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Copper Exposure Reduced the Resistance of the Catfish *Saccobranchus fossilis* to *Aeromonas hydrophila* Infection

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Infections remain a major cause of fish morbidity and mortality in aquacultural practices. Environmental pollutants such as heavy metals, industrial wastes and pesticides induced immunosuppression and could seriously increase this already high burden of fish ill-health (Sindermann 1979; Zeeman and Brindley 1981). The latest research, some of which has been conducted on fish (Stevens 1977; Vaile and Calamari 1984; Khangarot and Tripathi 1991; Tripathi 1993) indicates that heavy metals are highly immunosupprssive. It has been demonstrated that copper exposure induces immunotoxicity and thus reduces the resistance of the fish to protozoan, viral and bacterial infection (Hetrick et al. 1979; Knittel 1981; Khangarot et al. 1988). Toxic concentrations of Cu enter into the aquatic environment through wide variety of industrial effluents, mining wastes and metal industries (Niragu 1974).

The purpose of this study was to examine the exposure of sublethal concentrations of Cu for 14 days on the susceptibility of an Asian catfish Saccobranchus fossilis (Bloch) to Aeromonas hydrophila infection. & hydrophila is a gram negative bacterium widely distributed in freshwater environments, where it causes bacterial hemorrhagic septicemia (BHS) disease in many fish species (Hazen et al. 1978).

MATERIALS AND METHODS

Air-breathing catfish, S. fossilis (body length 5-8 cm, wet weight 10-12 gm) were collected from local sources and acclimatized to laboratory regimens in 60L glass aquaria for 14 days before Cu exposure. Fish were fed living daphnids, mosquito larvae and goat liver five days a week during acclimatization. Fish were exposed to 0.1 and 0.32 mg/L of Cu (as $CuSO_4 . 5H_2O$) and control for 14 days. Tests were run in duplicate with a group of 10 fish for each Cu concentration and control

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tests. The physico-chemical properties well water used as test water were determined using the procedure outlined in standard methods (APHA et al 1991). Test water characteristics are as follows: temperature 22-25°C; pH 7.8; dissolved oxygen 5.2-5.8 mg/L; total alkalinity 140-160 mg/L as $CaCO_3$; total hardness 210-230 mg/L as $CaCO_3$.

An isolate of fish pathogen A. hydrophila was used in fish infection experiments. The organism is gram negative, motile and has other biochemical characteristics identifying it as A. hydrophila. Slant cultures were maintained on trypticase soy agar under mineral oil at 4°C. Mass culture of bacteria to be used for challenge were grown for 24 hr at 37°C on nutrient agar. Harvested cells were suspended in sterile phosphate buffer saline (PBS). Ten-fold serial dilutions (10^{-2} to 10^{-8}) were then made from this suspension and the number of viable cells at each of the dilutions were determined by the pour colony counting method.

After 14 days of Cu exposure, fish were kept in well water for the next two days to observe delayed fish mortality, if any. Intraperitoneal injection (IP) route was used for fish infection. Fish were then challenged by a graded ten-fold dilution dose of live A. hydrophila and fish death was observed for 14 days. The moving average angle method (Harris 1959) was used to calculate LD_{50} of live A. hydrophila needed for Cu-exposed and control group fish. The median lethal time (LT_{50}), slope function (S), reaction time ratio (RR), slope function ratio (SR) and their 95% confidence limits (CL) were calculated as procedure outlined by Litchfield (1949) and Litchfield and Wilcoxon (1949). Dead fish were removed immediately and the kidney was examined within less than six hr to verify the presence of A, hydrophila. A negative control with phosphate buffer saline was carried out to determine whether laboratory transfer, intraperitoneal injection, trauma and delayed Cu-toxicity caused fish mortality without challenge to A, hydrophila pathogen, Test fish were not fed during the bacterial challenge period.

RESULTS AND DISCUSSION

Fish mortality data (Table 1) at various ten-fold dilutions of A. hydrophila indicate that Cu-exposed fish were more susceptible than Cu-unexposed fish to disease caused by this bacterial pathogen. Fish mortality was significantly related to the presence or absence of Cu exposure and to intraperitoneal injected (IP) A. hydrophila dose. In experiment 1, the per cent fish mortality for all the tested bacterial doses combined

Table 1. Mortality of control and Cu-exposed catfish in two weeks after intraperitoneal injection with live Aromonas hydrophila.

Experiment	Ten-fold dilution of A. hydrophila[a]	No. of dead fish (mg/L) of Cu		
		0.0	0.10	0.32
Exp.No. 1	10^{-2}	10[c]	10	10
	10^{-3}	10	10	10
	10^{-4}	10	10	10
	10^{-5}	2	9	10
	10^{-6}	1	4	7
	10^{-7}	-	2	3
	10^{-8}	-	1	2
	Diluent	-	-	-
Total mortality		33/80	46/80	52/80
Per cent mortality		41.25	57.5	65.0
LD_{50} (bacteria/mL)[b]		6.038	4.847	4.23
Exp. No. 2	10^{-2}	10[c]	10	10
	10^{-3}	10	10	10
	10^{-4}	10	10	10
	10^{-5}	3	8	10
	10^{-6}	2	6	8
	10^{-7}	-	4	2
	10^{-8}	-	-	-
	Diluent	-	-	-
Total mortality		35/80	48/80	50/80
Per cent mortality		43.75	60	62.5
LD_{50} (bacteria/mL)[b]		5.813	4.441	4.231

a= $\log_{10}$ dilution of A. hydrophila culture contain 7 x 10^{10} CFU/mL
b= $\log_{10}$ lethal dose
c= Number of fish tested (10)

was 65% in 0.32 mg/L of Cu, 57.5% in 0.1 mg/L of Cu, and 41.25% in the control group. The mean calculated log LD_{50} (bacterial dose causing 50% fish mortality) for A. hydrophila in control was 5.926, and for 0.1 mg/L and 0.32 of Cu; these values were 4.644 and 4.232, respectively. Cumulative per cent daily fish mortality of both the experiments suggest that Cu exposed fish died sooner than control groups. All the fish (100% mortality) died in 7.8 x 10^{-4} dose level after four days Of A. hydrophila challenge Cu exposed group. Fish were not fed during bacterial challenge period to reduce the chances of additional burden of pathogen challenge due to fouling of test water by the food.

Test for parallelism of dose per cent lines of control

Table 2. The median lethal time (LT_{50}) in hour, slope function (S), slope function ratio (SR) reaction time ratio (RR) and their 95 per cent confidence limits (CL) in control, 0.1 and 0.32 mg\L of Cu for different A. hydrophila dose levels.

Parameters	copper concentrations (mg\L)		
	0.0	0.1	0.32
	10^{-2} of A. hydrophila dose level		
LT_{50}	55	36	29
95% CL	(42-73)	(28-46)	(17-49)
S	1.65	1.62	2.44
95% CL	(1.32-2.06)	(1.31-1.98)	(1.68-3.54)
SR		1.02	1.42
95% CL		(0.87-1.91)	(1.2-2.06)
fsr		1.37	1.55
RR		1.53	1.83
95% CL		(1.06-2.22)	(1.03-3.4)
frr		1.45	1.82
	10^{-3} of A. hydrophila dose level		
LT_{50}	72	46	30
95% CL	(53-99)	(28-76)	(18-50)
S	1.68	2.21	2.3
95% CL	(1.33-2.12)	(1.6-3.1)	(1.6-2.3)
SR		1.32	1.37
95% CL		(0.9-1.93)	(0.88-2.12)
fsr		1.46	1.55
RR		1.57	2.4
95% CL		(1.31-1.88)	(1.21-4.44)
frr		1.2	1.85
	10^{-4} of A. hydrophila dose level		
LT_{50}	90	48	42
95% CL	(73-111)	(34-68)	(30-58)
S	1.61	2.24	2.19
95% CL	(1.39-1.87)	(1.71-2.19)	(1.71-2.8)
SR		1.39	1.36
95% CL		(1.03-1.88)	(1.02-1.81)
fsr		1.35	1.33
RR		1.88	1.73
95% CL		(1.25-2.81)	(1.15-2.4)
frr		1.5	1.5

and Cu-treated fish are compared to similar set of bacterial (A. hydrophila) dose levels. At 10^{-2} and 10^{-3} ten-fold dilution of A. hydrophila dose levels the slope function ratio (SR) is less than fsr, the curves are considered parallelism within experimental error. Copper exposure at 0.32 mg\L in case of 10^{-4} dilution dose level the SR exceeds the fsr, therefore, the curve deviate significantly from parallelism. The slope difference may be interpreted as evidence that the

course of the fish mortality has been altered by the IP injection of A. hydrophila at 0.32 mg\L of Cu exposure. The reaction time ratio (RR) at 10^{-2}, 10^{-3} and 10^{-4} bacterial dose levels are greater than the frr (see Table 2) in case of 0.1 and 0.32 mg\L of Cu. This suggest that Cu exposure significantly decreased the fish mortality time (LT_{50}) following A. hydrophila infection.

In our previous studies with S. fossilis it was found that sublethal Cu concentrations adversely affect the humoral and cell-mediated immune system parameters (Khangarot et al. 1988; Khangarot and Tripathi 1991). Copper ions suppressed the fish immune responses, therefore, in the present study the 14 days exposure of 0.1 and 0.32 mg/L of Cu increased the susceptibility of S. fossilis to bacterial pathogen A. hydrophila. The spleen and head kidney have been suggested as being center of antibody production in freshwater fishes (Ellis et al. 1976). Copper ions generally bind with proteins, nucleic acids, and other biological ligands (Keller 1980). The reduced resistance of S. fossilis to A. hydrophila observed could result from the action of Cu on one or several immunologic mechanisms. The results of the present study supported the previous studies carried out with salmon and other fish species, which has shown that sublethal exposure of Cu increased the susceptibility of salmonids to viral and bacterial pathogens (Hetrick et al. 1979; Knittel et al. 1981).

The increased susceptibility to bacterial infection after exposure to Cu ions is of paramount aquaculture significance. [There have been reports of higher levels (0.05 to 0.2 mg/L) of Cu in the vicinity of metal mines (Niragu 1974) .] A large number of cultured and wild fish populations are continuously exposed to an appreciable quantity of Cu and other toxic heavy metal ions which are released continuously from industrial effluents, mining and sewage wastes. The determination of actual effects of toxic heavy metals on fish immune systems and resistance to diseases under field conditions is urgently needed and such studies are in progress in our laboratory.

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