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Kinetics of Antibody Titers and Degree of Protection in Indian Major Carps, Catla (*Catla catla*) and Rohu (*Labeo rohita*) to *Aeromonas hydrophila* Antigen

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A B S T R A C T

The kinetics of antibody titers and degree of protection in Indian major carps, catla (*Catla catla*) and rohu (*Labeo rohita*) against *Aeromonas hydrophila* antigen were studied in details at four different age groups of 120, 150, 210 and 270 days old. The first injection (i.m) was given with 0.5 ml of 10^8 cells/fish of *A. hydrophila* antigen. The second injection was given on 20 days after the first injection with same dose. The peak antibody titer was observed on day 14 after first injection and on day 7 after second injection in all the age groups of both the species. Higher age groups of fish produced higher level of antibody titers after first as well as second injection. Antibody levels were found to decrease on day 21 after first injection and on day 14 after second injection. The secondary antibody titers were higher to that of antibody primary titers produced in all age groups and was significant ($p<0.05$). The level of antibody titers also increased with age within the species. Catla showed higher level of antibody titers compared to rohu in all the age groups after first and second injection. A considerable amount of protection was recorded on day 14 after first injection. High degree of protection was recorded on day 7 after second injection in all the age groups of fish in both the species. The protection levels were higher in older age groups after first as well as second injection and was significant ($p<0.05$). Catla showed higher degree of protection compared to rohu in all the age groups.

Introduction

Immune response has been studied in many fish species to a variety of antigens and recent investigations have shown that teleosts have the immunological capacity to respond to a variety of antigens (Smith *et al.*, 1967; Kidder *et al.*, 1973; Lamers and van Muiswinkel, 1985a; Grondel *et al.*, 1987; Karunasagar *et al.*, 1991; Azad *et al.*, 1999; Kalita *et al.*, 2006). *Aeromonas hydrophila* is a gram-negative bacterium, ubiquitous and heterogonous organism that produces disease known as motile aeromonad septicaemia (MAS) in fish under stress condition or in concert with infection by other pathogens. Protection in rainbow trout immunized with heat killed *A. hydrophila* cells against the homologous strain was reported upto 7 months after vaccination (Post, 1966). Lamers *et al.*,

(1985a) studied the humoral immune response and memory formation in carp (*Cyprinus carpio*) against formalin killed *A. hydrophila* and maximum antibody titer remained upto day 20. Heat killed bacterin *of A. hydrophila* induced higher antibody levels in carp (*Cyprinus carpio*) than formalin killed bacterin (Lamers and De Hass, 1983). Karunasagar *et al.,* (1991) reported very high antibody titers in catla followed by mrigal and rohu immunized by different routes against a haemolysin negative mutant *A. hydrophila*. The nature of antibodies produced in trout after injection, immersion and oral vaccination of live cells of *A. hydrophila* was studied (Loghothetis and Austin, 1994). The kinetics of the humoral response in bony fishes had studied in detail (Sailendri and Muthukkaruppan, 1975; Rijkers, 1982). Depending on the type of antigen and fish species, decrease in antibody titer may be fast or slow (Ellis, 1989) and also memory has been demonstrated in fish for cellular and humoral immune reactions (Botham *et al.,* 1980; Rizkars *et al.,* 1980a, Rizkers, 1982). Establishment of memory in carp took longer time than in mammals and remained for 8-12 months after primary stimulation (Lamers *et al.,* 1985c). The dependence of temperature on the immune response of poikilothermic vertebrates has been studied and reviewed (Rijkers *et al.,* 1980b). Thus, the present study was carried out to evaluate the kinetics of antibody titers and protective response against *A. hydrophila* antigen at different age groups of the two species of Indian major carps, catla (*Catla catla*) and rohu (*Labeo rohita*).

Materials and Methods

Fish: Hatchlings of 4 days old of Indian major carps, catla (*Catla catla*) and rohu (*Labeo rohita*) belonging to a particular brood stock were procured from the State Fish Seed Farm, BR project, Karnataka, India. Fishes were maintained in fish pond at college fish farm. Fish were fed twice daily and were segregated a specific interval and reared in same condition for 365 days. Four age groups (120, 150, 210 and 270 days old) of each species were considered for experimental purpose and average length and weight of different age groups are given in the Table 1.

Antigen: Virulent strain of *A. hydrophila* isolated from EUS affected *Sillago sihama* was used as antigen. The bacterium was grown in tryptose soya broth (TSB, Himedia, India) for 24 hrs at room temperature to a cell density of approximately 10^9 cells/ml. Cells were harvested by centrifugation for 10 minutes at 10, 000 rpm and washed three times in sterile phosphate buffered saline (PBS) and heat inactivated at 60°C for 1 hr in water bath. Inactivation was confirmed by spread plate count method. Antigen was kept at 4°C until used.

Immunization: 125 numbers of catla and rohu from each age group were acclimatized for 7 days in cement cisterns. After acclimatization, fishes were injected (i.m) with 0.1 ml of 10^8 cells/fish of *A. hydrophila* antigen and control group of fish received 0.1 ml/fish of phosphate buffer saline. The second injection was given 20 days after the first injection with same dose.

Antibody titers: Blood was collected individually from 5 numbers of fishes through caudal puncture at weekly intervals upto 3 weeks after first and second injection for estimating the antibody titers. Collected blood was stored overnight at 4°C. Serum was separated by centrifugation at 6000 rpm for 10 min, inactivated at 55°C for 30 min in a thermostat water bath. Doubling dilutions of the serum in sterile phosphate buffered saline (PBS pH 7.2) were taken in 'U' bottom micro-titer plate. Equal volume of heat inactivated suspension (10^9 cells/ml) of *A. hydrophila* was added with serum in each well. The plates were incubated for overnight at 4°C. The last dilution of the serum showing clear

agglutination was taken as the titer and expressed in log_2 values (Sundick and Rose, 1980). Challenge: Immunized and control fish were challenged with a fresh culture of *A. hydrophila grown* on TSB for 24hrs at room temperature. Ten numbers of fish in duplicate from immunized and control groups were challenged by injection (i.m) of 0.5 ml (10^6 cells/fish) of *A. hydrophila* at 14 and 21 days after first and also at 7, 14 and 21 days after second injection. The mortality was recorded daily upto 8 days post-challenge and relative percent survival (RPS) was analyzed as described by Amend (1981).

Results and Discussion

The kinetics of the primary and secondary antibody titers to *A. hydrophila* antigen at different age groups of catla (*Catla catla*) and rohu (*Labeo rohita*) is given (Table 2 and 3). Catla and rohu produced antibody titers at all age groups against the *A. hydrophila* antigen. The peak antibody levels recorded on 14 days after first injection were 8.25±0.25; 8.5±0.25; 8.75±0.25 and 9.16±0.30 for 120, 150, 210 and 270 day old groups of catla respectively. While, in case of rohu, the peak antibody titers recorded on 14 days following first injection against *A. hydrophila* antigen were 7.40±0.24, 7.42±0.20, 8.00±0.17 and 8.89±0.11 for 120, 150, 210 and 270 day - old groups of rohu respectively. The antibody levels were found to decrease 21 days after first injection in both catla and rohu. Higher age groups of fish recorded higher level of antibody titers with increase in age of the fish. The peak primary antibody titers increased with both the species are given (Table 2 and 3). The peak antibody titers produced in higher age groups were significant ($p<0.05$) compared to the lower age groups within the species. However catla showed higher level of primary and secondary antibody titers than rohu in all age groups. Similar observation was reported by Ellis (1989) that the antibody producing cells reached their peak numbers in 10-15 days and titers reached their maximum after 20-30 days. In common carp, Lamers *et al.*, (1985b) reported that the peak antibody titers reached on day 20 after primary (i.m) injection with high dose 10^9 cells/fish with *A. hydrophila* antigen. The peak primary antibody titer in the present study was observed little earlier on day 14 after first injection with dose of 10^8 cells/fish, which might have influenced by the higher ambient temperature during the experiment. Moreover, it is well known that in all poikilothermic vertebrates, the ambient temperature influences the kinetics of metabolic process.

Fishes were injected with heat killed *A. hydrophila* antigen by i.m injection and could produce high level of antibody titers after first injection in this study. Similar observations were made earlier by Lamers and De Hass (1983) in common carp that heat killed *A. hydrophila* cells were more immunogenic than the formalin killed cells. While, Ingram and Alexander (1976) had also shown in rainbow trout and Harris (1973) in dace, *Leuciscus leuciscus* reported that i.m. injection was more effective for induction of primary responses than i.p. injection.

Table.1 Average length-weight of Indian major carps (*Catla catla and Labeo rohita*) in different age groups

Days	*Catla catla*		*Labeo rohita*	
	Length (cm ± S.E.)	Weight (g ± S.E.)	Length (cm ± S.E.)	Weight (g ± S.E.)
120	12.80 ± 1.22	24.42 ± 0.81	13.10 ± 0.75	21.12 ± 0.42
150	14.80 ± 1.22	43.10 ± 1.86	14.50 ± 2.33	35.52± 0.37
210	19.40 ± 1.70	83.80 ± 1.46	17.70 ± 1.22	71.50 ± 0.65
270	23.40 ± 1.87	163.00 ± 3.74	21.62 ± 3.75	138.40 ± 3.23

Table.2 Antibody titers (log_2) in different age groups of *Catla catla* after injection immunization with *A. hydrophila* antigen

Age (days)	Test group	Antibody titer(log_2)					
		Days after first injection			Days after second* injection		
		7	14	21	7	14	21
120	I	6.25±0.25	8.25 ± 0.25	6.25 ± 0.25	9.00 ± 0.31	8.60 ± 0.33	6.66 ± 0.33
	Cont.	0.40± 0.22	0.40 ± 0.24	0.60 ± 0.36	0.50 ± 0.34	0.40 ± 0.24	0.40 ± 0.24
150	I	6.25± 0.22	8.50 ± 0.22	7.00 ± 0.57	9.25 ± 0.25	8.80 ± 0.20	7.25 ± 0.22
	Cont.	0.62 ± 0.37	0.50 ± 0.25	0.66 ± 0.49	0.33 ± 0.23	0.40 ± 0.24	0.50 ±0.50
210	I	7.25 ± 0.25	8.75 ± 0.25	7.42 ± 0.20	9.66 ± 0.33	9.60 ± 0.24	7.80 ± 0.37
	Cont.	0.60 ± 0.36	0.40 ± 0.24	0.50 ± 0.34	0.83 ± 0.40	0.60 ± 0.36	0.50 ± 0.26
270	I	8.33 ± 0.21	9.16 ± 0.30	8.80 ± 0.20	10.50 ± 0.22	9.75 ± 0.25	8.25 ± 0.25
	Cont.	0.60 ± 0.22	0.71 ± 0.18	0.71 ± 0.35	0.75 ± 0.41	0.50 ± 0.31	0.40 ± 0.22

*Second injection given on day 21 after first injection
I= Immunized

Table.3 Antibody titers (log_2) in different age groups of *Labeo rohita* after injection immunization with *A. hydrophila* antigen

Age (days)	Test group	Antibody titer (log_2)					
		Days after first injection			Days after second* injection		
		7	14	21	7	14	21
120	I	6.33 ± 0.71	7.40 ± 0..24	5.83 ± 0.30	8.00 ±0.57	6.66 ± 0.21	6.60 ± 0.40
	Cont.	0.71 ± 0.35	1.00 ± 0.30	0.42 ± 0.29	1.00 ± 0.47	0.75 ± 0.25	0.40 ± 0.31
150	I	6.40 ± 0.50	7.42 ± 0.20	6.66 ±0.33	8.80 ± 0.20	8.00 ± 0.57	7.00 ± 0.20
	Cont.	0.87 ± 0.29	1.25 ± 0.44	0.75 ±.41	0.75 ± 0.25	1.50 ±0.37	0.62 ±0.32
210	I	7.00 ± 0.57	8.00 ± 0.57	7.00 ± 0.40	9.60 ±0.24	8.33 ±0.21	7.42 ± 0.20
	Cont.	0.50 ± 0.24	1.25 ± 0.45	0.87 ±0.22	1.50 ± 0.37	1.00 ±0.23	0.75 ± 0.31
270	I	7.24 ± 0.20	8.89 ± 0.11	8.33 ±0.21	10.16 ± 0.16	9.50 ± 0.50	7.75 ± 0.25
	Cont.	1.12 ± 0.39	0.75 ±0.25	1.25 ± 0.31	0.75 ± 0.25	1.00 ± 0.42	0.50 ± 0.18

I = Immunized
C= Control

Table.4 Comparison of protective response (RPS) in injection immunized *Catla catla* and *Labeo rohita* in different age groups upon homologous challenge (10^6 CFU/fish i.m) with *A. hydrophila*

Species	Age group (days)	Protective response as RPS					
		Days after first injection			Days after second injection		
		7	14	21	7	14	21
Catla catla	120	ND	46.67	37.50	72.22	50.00	41.17
	150	ND	50.00	41.17	75.00	56.25	52.94
	210	ND	57.14	46.67	78.57	62.50	60.00
	270	ND	60.00	56.25	81.25	70.59	64.70
Labeo rohita	120	ND	38.46	28.57	64.28	46.15	38.46
	150	ND	46.15	35.29	66.66	53.84	41.66
	210	ND	53.84	42.86	73.33	57.14	46.67
	270	ND	58.33	46.67	76.92	66.66	58.38

RPS = Relative percent survival
ND = Not done

The peak antibody titers in catla measured on 7 days after second injection was higher in all age groups compared to the peak primary antibody titers on 14 days after the first injection. The antibody titers recorded were 9.00±0.54, 9.50±0.50, 9.66±0.33 and 10.50±0.22 for 120, 150, 210 and 270 days old catla respectively. While, the peak antibody titers in rohu measured on 7 days after second injection was higher in all age groups compared to the peak primary antibody titers measured on 14 days after the first injection.

The antibody titers recorded were 8.00±0.57, 8.80±0.20, 9.60±0.24 and 10.16±0.16 for 120, 150, 210 and 270 day-old respectively (Table 2 and 3). The levels of antibody titers were found to decrease after 14 days of second injection. The secondary antibody titers in rohu recorded 7 days after second injection were higher in all age groups. Higher age groups showed higher levels of antibody titers within the species and the peak secondary antibody titers were significant ($p<0.05$) compared to the peak primary antibody titers obtained in catla and rohu.

The secondary antibody titers were not significantly different between the age groups of the species. However, enhanced level of secondary antibody titers were observed with increase in age/size of the fish, which clearly indicates that size/age of the fish has a prominent effect on the secondary immune response. However, catla showed higher level of primary and secondary antibody titers than rohu in all age groups. Karunasagar *et al.,* (1991) reported that immunized fingerlings of the three species of Indian major carps against *A. hydrophila via* three different routes, catla showed the maximum antibody titers followed by mrigala and rohu. However, Azad *et al.,* (1999) documented that responses of catla and rohu showed similar trends with respect to antibody titers against A. *hydrophila* with different doses of oral vaccination.

Details of protective response (RPS) following challenge on 14 and 21 days after first injection and 7, 14 and 21 days after second injection in four different age groups are given (Table 4). Protective response obtained on day 14 was higher in all the age groups after first injection. The maximum protection (60.00%) was recorded for 270 day- old age group and minimum protection was (46.67%) for 120 day-old group. Higher protection level was obtained on day 7 after second injection in all the age groups. The maximum protection (81.25%) was recorded for the 270 day-old age group and minimum protection (72.22%) in 120 day-old age group of catla. In case of rohu, higher levels of protective response were recorded in all age groups on day 14 after first injection. The maximum protection (58.33%) was found for 270 day-old age and minimum protection (38.46%) for 120 day-old age groups. Higher protection level was recorded in all the age groups on day 7 after second injection. The maximum (72.22%) and minimum (64.78%) protection were found for 270 day and 120 day-old age groups of rohu respectively.

The protection level was higher in older age groups of catla and rohu after first as well as after second injection. The protective response was significant ($p<0.05$) in both the species due to second injection and with the age of the species. Catla showed higher degree of protective response compared to rohu in all the age groups. Earlier reports made by Khalifa and Post (1976) reported that 100% protection was achieved in rainbow trout at antibody titer of 1:32. The present study indicates that the enhanced antibody titers with increase in age/size might have affect in protective response in both the species. Karunasagar *et al.,* (1991) showed positive correlation of protection with the level of antibodies in all the three species of

Indian major carps, where a particular age group of fingerlings was used and reported that maximum protection was recorded in catla and lowest in rohu. However, catla showed higher level of protection in all the age groups than rohu after first as well as second injection in this study, which might be due to specific growth rate and innate mechanism of the species.

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