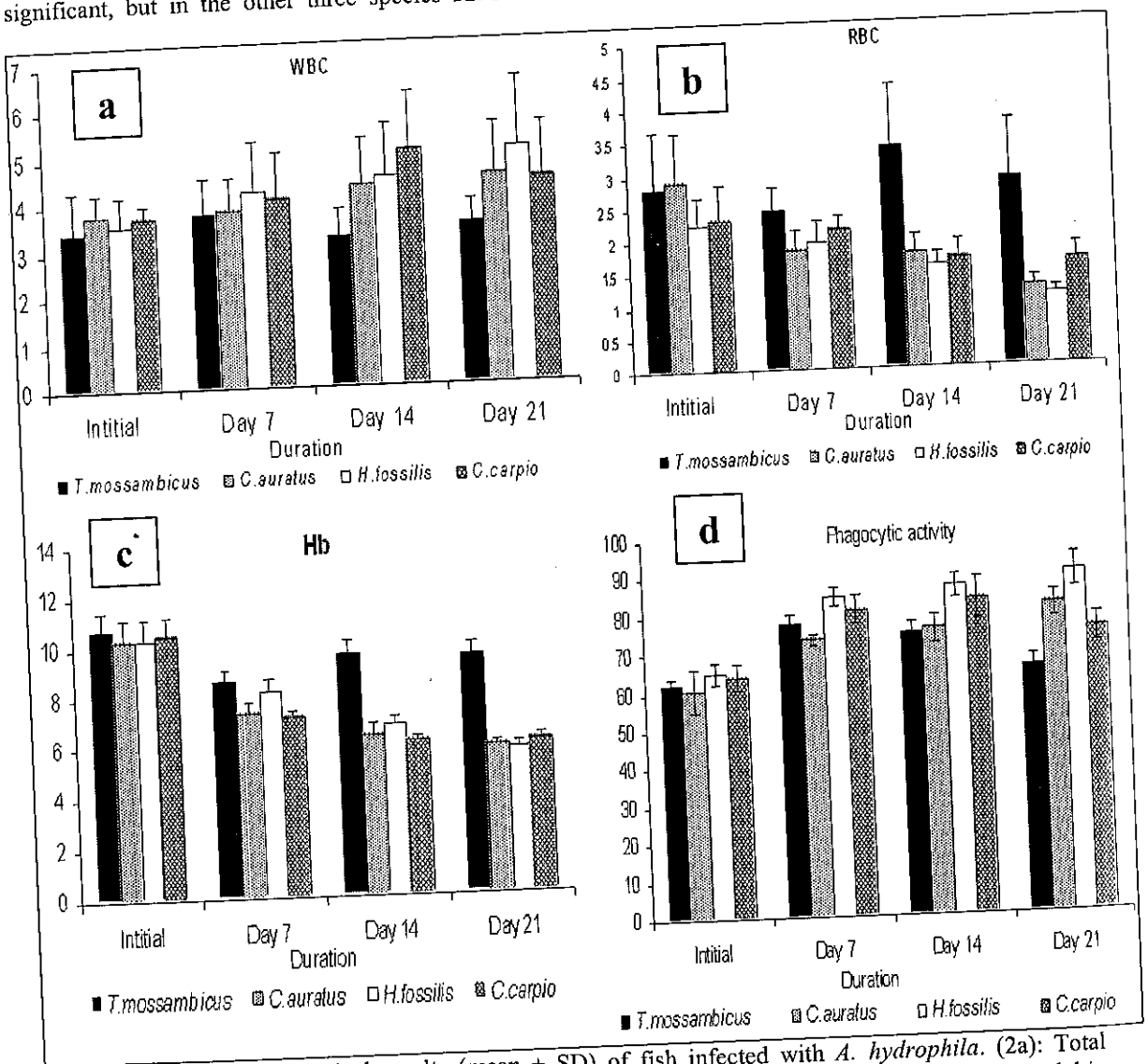

Figure 2. Haematological results (mean ± SD) of fish infected with *A. hydrophila*. (2a): Total leukocyte count (WBC; 10^4 mm^{-3}), (2b): Total erythrocyte count (RBC; 10^6 mm^{-3}), (2c): Haemoglobin content (Hb; g/dl) and (2d): Phagocytic activity (%) at day 1, 6, 14 and 21

DISCUSSION

The present study confirmed that *A. hydrophila* is the primary causative agent for haemorrhagic septicemia and secondary causative agent for EUS in all fishes. Bacterial concentration is responsible for the development of haemorrhagic lesions. A bacterial dose of at least 10^6 cfu/ml cells can induce dermomuscular lesions [18]. The present findings confirmed the statement of Lio-

Po *et al.* [18] and the dermomuscular lesions resembling to EUS. A variation in the intensity of the lesions and mortality pattern depends on the quality of the bacterial isolates. The variability in virulence among strains of *A. hydrophila* was similar in the present study as reported by Torres *et al.* [29] in grass loach. Severe dermomuscular lesion was observed in *H. fossilis,* showing the easy infection of catfish as suggested by Lio-Po *et al.* [19] in walking catfish induced with 10^6 cfu/ml [19]. Cumulative mortality was also comparatively high (100%) in *H. fossilis* on day 21 and it is confirmed that the catfish gets infected easily and leads to high mortality. Similarly the complete mortality was observed in *Clarius batrachus* induced with *A. hydrophila* at the concentration of 10^7 cfu/ml [20]. The present result also confirmed the previous reports about catfishes. Similarly *C. auratus* also showed severe infection in haemopoietic system, degeneration of the liver and mild necrosis of the kidney as reported by Brenden and Huizinga [6] in the same species. *A. hydrophila* is virulent on *Osphronemus gouramy* and non-virulant on *C. carpio* [27]. In the present results it was most virulent on *H. fossilis*, comparatively less on *C. auratus and C. carpio*, but non-virulent *O. mossambicus*.

The WBC level of four infected species initially increased from the initial level and after day 14 and day 21 it increased significantly ($p < 0.05$) in *H. fossilis, C. auratus* and *C. carpio*. But in *O. mossambicus* it decreased upto day 14 and reached near the initial level at the end of the trial (Day 21). The infection increased the leukocyte count in blood parameter as a mechanism of defense against the pathogens which is well understood [3, 8, 31]. In almost all infected species, the homeostatic processes are extended beyond the normal level due to stress [23]. Due to *A. hydrophila* infection in all group fishes, the RBC count and haemoglobin level decreased ($P > 0.05$) from day 7. In the case of Tilapia RBC count and Hb content did not show any significant difference on day 21. In *H. fossilis, C. auratus* and *C. carpio* the RBC and Hb level significantly decreased from day 7 to day 21. The result of decreased haemoglobin and RBC may be due to the poor mobilization of haemoglobin from the spleen and other haemopeotic organ in *Ictalurus punctatus* [26]. In the present study, a significant decrease in erythrocyte and haemoglobin content was observed which is possibly due to hypochromic microcytic anemia caused by *A. hydrophila* infection. Haemoglobin content and erythrocyte number may be severely affected by bacterial infections [4]. Experimental infection of *Edwardsiella tarda* clinically changed the blood parameters. A significant reduction ($p < 0.05$) in haemoglobin and erythrocytes and significant increase ($p < 0.05$) in the total leukocyte level was observed in infected tilapia [5]. In the present finding, similar changes in blood parameters were observed in all species, but in tilapia the changes occurred reversibly. Decrease in RBCs and Hb concentration indicate that RBCs are being destroyed by the leukocyte activity in an erythrocyte anemia with subsequent erythroblastosis [11]. The EUS infected pearl spot fish *Etroplus suratensis* with anemic condition followed by a significant reduction in RBC, Hb and PCV [22]. The phagocytic activity was significant ($p < 0.05$) in *H. fossilis, C. auratus* and *C. carpio* and not significant in tilapia. Limited reports were available on alteration of blood parameter in tilapia, gold fish, catfish and common carp infected with *A. hydrophila* especially in erythrocyte count and phagocytic activities [1, 4, 21].

Conclusively among the four freshwater fishes, the experimental infection by *A. hydrophila*, stinging catfish *H. fossilis* was affected quickly and infected severely. So in farm conditions, intensive care is necessary for catfish culture. Further tilapia is a strong and hardy fish, so it could be suggested that either tilapia is resistant to the pathogen or the dosage was insufficient.

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