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PROTECTIVE RESPONSE OF COMMON CARP ORALLY VACCINATED WITH BIOFILM AND FREE CELLS OF *AEROMONAS HYDROPHILA* CHALLENGED BY INJECTION AND IMMERSION ROUTES

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ABSTRACT

Fingerlings of common carp (*Cyprinus carpio* Linn.) were orally vaccinated using biofilm (BF) and free cells (FC) of *Aeromonas hydrophila* incorporated diets for 15 and 20 days. The antigen dose was 10^{10} CFU/fish/day. Serum agglutination titres of BF fed carp was significantly higher that that of the FC fed group. Peak titres (4.5 ± 0.29) were recorded in BF fed carp at 60 dpv in 20 day vaccination group. Cent percent protection was achieved by both injection and immersion challenge of 15 day BF vaccination at 60 dpv. It produced better protective response than the injection challenge.

Key words: Common carp, oral vaccine, *Aeromonas hydrophila,* biofilm, challenge, injection, immersion, relative percent survival.

Attempts of vaccination against infections by *Aeromonas hydrophila* in fish have produced inconsistent and highly variable results (Post, 1966; Schachte, 1978). Variability of responses to oral vaccination has been attributed to the destruction of antigenic epitopes by the digestive acids and enzymes (Rombout *et al.*, 1985). Methods of delivering oral antigens, bypassing the foregut environment, have produced encouraging results (Rombout *et al.,* 1985; 1986). biofilm of *A. hydrophila* has been successfully used in oral vaccination of carps (Azad *et al.*, 1997). Commenting on the route of challenge to assess the potency of a vaccine, Ellis (1988) suggested that the challenge system should resemble a natural exposure. Since the vaccine was delivered through oral routes, we felt that an immersion route of challenge would help in assessing the vaccine, better. Thus, the present study was carried out to assess the

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influence of route of challenge on the protective response of common carp vaccinated with BF and FC *A. hydrophila.*

MATERIALS AND METHODS

Fish: Fingerlings of common carp (*Cyprinus carpio* L.) were procured from the State Fish Seed Farm, BR Project, Karnataka India. Healthy fish were maintained in cement cisterns and fed with pelleted carp diet till the start of the experiment.

Bacterium: *A hydrophila* (SAH 93) isolated from EUS affected *Sillago sihama* was used.

Vaccination: Heat inactivated BF and FC and *A. hydrophila* were used in vaccine preparation following the method described by Azad *et al.* (1997). Briefly, BF was developed on chitin suspended @ 0.3% (w/v) in 0.225% (w/v) tryptic soy broth (TSB) and incubated on mechanical rocker (180 rpm) maintained at room temperature for four days. Biofilm cells were harvested from the substrate by vortexing on a cyclomixer for three min. Free cells were cultured in TSB for 24 h. Heat inactivation of BF and FC was caried out at 90°C and 60°C for 30 min, respectively. Feed ingredients (Varghese *et al.*, 1976), except vitamins, were mixed, cooked (121°C for 15 min, in a pressure cooker) and cooled. Vitamins and cell suspensions (BF & FC) were incorporated in the dough before pelletization. Control diet (C) was prepared, similarly without the addition of bacterial preparation. The cell density in the pellet was so adjusted as to deliver 10^{10} CFU/fish/day. The feed pellets were sun dried and packed in polythene bags.

Common carp fry (3.84 ± 0.32 g), stocked separately in one m^3 cement tubs @ 300 fingerlings/tub (Table 1), were acclimatized to control diet for a week. Acclimatization was carried out to ensure better utilization of the artificial diet. The fish were, then, starved for a day before starting the experiment. Designated diets (BF,FC and C) were offered in hanging trays, two times a

Table 1. Details of oral vaccination (10^{10} CFU/fish/d) of common carp with varying durations (15 and 20 d) of feeding with BF and FC

Particulars	15 d			20 d		
	BF	FC	C	BF	FC	C
No. vaccinated	300	300	100	300	300	100
No. survived	294	291	97	258	273	88
% survival	98	97	97	86	91	88
Initial average weight (g ± S.E.)	4.97 ± 0.10	4.82 ± 0.15	4.92 ± 0.16	4.95 ± 0.10	4.87 ± 0.11	4.95 ± 0.15
Final average weight (g ± S.E.)	6.03 ± 0.82	6.11 ± 0.87	6.00 ± 0.95	5.98 ± 0.89	6.07 ± 0.95	6.13 ± 0.81
Antigen dose (CFU/Fish/day)	1.39 × 10^{10}	1.5 × 10^{10}	–	1.39 × 10^{10}	1.5 × 10^{10}	–

BF: Biofilm; FC: Free cell; C: Control; CFU: Colony Forming Units

day, @ 10% of the body weight, Bacterin incorporated diets (BF or FC) were given for 15 and 20 days. Vaccinated fish were shifted to 25 m^3 cement cisterns and maintained on control diet. Over 75% of the water was exchanged daily.

Serum agglutination titers: Serum agglutinin titers against *A. hydrophila* were measured from zero day post vaccination (dpv). Blood from 5–10 fish of BF, FC and C was drawn through the caudal vein and 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 and saved for determining agglutinin titers. Doubling dilutions of the serum in sterile phosphate buffered saline (PBS pH 7.2) were taken in 'U' bottom microtiter plates. Equal volume of heat inactivated suspension (10^9 CFU/ml) of *A. hydrophila* was reacted with the serum in each well. The last dilution of the serum showing clear agglutination was taken as the titer and expressed in $\log_2$ values. Challenge: Vaccinated and control fish were challenged via injection and immersion routes at 60 dpv. The time of challenge was fixed based on the time taken for the growout of fry to fingerling. A total of 25 fish from each treatment group was challenged with 24 h culture of *A. hydrophila,* corresponding to its log phase of growth. Injection (intramuscular) with 10^6 CFU per fish and immersion in bacterial suspension of 10^7 CFU/ml for 30 min were carried out. Challenged fish were maintained in aerated aquarium tanks (50 l) for a week. Aseptic kidney samples for the moribund fish were plated on Aeromonas selective media (Hi Media, India) and only specific mortalities were used in computing relative percent survival (RPS) following Amend (1981).

RESULTS AND DISCUSSION

Serum agglutination titres of carp orally vaccinated for 15 and 20 days are given in Table 2. Preliminary studies on the use of biofilm of *A. hydrophila* as oral vaccine in carps has indicated that the duration of feeding exceeding 10 days, would result in better titres and survival upon challenge (Azad *et al.*, 1997). Hence, a 15 and 20 day duration was selected in the present study. Vaccinated fish showed a significant raise in the titres from 40 dpv. Both, BF and FC, registered peak titres (4.00 ± 0.17 and 3.65 ± 0.18, respectively) at 60 dpv in 15 day vaccination group. Similar responses were observed in the 20 day vaccination group. Titres varied significantly ($p<0.05$) with holding time (dpv) and with nature of the vaccine (BF/FC). Titres of the BF and FC vaccinated carps were detectable only at 30 dpv when the carps were fed @ 10^{11} CFU/fish/day for 10 days (Azad *et al.*, 1997). These findings have shown that the BF form of the bacterin are better in eliciting the titres than the FC. Titers recorded in the present study are lower than those reported by Lamers and Mulswinkel (1986) who used both heat killed and formalin inactivated *A. hydrophila* for injection vaccination of carp. This difference is due to the route of antigen delivery. Injection, both intramuscular and intraperitoneal,

Table 2. Serum agglutination titres (log_2) in common carp with varying durations of oral vaccination using BF and FC

Vaccination	Serum agglutination titers (log_2)					
	10 dpv	20 dpv	30 dpv	40 dpv	50 dpv	60 dpv
15-day vaccination						
BF	1.00 ± 0.33	0.67 ± 0.33	1.33 ± 0.33	2.67 ± 0.33	3.25 ± 0.25	4.00 ± 0.17
FC	0.33 ± 0.33	0.33 ± 0.33	1.00 ± 0.50	2.00 ± 0.58	3.40 ± 0.41	3.56 ± 0.18
C	ND	ND	0.33 ± 0.33	0.33 ± 0.33	0.50 ± 0.33	0.57 ± 0.20
20-day vaccination						
BF	0.67 ± 0.33	0.75 ± 0.48	1.75 ± 0.25	3.00 ± 0.32	3.80 ± 0.37	4.50 ± 0.29
FC	0.50 ± 0.50	0.50 ± 0.28	1.00 ± 0.41	2.00 ± 0.41	2.25 ± 0.48	3.25 ± 0.37
C	ND	ND	0.25 ± 0.25	0.50 ± 0.29	0.25 ± 0.25	0.50 ± 0.29

ND: Not detected; BF: Biofilm; FC: Free cell; C: Control; dpv: Days post vaccination

vaccination has been known to elicit higher serum agglutinin titers compared to either immersion or oral vaccination (Ellis, 1988).

Protective response measured as RPS of vaccinated fish is given in Table 3. Cent per cent protection was achieved through both routes of challenge in carp fed BF for 15 days. However carps challenged by injection were protected to a lesser extent. Farm reared fry of carp are, generally, moved to grow-out facilities after a rearing period of 45–60 days. This shift over phase is very crucial from the infectivity point of view. Hence, 60 dpv was selected for testing the protective response in the present study. Bath challenge of direct immersion (Di) and spray vaccinated coho salmon produced mortalities ranging from 1 to 3% (Amend and Johnson, 1981). Injection (ip) challenge produced much higher mortalities in direct immersion vaccinated fish (Tebbit and Goodrich, 1983; Newman and Majnarich, 1982). Protective response elicited by vaccines can vary with the test protocol to measure RPS. This was clearly demonstrated in Atlantic salmon against furunculosis (Nordmo and Ramstad, 1997). They recorded quicker and higher mortalities in salmon challenged by injection routes compared to those of bath and cohabitation challenges.

Table 3. Protective response in orally vaccinated (10^{10} CFU/fish/d) common carp upon injection and immersion challenges at 60 dpv

Treatment	No. challenged	% Mortality	RPS	% Mortality	RPS
		15 d vaccination			
BF	25	16.00 ± 0.45	100.00	0	100.00
FC	25	16.03 ± 0.64	76.41	11.85 ± 0.52	80.86
C	25	67.95 ± 1.28	–	61.92 ± 1.92	–
		20 d vaccination			
BC	25	11.86 ± 0.53	82.63	8.01 ± 0.32	87.50
FC	25	16.03 ± 0.35	76.62	11.86 ± 0.53	81.50
C	25	68.27 ± 6.73	–	64.10 ± 2.56	–

The isolate of *A. hydrophila* has been known to produce hemolytic and necrotic changes in the musculature at the site of injection in carps (Azad *et al.*, 1997). We tried to compare injection and immersion routes of challenge as *A. hydrophila* is known to cause enteropathogenic disorders by entry through contact. The amount of antigen uptake by a fish during immersion vaccination has been reported to be from 0.01 to 0.2% of the initial bath vaccine concentration (Tatner and Horne, 1993). This, probably, is the reason for the reduced mortalities in immersion challenge. Challenge route plays an important role in determining the potency of a vaccine. Also, period of observation following the challenge should be determined based on the incubation period of the pathogen. Longer than optimal periods of observation may increase pathogen load in the challenge medium, thus, leading to distorted inferences. Oral route, unlike the injection route, has been known to elicit very slow or poor systemic responses (Kawai *et al.*, 1981). However with prolonged periods of vaccination and improved methods of antigen delivery, oral vaccinations elicit significant systemic response (Rombout *et al.*, 1986).

The present study revealed no difference between the 15 and 20 day vaccination regimes producing comparable humoral and protective responses. The longevity of response has to be further studied for exploring the possibilities of using the BF form of *A. hydrophila* in oral vaccination of carps.

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