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Biofilm vaccine of *Aeromonas hydrophila* – standardization of dose and duration for oral vaccination of carps

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Humoral and protective responses to different doses and duration of oral administration of an *Aeromonas hydrophila* biofilm vaccine in three species of carp, catla (*Catla catla* Ham.), rohu (*Labeo rohita* Ham.) and common carp (*Cyprinus carpio* Lin.) were studied. Among the three doses (10^7, 10^{10} and 10^{13} cfu g^{-1} fish d^{-1} administered for 15 days, 10^{13} cfu g^{-1} fish d^{-1}, elicited the highest serum antibody titre and protective response in all the three carp species. Of the three vaccination durations studied (10, 15 and 20 d at 10^{10} cfu g^{-1} fish d^{-1}), 15 and 20 d induced higher responses than 10 d. Among the three carp species, catla produced the highest antibody and protective response followed by rohu and common carp. Independent of dose and duration, the antibody titre and protective response increased with time following vaccination up to 60 d.

Key words: Biofilm vaccine, *A. hydrophila*, carps.

I. Introduction

Aeromonas hydrophila is an ubiquitous bacterium responsible for stress-associated pathogenicity in warm water fish. Vaccination strategies employed against *A. hydrophila* have included heat-killed cells (Post, 1966), heat or formalin-inactivated bacterial extracts (Song & Kou, 1981), sonicated preparations (Thune & Plumb, 1982) and live cells (Logothetis & Austin, 1994).

Among the various methods of vaccination, the oral route is simple, cheap and ideal for mass administration to fish of all sizes. However, attempts to orally vaccinate against motile aeromonad septicemia (Post, 1966; Schachte, 1978), vibriosis, yersiniosis and furunculosis (Johnson & Amend, 1983; Nelson *et al.*, 1985; Michel, 1979) have either yielded mild and short lived or inadequate responses. One of the important factors for the inconsistency and poor response to oral vaccination is the digestive degradation of antigens in the foregut, before the vaccine reaches immune-responsive areas in the hind-gut and other lymphoid organs (Johnson & Amend, 1983; Rombout *et al.*, 1985). This view is further supported by better performance of vaccines when administered by anal intubation which can produce high systemic antibody titres

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against *Vibrio anguillarum* (Rombout *et al.*, 1986) and a protective response against *V. anguillarum* and *Yersinia ruckeri* (Johnson & Amend, 1983).

Strategies that have been explored for improving oral vaccination have included protected antigens such as the enteric coated microspheres (Piganelli *et al.*, 1994; Polk *et al.*, 1994; Dalmo *et al.*, 1995), encapsulated antigens (Lillehaug, 1989), enteric coated vaccine (Wong *et al.*, 1992), adjuvants (Jenkins *et al.*, 1994) and bioencapsulation of vaccine in live feed (Kawai *et al.*, 1989).

We have developed and evaluated a biofilm of *A. hydrophila* for oral vaccination of carp which induced significantly higher antibody titres and protection compared to a free cell vaccine (Azad *et al.*, 1997). Certain bacteria form biofilms on substrates (Costerton, 1984), and these have been found to be resistant to antibiotics (Anwar & Costerton, 1990), phagocytosis and the killing effect of whole blood and serum (Anwar *et al.*, 1992) due to a protective glycocalyx layer. The glycocalyx matrix of the biofilm vaccine is believed to protect the antigens against gastric destruction. Here we present further studies on standardization of dose and duration of oral vaccination with a biofilm vaccine of *A. hydrophila* in catla (*Catla catla*), rohu (*Labeo rohita*) and common carp (*Cyprinus carpio*).

II. Materials and Methods

PREPARATION OF VACCINE

The biofilm vaccine of *A. hydrophila* (SAh 93) was prepared according to Azad *et al.* (1997). Briefly, the isolate was grown on chitin flakes suspended in tryptone soy broth (TSB) and the biofilm was harvested and heat inactivated at 90° C for 30 min. Inactivated biofilm was then mixed with cooked and cooled ingredients of feed, pelletized and sun dried. Along with biofilm-incorporated feed (BF), a control feed (C) without vaccine was prepared.

VACCINATION OF FISH

Standardization of dose

Three groups, each of 200 catla fry ($3{\cdot}75 \pm 0{\cdot}76$ g), were stocked into 1 m^3 cement tubs and acclimatized with control feed (C) for a week followed by starvation for 24 h. The groups were fed with BF in feeding trays suspended in tubs at 10^7, 10^{10} and 10^{13} colony forming units (cfu) g^{-1} fish d^{-1} respectively. Three groups of fry of rohu ($4{\cdot}35 \pm 0{\cdot}70$ g) and common carp ($4{\cdot}0 \pm 0{\cdot}65$ g) were vaccinated similarly. A control group consisting of 200 fry of each species was given feed C. In all the tubs, the feed provided was consumed by fish within 15–20 min. After 15 d feeding, vaccinated and control fish were shifted to separate cement cisterns (25 m^3) and reared on feed C at 5% of body weight per day. Water in the experimental cisterns was replenished to an extent of 20–25% daily.

At 10 d intervals, starting from day 0 post-vaccination (dpv), ten fish from each treatment were sampled. The fish were anaesthetized (10 ppm benzocaine), bled from the caudal vein and the blood stored at 4° C overnight. The serum was separated by centrifugation at $3600 \times g$ for 10 min and inactivated

at 50° C for 30 min. Serum agglutination titres of pooled serum were determined with heat-inactivated cells of *A. hydrophila* (SAh 93) according to Azad *et al.* (1997).

Twenty-five fish in each treatment were challenged by intramuscular injection of 10^6 cfu *A. hydrophila* (SAh 93) per fish on 20, 40 and 60 dpv. The challenged fish were maintained in well aerated aquarium tanks. Moribund fish were tested for *A. hydrophila* by reisolating the pathogen from the kidney on Rimler Shott's medium. Post-challenge mortalities were recorded for 7 d and specific mortalities were used for computing relative percent survival (RPS).

Standardization of duration

Three groups, each of 200 catla fry ($4{\cdot}16 \pm 0{\cdot}13$ g) were acclimatized for 7 d and starved for 24 h before oral vaccination as described above. Fish were fed with BF at 10^{10} cfu g^{-1} fish d^{-1} for 10, 15 and 20 d respectively. Fry of rohu ($4{\cdot}87 \pm 0{\cdot}11$ g) and common carp ($3{\cdot}84 \pm 0{\cdot}32$ g) were vaccinated similarly. Vaccinated and control fish were maintained separately in 25 m^3 cement cisterns and fed on feed C. Serum agglutination titres and RPS were determined as described above.

Serum agglutination titres and RPS were subjected to ANOVA and Duncan multiple range tests. The water temperature during the experimental period ranged from 26–32° C.

III. Results

STANDARDIZATION OF DOSE

Of the three doses, 10^{13} and 10^{10} cfu g^{-1} fish d^{-1} elicited significantly ($P=0{\cdot}05$) higher antibody titre than 10^7 cfu g^{-1} fish d^{-1} in all the three species (Fig. 1a–c). However, the titres of 10^{13} cfu g^{-1} fish d^{-1} did not differ significantly ($P=0{\cdot}05$) from that of 10^{10} cfu g^{-1} fish d^{-1}. With all the three doses, antibody titres increased with time following vaccination and this trend continued up to 90 dpv in catla, 50 dpv in rohu and 60 dpv in common carp. Antibody titres in the control groups remained below 2 during the experimental period. Between the three species there were clear differences in the onset of antibody production following vaccination. In catla, antibodies could be detected from 0 dpv with all the three doses, while in rohu even though antibodies could be detected from 0 dpv at higher doses, at 10^7 cfu g^{-1} fish d^{-1}, antibodies could only be detected from 30 dpv. Surprisingly, in common carp, with all the doses, antibodies could be detected only from 30–40 dpv.

All the three doses induced protection which was dose-dependent (Fig. 2a–c). In general RPS was significantly ($P=0{\cdot}05$) higher at 10^{10} and 10^{13} cfu g^{-1} fish d^{-1} than at 10^7 cfu g^{-1} fish d^{-1}. However, protection achieved at 10^{10} cfu g^{-1} fish d^{-1} was not significantly different ($P=0{\cdot}05$) from that at 10^{13} cfu g^{-1} fish d^{-1}. All the post-challenge mortalities were positive for *A. hydrophila*. In all three species, the protection increased with time. Overall, the RPS of carp species did not vary significantly from one another. In common carp, although detectable antibody titre was absent up to 20 dpv, there was good protection at this time.

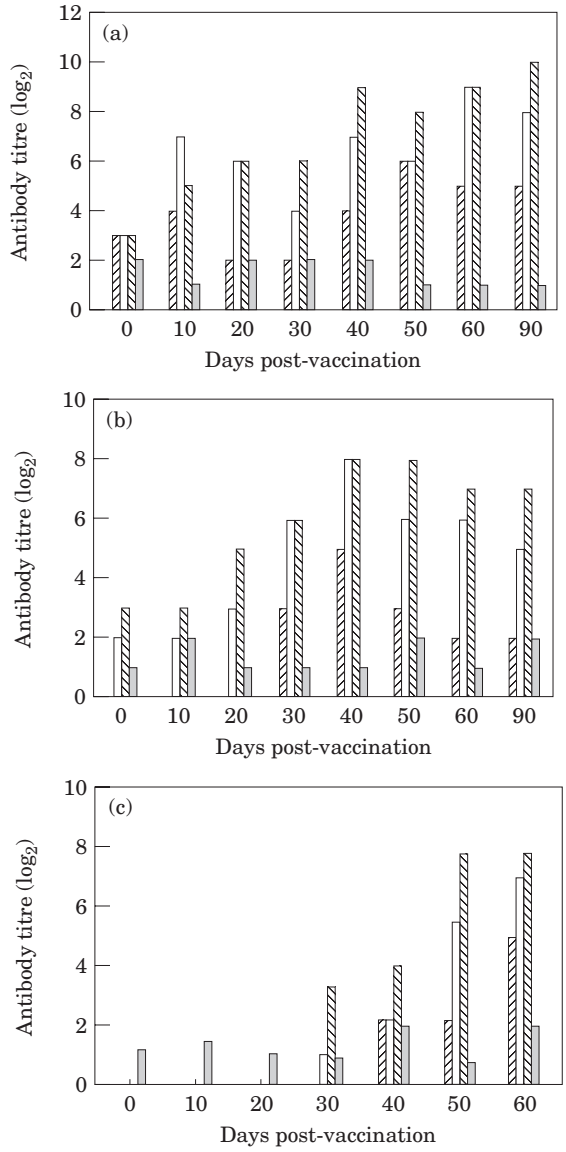

Fig. 1. Antibody titres in catla (a), rohu (b) and common carp (c) vaccinated at three doses of *A. hydrophila* biofilm vaccine for 15 d; 0=control (□), 7=10^7 (▨), 10=10^{10} (□), 13=10^{13} (▧), cfu g^{-1} fish d^{-1}.

STANDARDIZATION OF DURATION

In all the three species, duration of vaccination had a significant (P=0·05) effect on antibody titre. The longer the duration of vaccine administration, the higher was the antibody titre. Higher titres were recorded with the 15 and 20 d schedules (Fig. 3a–c). In all three species antibody titre was significantly (P=0·05) higher with 20 and 15 d vaccination than with 10 d. However, in

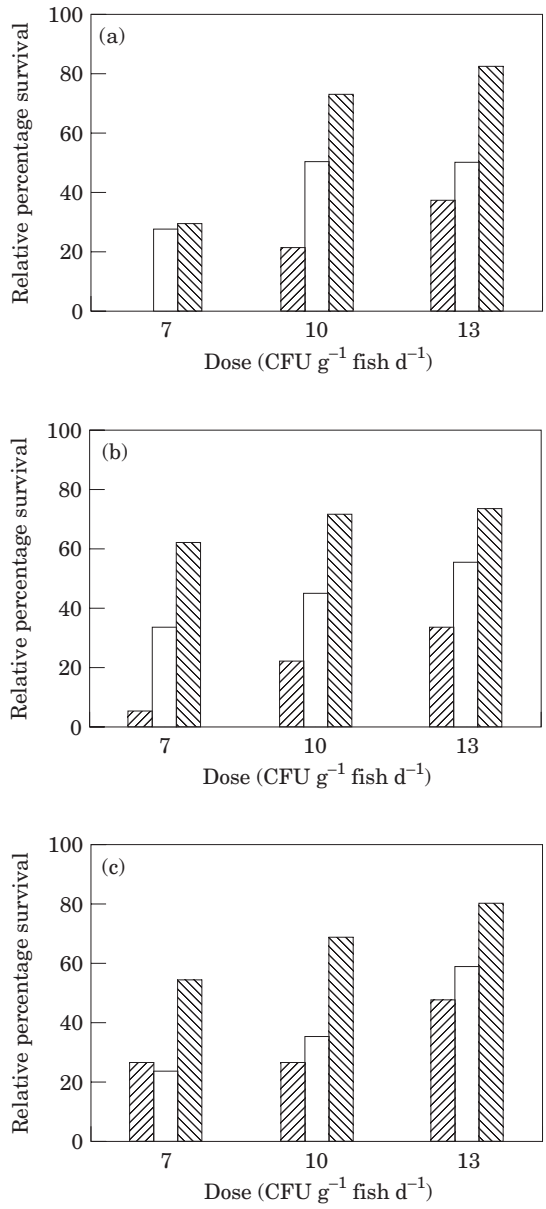

Fig. 2. Relative percent survival (RPS) in catla (a), rohu (b) and common carp (c) vaccinated at three doses of *A. hydrophila* biofilm vaccine; 7=10^7, 10=10^{10}, 13=10^{13} cfu g^{-1} fish d^{-1}. DPV=Days post-vaccination. (▨) 20 DPV, (□) 40 DPV, (▧) 60 DPV.

catla and common carp there was no difference between 15 and 20 d vaccination schedule. Irrespective of duration of vaccination the titre showed an increasing trend with duration of time following vaccination up to 60 dpv. Of the three species, titres in catla and rohu were higher compared to common

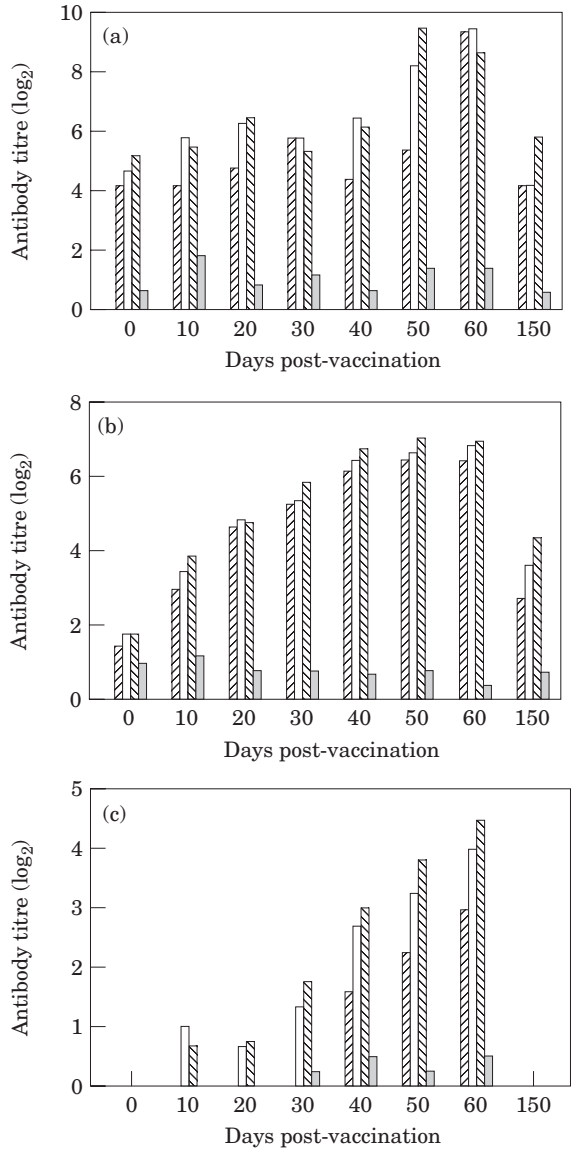

Fig. 3. Antibody titres in catla (a), rohu (b) and common carp (c) following 0 (□), 10 (▨), 15 (□) and 20 (▧) d of vaccination at 10^{10} cfu g^{-1} fish d^{-1} of *A. hydrophila* biofilm vaccine.

carp. In common carp, titres were detectable only after 40 dpv in the 10 d duration vaccination group.

Similar to antibody titre, protection also increased with duration of vaccination (Fig. 4a–c). The longer the duration of vaccination the higher was the protection achieved. RPS with 20 d vaccination was significantly higher than that with 15 d except in common carp. In general, in all the vaccination groups, higher protection was achieved with time up to 60 dpv.

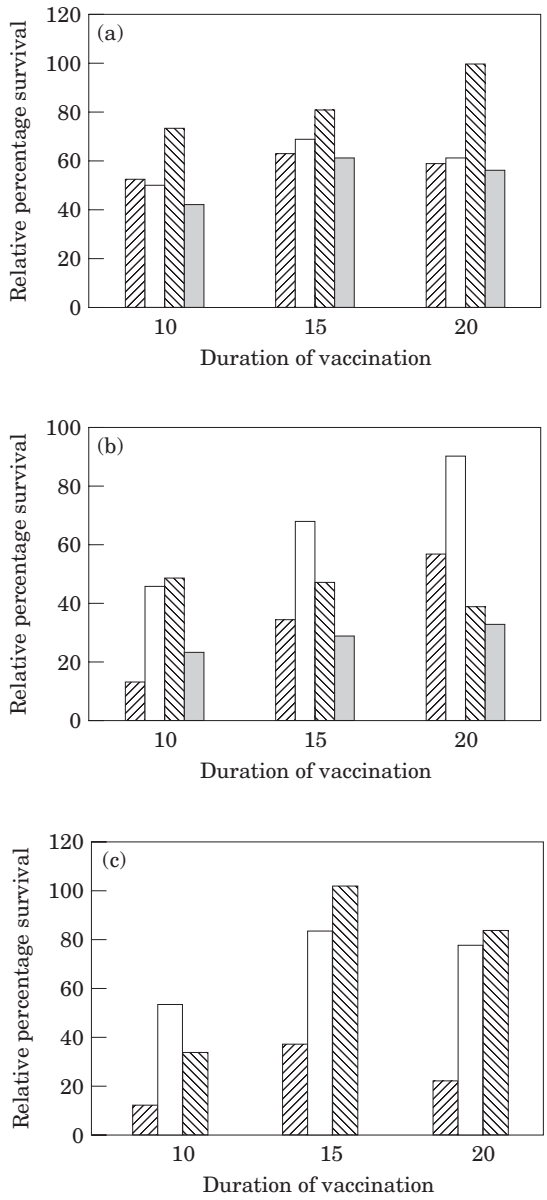

Fig. 4. Relative percent survival (RPS) in catla (a), rohu (b) and common carp (c) fed with *A. hydrophila* biofilm vaccine at 10^{10} cfu g^{-1} fish d^{-1} for 10, 15 and 20 d. DPV=Days post-vaccination. (▨) 20 DPV, (□) 40 DPV, (▧) 60 DPV, (▣) 150 DPV.

IV. Discussion

Earlier studies have shown that oral vaccination of carp with *A. hydrophila* biofilm vaccine is better than free cell vaccine in terms of antibody titre and protective response (Azad *et al.*, 1997). The ability of the biofilm oral vaccine to induce antibody production and protection in carp as demonstrated in this

study, further confirm the earlier findings. In the present study, the *A. hydrophila* biofilm vaccine elicited a dose dependent antibody response with the highest titre and protection (RPS) at 10^{13} followed by 10^{10} and 10^{7} cfu g^{-1} fish d^{-1} in all the three carp species. High dose priming is normally believed to elicit greater humoral response and memory in carp (Lamers *et al.*, 1985) and this appears to be true also in the case of orally administered biofilm vaccine. As there is no significant difference between 10^{10} and 10^{13} cfu g^{-1} fish d^{-1}, the former dose is considered optimum for vaccination. Enhanced antibody titres and RPS were recorded with extended duration of vaccination. Among different durations of vaccination, 15 and 20 d were found to be effective with no significant difference between the two in eliciting protection. However, vaccination for more than 15 d did not produce further improvement in protection or agglutination titres. Similar views have been expressed by earlier studies on free cell vaccine (Fryer *et al.*, 1978).

It appears that oral vaccination with the biofilm vaccine is capable of inducing a specific systemic immune response. However, earlier studies have given contradictory views about the relationship between systemic immune response and oral vaccination, ranging from lack of systemic immune response (Kusuda *et al.*, 1978; Kawai *et al.*, 1981) to positive response (Ainsworth *et al.*, 1995). The responses of catla and rohu showed similar trends with respect to antibody titres and protection with different dose and duration of vaccination. However, common carp, with no detectable titres up to 30 dpv, showed a protective response more or less equal to those of catla and rohu. In the case of control common carp, low agglutinating titres were detected throughout the experiment, but following vaccination no titres were detected until 30 dpv. This could be possibly due to absorption of background levels of cross-reacting antibody with vaccinating antigen and production of specific antibody taking longer in common carp than in catla and rohu. Consistent and high serum agglutination titres in catla and rohu, observed in the present study, may be related to these species having a longer intestine making it possible to absorb and transfer more of the antigens from the gut lumen. Hence, there appears to exist differences in the immune response to oral vaccination between Indian major carps and common carp.

A progressive improvement in serum antibody titre and protective response with time following biofilm oral vaccination was observed here. This is probably due to the enhanced uptake and longer retention of the biofilm antigens compared to that of free cell vaccine (Azad *et al.*, 1997). The possibility of biofilm antigen being available at the immune responsive hindgut with minimally altered immunogenic epitopes could have contributed to the observed higher titre and subsequent protection. The glycocalyx of biofilm, is a polymer of neutral hexoses (Costerton *et al.*, 1981) which encapsulates and possibly protects the bacterial surface antigens from digestion in the gut. The ability of *A. hydrophila* antigens to remain in lymphoid tissue for 12 months has been demonstrated with injection vaccination (Lamers *et al.*, 1985).

In addition to protection against gastric destruction of antigens, presenting the vaccine in a biofilm form may have an added advantage. Glycocalyx *per se* might act as an immunogenic antigen. A glycocalyx-like capsular

polysaccharide in *A. salmonicida in vivo* has been shown to confer resistance to a variety of serum factors and fresh peritoneal fluids of salmonid fish (Garduño *et al.*, 1993). Antibodies to glycocalyx may prevent biofilm formation by the pathogen which is believed to be the first step in establishing infection in a host. This aspect, however, needs further study.

Reports on the immunogenic and protective responses of glycocalyx as an antigen in fish vaccination are not available. However, a comparison with similar work on human health can be drawn. Capsular glycocalyx of *Haemophilus influenza* type b was found to be highly immunogenic, as it elicited antibodies during infection and a vaccine directed against the surface components of the pathogen were highly protective (Costerton *et al.*, 1981). A recent study of immune response and protection induced by an extracellular polysaccharide having a similar composition as the capsular polysaccharide of *A. salmonicida* (Bricknell *et al.*, 1997) also lends support to the notion that the role of the glycocalyx surface layer is important in protection.

The present approach of exploiting the natural resistant properties of bacterial biofilms for development of effective oral vaccines is simple and cheap and merits further studies.

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