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Disease Management Options in Captive Reared Clownfish, *Amphiprion sebae* Bleeker 1853: Application of Chemotherapy, Marine Natural Products and Autogenous Vaccines

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Abstract

Disease conditions in aquarium captive-reared clownfish, *Amphiprion sebae* Bleeker 1853, were caused by bacterial pathogens belonging to *Vibrio* sp., *Alacaligenes* sp., *Serratia marcescens* and *Flavobacterium* sp. An emerging pathogen, *Serratia marcescens* was noted to cause severe ulcerations in the aquarium fish in a repetitive manner. Disease management studies conducted with antibiotics, extracts from marine macroalgae and sponges and immunoprophylaxis using formalin-killed *S. marcescens* cells gave promising results. Among the antibiotics, ciprofloxacin had a high inhibitory zone of 32 mm followed by oxytetracycline, trimethoprim and co-trimoxazole with 24 mm zones each. The methanol extract of the macroalga, *Gracilaria corticata* produced 36 mm inhibition zone, while the extracts of two sponge species, *Callyspongia diffusa* and *Sigmadocia carnosa* produced 26 mm and 24 mm zones respectively; suggesting scope for the use of extracts from maroalgae and sponges (marine natural products) for disease management in marine aquaria. The autogenous vaccine when administered in fish at 1×10^5 cells.g^{-1} resulted in increased relative percentage survival after the 15th day and continued with 100 % protection at 35 and 50 days, on challenge with *S. marcescens*. These studies suggest that proactive management strategies can be successfully adopted for managing *S. marcescens* infection in clownfish.

Keywords: marine ornamental fish, *Serratia marcescens*, marine natural products, bacterin, relative percentage survival

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Introduction

The incidence of diseases due to bacterial pathogens is a major cause of economic loss in commercial fish culture. Consequently, the prevention and control of diseases have become global priorities in aquaculture, along with the issue of antimicrobial resistance (AMR). In view of the animal health risks associated with AMR, various intergovernmental agencies such as Food and Agriculture Organisation (FAO), World Organisation for Animal Health (OIE) and World Health Organisation (WHO) recommend reducing the overuse and misuse of antimicrobials (WHO 2006). Therefore, investigations on disease management, of late, have been focused on eco-friendly and cost effective alternative strategies (Reverter et al. 2014; Gudding 2014). Among the prophylactic measures of disease management, immunisation has become a reliable tool in integrated and comprehensive programmes of aquatic animal health management worldwide. At present, it is a significant part of biosecurity in aquaculture (Gudding 2014). As in food fish of commercial value, immunisation could serve as a good alternative in controlling the occurrence of potential bacterial pathogens in aquarium ornamental fish as well. The development of vaccines for fish as an important disease management measure has been stressed by several authors (Gudding and Van Muiswinkel 2013; Brudeseth et al. 2013). 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 emphasise the importance of vaccine development.

Apart from immunoprophylaxis, use of extracts (natural products) from marine macroalgae (seaweeds) and sponges is another suitable alternative for eco-friendly disease management in aquaculture (Kim and Dewapriya 2012; Reverter et al. 2014; Beesoo et al. 2017). Marine secondary metabolites (MSMs) from these resources can be promising elements for developing better management practices for food fish culture. Application of such novel practices as well as conventional antibiotics in controlling bacterial diseases in marine aquarium is a potential research area, considering the high value of the aquarium fish trade. *Serratia marcescens*, an emerging pathogen was found to be consistently associated with repetitive occurrences of severe ulcerations in pomacentrids. In the present paper, results of preliminary experiments on protection against *S. marcescens* using both chemotherapy and prophylactic methods in a commercially important marine ornamental clownfish, *Amphiprion sebae* Bleeker 1853, are presented.

Materials and Methods

Bacterial pathogen

The bacterial species frequently causing infection with severe ulcerations in pomacentrids was identified as *S. marcescens*, based on culture, physical and biochemical characteristics. The red pigment producing isolates were positive for H_2S production, gelatine liquefication, citrate utilisation, as well as for lysine and ornithine reactions. It was negative for the methyl-red test as well as for arginine reactions. The isolate exhibited acid and gas production with glucose, sucrose and sorbitol.

Susceptibility to antibiotics

Kirby-Bauer disc diffusion method described by Bauer et al. (1966) was used to estimate the sensitivity pattern of 25 different antibiotics, as shown in Table 1, against the *S. marcescens* isolates from diseased marine aquarium fish. Randomly selected isolates were used for disc diffusion test in duplicate.

Susceptibility to marine natural products

Eight different extracts of sponges and seaweeds were used for the *in vitro* inhibitory studies against the randomly selected isolates of *S. marcescens* in duplicate by the well diffusion method. Wells were cut in nutrient agar (HiMedia, India) plate swabbed with a fresh culture of the isolate and were sealed with 1 % agar. The extracts were added to the wells and incubated at 37 $^{\circ}$C. Methanol was added to the control wells. Following incubation, zones of inhibition around the wells were measured and recorded.

Immunoprophylactic studies

Bacterin (autogenous vaccine) was prepared from *S. marcescens* by activating the slant culture in broth. The actively growing culture was inoculated into the fresh broth and incubated for 18 h after which the culture was spread plated in nutrient agar plates. After incubation, the cells were harvested in sterile tubes and suspended in phosphate buffered saline (PBS) containing 0.3 % formalin. The cells were then centrifuged and the supernatant discarded. The pellet was serially diluted in PBS and the cells counted using a haemocytometer before use for intraperitoneal injection.

Experimental design

Three months old clownfishes of same brood were stocked in three 5 ton tanks. The tanks were provided with biofilter and water recirculation was maintained by airlift. The fish were fed twice daily with boiled mussel meat. The prepared bacterin was administered via the intraperitoneal route to the whole stock maintained in two tanks at the rate of 10^5 cells.g^{-1} which was the lethal dose estimated from pathogenicity studies conducted earlier with the isolate. The fishes were monitored continuously for mortality and response pattern.

Challenge studies

For challenge studies, the fish were transferred to experimental fibreglass reinforced plastic (FRP) tanks of one ton capacity. After 15, 30 and 50 days of administration of bacterin, challenge studies were conducted by intraperitoneal injection of live pathogenic cells to the experimental groups of six fish each in triplicate at the lethal dose, which was determined earlier as10^5 cells.g^{-1}. A set of control fish was given 0.85 % saline injection. After injection, the fishes were monitored for mortality, clinical signs and behavioural responses.

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

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

Results

The antibiotic sensitivity pattern of the pathogenic isolate, *S. marcescens* is given in Table 1. The isolate was sensitive to compounds including ciprofloxacin, trimethoprim, co-trimoxazole, gentamycin, tetracycline, nalidixic acid, erythromycin, chloramphenicol, streptomycin and nitrofurantoin followed by erythromycin, furazolidone, sulphadiazine, neomycin and kanamycin with intermediate sensitivity. Mean diameters of the zones of inhibition corresponding to the sensitivity of isolates are shown (Table 1).

Table 1. Antibiotic sensitivity pattern of *Serratia marcescens*.

Antibiotic used per disc (µg)	Concentration	Response of *S. marcescens*	Diameter of zone of inhibition (mm)
Penicillin	2.5 I.U.	R	-
Ampicillin (A^{25})	25	R	-
Cloxacillin (Cx^{10})	10	R	-
Neomycin (N^{30})	30	I	16
Kanamycin (K^{30})	30	I	16
Gentamycin (G^{10})	10	S	23.5
Erythromycin (E^{15})	15	I	20
Streptomycin (S^{10})	10	S	17
Chloramphenicol (C^{25})	25	S	20
Rifampicin (R^{15})	15	R	-
Nalidixic acid (Na^{30})	30	S	22
Amphotericin B (Ap^{100})	100	R	-
Bacitracin (B^{10})	10	R	-
Doxycycline (Do^{30})	30	R	-
Co-trimoxazole (Co^{25})	25	S	24
Metronidazole (Mt^{5})	5	R	-
Sulphamethizole (Sm^{100})	100	R	-
Sulphadiazine (Sz^{100})	100	I	16
Trimethoprim (Tr^{5})	5	S	24
Ciprofloxacin (Cf^{10})	10	S	32
Furazolidone (Fr^{50})	50	I	18
Nitrofurazone (Nr^{100})	100	R	-
Nitrofurantoin (Nf^{300})	300	S	17
Tetracycline (T^{30})	30	S	23
Oxytetracycline (O^{10})	10	S	24

R – resistant; S – susceptible; I – intermediate

Majority of the *S. marcescens* isolates were resistant to four antibiotics (resistance index 0.16) followed by resistant to 10 antibiotics (resistance index 0.4). Maximum numbers of isolates were sensitive to 14 antibiotics (sensitivity index 0.56).

The bioactive potential of some of the tested seaweed and sponge extracts could be visualised as zones of inhibition where the isolate failed to grow. The inhibitory activity of the extract of *Gracilaria corticata*, a red seaweed and sponge extracts (*Callyspongia* and *Sigmadocia carnosa*) were found to be high (Fig. 1) as indicated by the diameters of clear zones of inhibition of growth of the pathogen (Table 2).

Table 2. Susceptibility pattern of *Serratia marcescens* towards extracts of seaweeds and sponges.

Extract source *	Sensitivity	Zone diameter (mm)
Hypnea musciformis	+	14
Valoniopsis pachynema	+	24
Chnoospora maxima	+	10
Caulerpa scalpelliformis	-	-
Gracilaria corticata	+	36
Gracilaria fergusonii	-	-
Sigmadocia carnosa	+	24
Callyspongia	+	26
PVR-Sponge extract	-	25

+ sensitive; - resistant

*Source- ICAR ad-hoc project, Vizhinjam Research Centre of CMFRI.

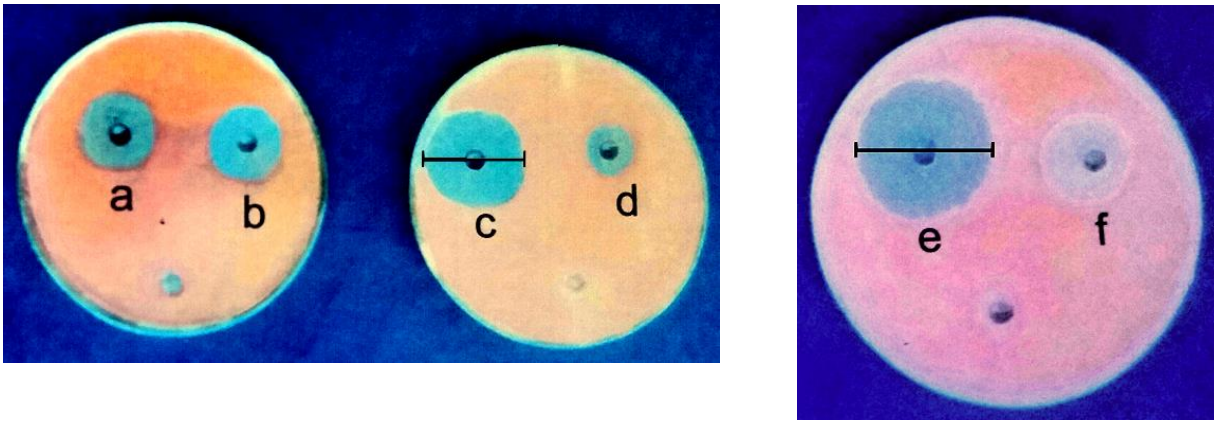

Fig. 1. Inhibiton zones against *Serratia marcescens* by marine extracts from (a) *Sigmadocia carnosa*, (b) *Valoniopsis pachynema*, (c) *Callyspongia*, (d) *Hypnea musciformis*, (e) *Gracilaria corticata*, and (f) PVR sponge extract [bar = 25 mm (c); 36 mm (e)].

Results of the experiments using bacterin conducted *in vivo* indicated that the administration of formalin-killed cells (bacterin) could provide protection even on the 50th day post immunisation, without any booster dose in between. The RPS rendered by the administration of bacterin to the experimental fish was seen to increase after the 15th day and provided 100 % protection at 35th and 50th days. The non-immunised fish showed mortality percentages of 80 and 50 at 15th day and on 35th and 50th days, respectively (Fig. 2).

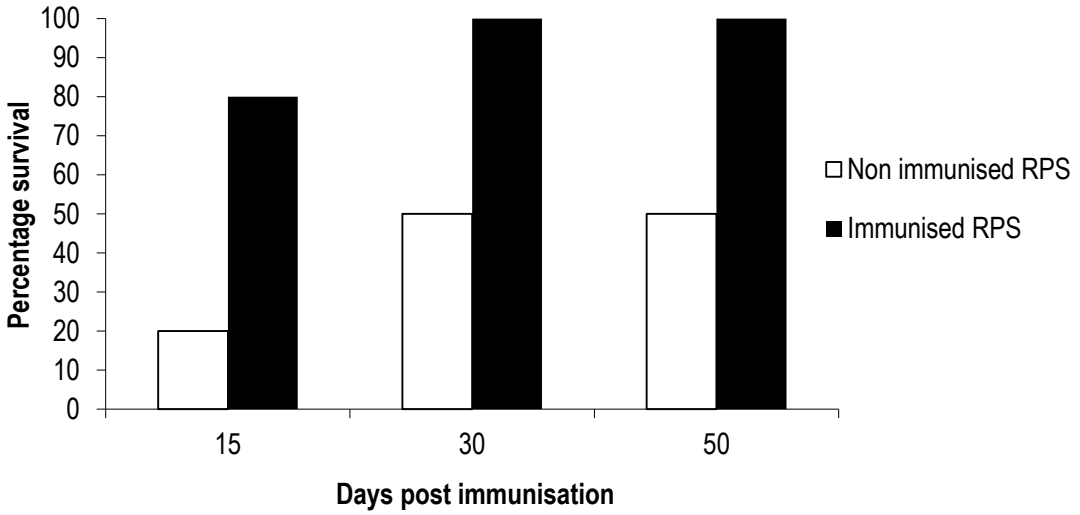

Fig. 2. Relative percentage survival in immunised and non-immunised fish after intraperitoneal challenge with live cells of *Serratia marcescens*.

Discussion

Since the documentation related to research on management of diseases in marine ornamental fish using antibiotics is limited, a comparative account on antibiotic sensitivity pattern of the tested pathogen was not attempted. Reports on the influence of antibiotics in reducing bacterial infections in marine rearing facilities by Novotny (1978) and Austin et al. (1981) recommended the use of limited number of compounds. Subasinghe (1992) reported the use of antibiotics such as tetracycline, potentiated sulphonamides and chloramphenicol in the ornamental fish industry in Sri Lanka. Sonia and Lipton (2012) reported the antibiotic sensitivity of pathogenic strains of vibrios infecting captive-reared ornamental blue damsel fishes and stated that they were highly susceptible to most broad-spectrum antibiotics.

Serratia marcescens isolated and characterised in the present study followed a regular sensitivity pattern with varying degrees of susceptibility to the antibiotics tested. In the experiment by Baya et al. (1992) two strains of *S. marcescens* were found to be sensitive to trimethoprim and oxolinic acid although they were resistant to most of the antibiotics used in their studies. Sensitivity to trimethoprim was exhibited by the isolate used in the present study as well. McCarthy et al. (1974) stated that sulphonamides when used in combination with trimethoprim were effective in inhibiting Gram negative pathogens. Bacteriostatic effects of the above individual drugs are well known which when used in combination have bactericidal effect. Vigneulle and Baudin Laurencin (1995) and McIntosh and Austin (1990) have reported the sensitivity of a highly proteolytic species, *Serratia liquefaciens* towards several antibiotics. Similar observations were made in the present investigations suggesting the feasibility of chemotherapeutic measures for disease control in marine aquaria. However, owing to the environmental concerns associated with the use of antibiotics, less harmful biotechnological methods are advisable for disease management. Further, any recommendations on the use of antibiotics should take into consideration the negative effects of the build-up of antimicrobial resistance.

There are several reports on the effectiveness of marine natural products in aquaculture as a disease management strategy (Rao et al. 1991; Mary et al. 1994; Jung et al. 1995; Selvin et al. 2004b). In the present study, methanol extracts from several seaweeds and sponges exhibited high inhibitory activity against the tested pathogen. The red seaweed, *G. corticata* and green seaweed, *V. pachynema* and sponges, *Callyspongia* and *S. carnosa* were found to have considerably high activity (Fig. 1). Studies by Lipton (2001) indicated comparable results. Significant observations were made in the studies conducted by Selvin and Lipton (2004) and Selvin et al. (2004a), based on which secondary metabolites isolated from green alga *Ulva fasciata* and a marine sponge *Dendrilla nigra* were developed as antimicrobial agents for the control of bacterial diseases. In a recent study, Maftuch et al. (2016) demonstrated the antibacterial activity of *Gracilaria verrucosa* fraction obtained by column chromatography against fish pathogens such as *Aeromonas hydrophila*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Vibrio harveyi* and *Vibrio alginolyticus*. Similarly, Mohan et al. (2016) reported the antimicrobial properties of sponge endosymbiotic bacteria on several virulent marine ornamental fish pathogens.

The above reports suggest that the novel products from seaweeds and sponges are possible proactive disease management tools for application in fish culture. Although the bioactive principles were not isolated in the present study, the findings point out the potential of these extracts as a valuable management option against bacterial diseases in marine ornamental fish. Results of preliminary screening studies offer scope for further investigations aiming at the development of antimicrobial agents to replace antibiotics for therapy of diseased cultured fish. Marine plants, invertebrates and a wide array other resources offer immense scope for antibacterial, antifungal, antioxidant and other biological activities such as immunomodulation that can be used for drug development. The biotechnological implications of these resources need to be assessed with the help of molecular biological tools for application in fish health management. Further, the gap in the field of commercial application of fish vaccines requires more studies on development and testing novel prophylactic antimicrobials from marine organisms. Hence, this can be a thrust area for disease management research particularly in non-food fish and commercial aquarium fishes.

Vaccination is considered as a reliable tool for maintaining aquaculture systems free of specific pathogens. Vaccination procedures for the prevention of specific bacterial diseases affecting commercially reared fish species have had a significant impact on aquaculture industry, particularly in the intensive farming of high-value species such as salmon and trout (Sommerset et al. 2005). Vaccine development status for major bacterial diseases in aquaculture has been reviewed by Pridgeon and Klesius (2012). However, the literature on immunisation studies of marine ornamental fish is too scanty to provide comparative accounts. Hence, the results of the present study are analysed based on immunisation studies in other marine finfish. Almost all the fish vaccines available are bacterins or formalin-inactivated whole cell suspensions, with or without adjuvants. In the present study, formalin-killed cells were injected via the intraperitoneal route to determine the role of immunisation in protecting 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 was enough to kill the cells, without losing antigenic properties. Besides, the above dose gave up to 100 % protection to the experimental group. From immunisation studies conducted earlier in fishes, it was established that one of the most effective routes of delivery of vaccine/bacterin is by injection, as this ensures the administration of an exact dose (Sommerset et al. 2005; Brudeseth et al. 2013). Dosage and mode of administration are of particular relevance in the case of marine ornamental fish, where individual fish command high market rate.

McIntosh and Austin (1990) demonstrated the efficacy of immunoprophylactic procedures against *Serratia* species in one among the very few studies on this species. They reported the effectiveness of vaccination regimes of *S. liquefaciens*, a highly proteolytic species, on Atlantic salmon. Survival rate due to the vaccine administration was identified 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. The results on survival of non-immunised fish obtained in the present study against the emerging pathogen, *S. marcescens* are comparable to the above report. In addition, the pathogenic conditions caused by both *S. marcescens* and *S. liquefaciens* were similar pointing at the high proteolytic property shown by both. The above authors have recorded that the species, *S. liquefaciens* was extremely proteolytic and pathogenic on Atlantic salmon. The bacterial pathogen, *S. marcescens* employed in present work also was found highly proteolytic in nature, ascertained by biochemical studies such as gelatine liquefaction.

The effectiveness of vaccination in juvenile fish and seed is another area that can be explored further. Quentel and Ogier de Baulny (1995) found that intraperitoneal injection protected juvenile turbot, *Scophthalmus maximus* (Linnaeus 1758) against vibriosis during a challenge performed one month after a single immunisation. The relative percentage protection was the same when the vaccination was done 62, 76 or 104 days post-hatching. They recorded that the juveniles were protected even after 2 months of immunisation and also that the RPS values were higher than those obtained 1 month after vaccination. This result is tallying with that of the present study, where the RPS obtained was the same, 35 and 50 days post immunisation in three months old clownfish.

The findings of Bakopoulos et al. (2003) also need mention, which explains the efficacy of various vaccination modalities in two size groups of sea bass, *Dicentrarchus labrax* (Linnaeus 1758) against *Photobacterium damselae* subsp. *piscicida*, the causative agent of pasteurellosis. They reported different levels of protection in different size groups vaccinated with novel vaccine mixtures, using bath, intraperitoneal and oral routes and challenged by bath, immersion and intraperitoneal routes, 6 and 12 weeks post immunisation. These records suggest possible options for vaccination methods in fish belonging to various size groups. The very high protection obtained in the challenged fish at 35 and 50 days post immunisation in the present study substantiates the suitability of immunisation of marine ornamental fish as an ideal disease control option. In view of these encouraging results, further investigations could be considered on the use of vaccination in the marine ornamental fish industry.

High levels of protection were recorded by van Gelderen et al. (2009) in the experimental vaccination studies of Atlantic salmon (*Salmo salar*) against marine flexibacteriosis. Similarly, Lin et al. (2006) demonstrated successful vaccination in the case of three pathogens of cobia, *Rachycentron canadum* (Linnaeus 1766), such as *Vibrio alginolyticus*, *Vibrio parahaemolyticus* and *Photobacterium damselae* subsp. *piscicida*, using a combined three inactivated bacterin-antigen preparation. They recorded that the vaccine gave RPS values 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, 100 % protection was conferred on clownfish in the present investigations via intraperitoneal injection, which is promising. Li et al. (2016) conducted studies on evaluation of various vaccination modalities and reported that out of the several whole cell bacterin preparations such as formalin-killed, phenol-killed, heat-killed and chloroform-killed bacterins, formalin killed bacterin displayed the maximum degree of protection against vibriosis of farmed marine fish. They could also observe that intraperitoneal injection imparted the 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 findings of the above authors.

The efficacy of intraperitoneal injection of formalin-killed bacterin as the appropriate strategy was convincingly proven in the present work, as the degree of protection conferred by single vaccination was 100 %. Vaccination studies against *Vibrio salmonicida* in Atlantic salmon by Hjeltnes et al. (1989) showed that fish vaccinated twice appeared to be better protected than fish vaccinated once, the degree of protection being dependent on the route of administration of the vaccine. However, in the present experiment, 100 % protection was noted without any booster dose. This observation could be of significance in immunoprophylaxis of marine ornamentals where additional parameters such as size and sensitiveness of the fish may be considered for future research.

Conclusion

The pioneering work on disease management using marine natural products as well as vaccination in high-value marine ornamental fish such as clownfish gave promising results. Since the bioactive potentials of secondary metabolites from marine sources are well documented, advanced research should include the development of proactive management strategies in the form of synergistic formulations for use in aquaculture. This can form a part of integrated disease management (IDM) in aquaculture or aquarium keeping. The high RPS of up to 100 % obtained in the present study showed the potential of vaccination strategies in combating bacterial diseases in marine ornamentals by intraperitoneal administration of formalin-killed bacterin. In comparison to the results of previous studies on immunisation in other marine fish, the present investigation gave improved results. In view of the homogeneity in immune system of fishes, immunisation can be recommended as a potential disease management option for various marine ornamental fish groups.

Studies on improvements in the mode of application, types and composition of vaccines are other areas that need special attention in developing efficient vaccines, in addition to gaining better knowledge of the fish immune system. Caipang et al. (2014) have recorded that although most bacterial vaccines were found efficient in candidate species such as Asian seabass, the production of truly effective teleost vaccines could be achieved only through a thorough understanding of the immune processes in the host during vaccination. Though mono and multivalent vaccines have been developed against several bacterial diseases in fish, the applications of such vaccine preparations/bacterins are yet to find successful commercial level applications. These could be thrust areas of research on disease management of captive marine ornamental fish as well.

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