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Immunisation trials on clownfish, *Amphiprion sebae* against a potential pathogen, *Serratia marcescens*

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Short Communication

Abstract

Immunisation studies on an emerging pathogen, *Serratia marcescens* were conducted in commercially important marine ornamental fish, *Amphiprion sebae*. *In vivo* experiments were conducted using formalin killed cells (bacterin) of *S. marcescens* in the 3 months old juveniles of *A. sebae* from the same brood. Bacterin was administered at the rate of 10^5 cells per gram body weight of fish which was the lethal dose estimated from pathogenicity studies carried out earlier. After 15, 30 and 50 days of administration of bacterin, experimental fish were subjected to challenge studies. The relative percentage survival (RPS) rendered by administration of bacterin increased after 15th day and 100% protection was achieved from challenge on 35th and 50th days.

Keywords: *Serratia marcescens, Amphiprion sebae, immunisation, bacterin, relative percent survival (RPS)*

Introduction

Immunisation became a proven and accepted tool in integrated and comprehensive programme of aquatic animal health management worldwide since its inception over 50 years ago (Busch, 1994) and has gained importance as a part of biosecurity in aquaculture (Gudding, 2014). Majority of the studies of the past on immunisation of fish concentrated on food fish of commercial value. The bacterial pathogens of interest are *Aeromonas* (Newman, 1993; Thornton, 1995), *Vibrio* (Cardella and Eimers, 1990; Sindermann, 1990), *Renibacterium* (McCarthy *et al*., 1974), *Flexibater* (Schachte Jr., 1978), and in recent years *Pasteurella* (Mazzolini *et al*., 1998), *Flavobacterium* (Birnbaum, 1998) etc. Similarly, in aquarium reared ornamental fish, immunisation could serve as a good alternative to control the occurrence of potential bacterial pathogens. The development of vaccines for fish as an important management measure has been stressed by Sadao (2004). Evelyn (1997) opines that the high cost of application of chemotherapy and the short term nature of protection conferred via antibiotics, along with environmental concerns further stress the importance of vaccine development. The present study is the preliminary experiments on protection via immunisation against an emerging pathogen, *Serratia marcescens* in a commercially important marine ornamental clownfish, *Amphiprion sebae* which were conducted at the Mandapam Regional Centre of CMFRI. *S. marcescens* is a red pigmented bacterial pathogen, which repetitively caused severe ulcerations leading to mortality in aquarium-held and captive-reared clownfish. Experimental infection studies were conducted to confirm its pathogenicity (Pramila, 2003).

Material and methods

Bacterin (autogenous vaccine) was prepared from the isolate of *S. marcescens* as per the following scheme:

Culture in slant
↓
Activation in broth
↓
Inoculation in fresh broth
↓
Incubation for 18 hours
↓
Spread plating in nutrient agar plates
↓
Harvesting of cells in sterile tubes
↓
Harvested cells suspended in sterile phosphate buffered saline (PBS) containing 0.3% formalin
↓
Centrifugation at 4000 rpm
↓
Supernatant discarded
↓
Pellet serially diluted in PBS
↓
Cells counted in hemocytometer
↓
Intraperitoneal injection

Experimental set up

Same brood of 3 months old clownfish (*A. sebae*) was stocked in three 5 ton tanks, each containing 50 fishes. The tanks were provided with biofilter and water recirculation was effected by airlift, which maintained the water quality parameters within safe limits. The fish were fed twice a day with boiled mussel meat. The prepared bacterin was administered via intraperitoneal route to the whole stock maintained in two tanks at the rate of 10^5 cells per gram body weight of fish which was the lethal dose estimated from pathogenicity studies conducted earlier with the isolate (Pramila, 2003). The fish were monitored continuously for mortality and response pattern.

Challenge studies

For challenge studies, the fish were transferred to experimental FRP tanks of 1 ton capacity. After 15, 30 and 50 days of administration of bacterin, challenge studies were conducted by intraperitoneal injection of live pathogenic cells to experimental groups of six fish each in triplicate, at the lethal dose which was determined earlier as 10^5 cells from pathogenicity studies. A set of non immunised control fish was given injection of 0.85% saline. After injection fish were monitored for mortality as well as clinical signs and behavioral responses.

The relative percentage survival (RPS) rendered by the administration of bacterin was calculated by the formula:

$$\text{Relative Percent Survival (RPS)} = 1 - \frac{\text{Percentage of dead (immunised)}}{\text{Percentage of dead (non immunised)}} \times 100$$

Results and discussion

Results of the experiments using bacterin conducted *in vivo* indicated that the administration of formalin-killed cells (bacterin) was capable of providing immunity even on 50th day of administration without any booster dose in between.

It was found that the RPS rendered by the administration of bacterin to the experimental fish went on increasing after 15th day and 100% protection from challenge was obtained on 35th and 50th days. The non-immunised fish showed 80% mortality on 15th day and 50% each on 35th and 50th day of administration (Fig. 1).

Vaccination is considered as a reliable tool for maintaining aquaculture systems free of specific pathogens. Vaccination of fish for the prevention of specific bacterial diseases affecting commercially reared fish species has a significant impact on aquaculture industry. This is exemplified in a surveillance of health status of marine rainbow trout farms made by Pedersen *et al.* (2008), where both mortality and numbers of antimicrobial treatments during the period of observation were considerably higher in unvaccinated fish compared with vaccinated fish. But, in spite of the fact that numerous fish vaccines were developed, relatively less amount of work was done with marine ornamental fishes to make comparative accounts possible. Hence the results need analysis based on immunisation studies in other marine finfish.

Almost all fish vaccines available are bacterins or formalin-inactivated whole cell suspensions, some with adjuvants. In the present study, protection obtained by intraperitoneal injection of formalin-killed cells was the technique used to determine

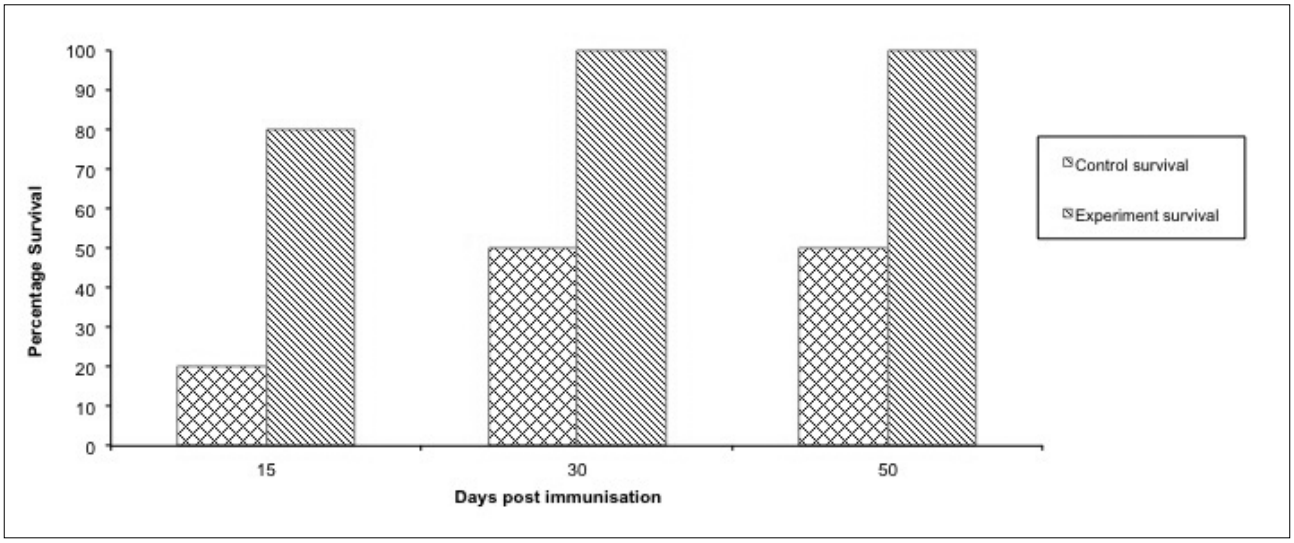

Fig. 1. Relative Percent Survival (RPS) in immunised and non immunised fish on challenge with formalin killed bacterin of *Serratia marcescens*

the role of immunisation in providing protection to fish, when challenged with the pathogenic isolate of *S. marcescens*. Formalin at the rate of 0.3% to 0.5% is usually used to prepare the killed bacterin (Fryer *et al.*, 1978). The concentration used in the present study was 0.3%, which brought about the killing of the cells, without losing antigenic properties and imparted protection to the experimental group of fish. Further, it is already established that in order to administer an exact dose of vaccine/ bacterin, it is advantageous to inject the fish. This is particularly important in the case of marine ornamental fish, which command high market value. Studies on evaluation of various vaccination modalities conducted by Li *et al.* (2016) proved that out of the several whole cell bacterin preparations such as formalin, phenol-killed, heat-killed and chloroform-killed bacterins, formalin killed bacterin displayed maximum degree of protection against vibriosis of farmed marine fish. In the same study, intraperitoneal injection exhibited best protection among the various administration routes tried, with RPS values of 85% to 100%. The modalities followed and found successful in the present study are equivalent to the above work.

Very few studies were conducted in the past on the immunisation studies of *Serratia* species. McIntosh and Austin (1990) reported the effectiveness of vaccination regimes of *Serratia liquefaciens* on Atlantic salmon proved by challenge studies via intraperitoneal route in fish vaccinated with whole cell and a toxoid preparation. In their study, the unvaccinated control fish suffered 50% mortality compared to the immunised ones. Similar results on survival percentage were obtained in the vaccination studies with the emerging pathogen, *S. marcescens* in the present study. McIntosh and Austin (1990) also recorded that *S. liquefaciens* is extremely proteolytic and pathogenic on Atlantic salmon. The bacterial pathogen, *S. marcescens* employed in the present work was also found highly proteolytic in nature ascertained by biochemical studies conducted prior to the disease management attempts (Pramila, 2003).

From the studies of Quentel and Ogier de Baulny (1995), it was found that intraperitoneal injection protected the juvenile turbot during a challenge performed one month after a single immunisation. The relative percentage protection was the same when the vaccination was done during 62, 76 or 104 days post hatching. Two months after the first immunisation, the juveniles were still protected and RPS values were higher than those obtained one month after vaccination. This result agrees with the results of the present study, where the RPS obtained was the same, 35 and 50 days post-immunisation. The results of the present study are comparable with those obtained by Bakopoulos *et al.* (2003) as well, who found that intraperitoneal challenge with the pathogen resulted in protection of two size groups of fish vaccinated with novel vaccine mixtures.

In the current experiment, 100% protection was obtained on challenge, 35 and 50 days post-immunisation. Such higher levels of protection were recorded in recent works by Van Gelderen *et al.* (2009) in experimental vaccination of Atlantic salmon (*Salmo salar L.*) against marine flexibacteriosis and in the case of vaccination with three inactivated pathogens of cobia (*Rachycentron canadum*), namely *Vibrio alginolyticus, Vibrio parahaemolyticus and Photobacterium damselae* subsp. *piscicida*, when a combined inactivated bacterin antigen preparation was used (Lin *et al.*, 2006). In the latter study, the vaccine gave a relative percentage survival of 93.8%, 91.1% and 84.7% after challenge with *V. alginolyticus, V. parahaemolyticus* and *P. damselae* subsp. *piscicida*, respectively. In comparison to the above studies,

100% protection conferred on clownfish via intraperitoneal vaccination in the present study shows the potential role it has in the marine ornamental fish industry.

In a study by Hjeltnes *et al.* (1989), on vaccination against *Vibrio salmonicida* in the Atlantic salmon, fish vaccinated twice appeared to be better protected than fish vaccinated once, where the degree of protection depended on the route of administration of the vaccine. But in the present experiment, 100% protection could be noted without any booster dose. Studies in this regard may be taken up in the other marine ornamentals, with emphasis on the size and sensitiveness of the fish.

In conclusion, the pioneering work in the field of vaccination of high value marine ornamental fish such as clownfish shows promising results. The high RPS of up to 100% obtained in the present study shows the potential of vaccination strategies by administering the formalin killed bacterin via the intraperitoneal route, which proved better than several results recorded earlier in marine fish. Further, in view of the homogeneity in immune system of fishes, the results could be applicable to other groups of ornamental fish as well. Experiments on improvements in the delivery system and composition of vaccine may be taken up so as to develop efficient vaccines against bacterial diseases of ornamental fish.

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